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ORIGINAL PAPER

Evaluation of the Glasgow-Blatchford score in predicting clinical outcomes in upper gastrointestinal bleeding

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ABSTRACT

Introduction and aim. Acute upper gastrointestinal bleeding is a common cause of emergency admissions with potentially serious outcomes. Early evaluation of patients is crucial to predict morbidity, recurrence of bleeding, and mortality. The Glasgow Blatchford score (GBS) is a validated scoring system used to predict the need for medical interventions such as blood transfusion, endoscopy, and surgery. This study aimed to explore the correlation of GBS with prognostic markers in patients with upper gastrointestinal bleeding.

Material and methods. This retrospective study included patients >18 years old admitted to Hitit University Corum Erol Olcok Training and Research Hospital due to upper gastrointestinal bleeding between December 2022 and May 2023. Exclusion criteria were insufficient endoscopy or data or pregnancy. GBS scores were calculated at the initial presentation for each patient and their association with prognostic markers and mortality was analyzed. Comparison of numerical measurements between independent groups was evaluated using the Mann-Whitney U test and categorical variables were evaluated using the Chi-square test. Spearman coefficients were used for correlations. ROC analysis was used to determine the sensitivity and specificity of GBS to predict endpoints. The predictive factors for the endpoints were investigated using logistic regression analysis.

Results. A total of 140 patients were enrolled in the study. GBS was significant in predicting the need for blood transfusion (OR: 1.493, 95% CI: 1.297–1.719, p<0.001), need for endoscopic intervention (OR: 1.248, 95% CI: 1.089–1.430, p=0.001), and preference for ward/intensive care unit (OR: 0.869, 95% CI: 0.790–0.953, p=0.003). For predicting mortality, Charlson Comorbidity Index (OR: 1.023, CI=1.008–1.437, p=0.046) was significant. GBS was not significant for predicting mortality (p=0.582).

The area under the curve (AUC) of GBS with a cut-off of 9.5 for mortality was 0.64 (95% CI 0.513–0.775, p=0.032) with a sensitivity of 68.2% and specificity of 52.5%, AUC 0.752 (95% CI 0.653–0.851, p<0.001) for the need for endoscopic intervention with a sensitivity of 90% and specificity of 50.8%, AUC 0.729 (95% CI 0.646–0.812, p<0.001) for admission to intensive care with a sensitivity of 70.1% and specificity of 58.9% and AUC 0.853 (95% CI 0.782–0.924, p<0.001) for the need for blood transfusion with a cut-off of 8.5 with a sensitivity of 84.9% and specificity of 75.5% for the selected.

Conclusion. The GBS did not predict mortality, but effectively predicted the need for blood transfusion, endoscopic intervention, and intensive care unit admission. The Charlson comorbidity index was predictive for mortality in this study group.

Keywords. Glasgow-Blatchford score, prognosis, upper gastrointestinal bleeding

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Introduction

Upper gastrointestinal system (GIS) bleeding refers to bleeding anywhere in the gastrointestinal system proximal to the Treitz ligament. It is one of the leading causes of hospital admissions and is associated with significant morbidity and mortality. The key priorities in managing GIS bleeding include rapid patient evaluation, stabilization, identifying the site of bleeding, and preventing recurrent bleeding, which notably increases mortality.[1]

Various risk scoring systems have been developed to assess prognostic factors such as mortality, the need for early intervention, the risk of rebleeding, and length of hospital stay.[2] Recently, there has been considerable interest in pre-endoscopy risk scores for upper GIS bleeding that can be calculated after hospital admission. One of the most commonly used scores is the Glasgow Blatchford score (GBS).[3] These scoring systems use clinical, hemodynamic, and laboratory variables and can also identify low-risk patients who could be managed as outpatients.[4] Additionally, it has been suggested that these scores can identify higher-risk patients who may require urgent endoscopy or intensive care.[3,4]

The GBS indicates the need for intervention to control bleeding. It incorporates variables such as hemoglobin (g/dL), systolic blood pressure (mmHg), presence of syncope, melena, and history of heart or liver failure, adjusted by age.[4] However, additional prognostic markers have been explored to further refine risk stratification in patients with bleeding from the upper GIS. One such marker is the Charlson Comorbidity Index (CCI), which is a widely used tool to quantify comorbid conditions and predict long-term mortality.[5] The CCI assigns weighted scores to various chronic diseases, providing an overall comorbidity burden score. In the context of upper GIS bleeding, higher CCI scores may have been associated with increased mortality and worse clinical outcomes. Despite its established prognostic value in other medical conditions, its role in upper GIS bleeding risk assessment has not been fully elucidated.

Aim

The aim of this study was to investigate the relationship between the Glasgow Blatchford Score and prognostic markers, including the Charlson Comorbidity Index, in patients with upper GIS bleeding. By assessing the prognostic value of these markers, we aim to enhance the risk stratification process and improve clinical decision-making in this patient population.

Material and methods

Study group

Data related to the study were collected from hospital records of patients admitted to Hitit Training and Research Hospital between December 2022 and May 2023 with upper GIS bleeding.

Inclusion criteria for the study were patients 18 years and older who were admitted to our hospital due to upper GIS bleeding. Exclusion criteria included patients with insufficient endoscopy or data, and pregnant patients. The diagnoses were made after an endoscopy, and all subgroups of upper gastrointestinal bleeding (UGIB), including esophageal variceal bleeding, were included.

Demographic data such as age, sex, presenting complaints, chronic illnesses, medications, surgical history, smoking and alcohol history, and previous episodes of gastrointestinal bleeding were retrospectively evaluated from discharge summaries. Data on intensive care and ward admission status, intubation status, initial vital signs at emergency department admission, rectal examination findings of bleeding, syncope status, need for blood transfusion, time of endoscopic intervention, presence of active bleeding during endoscopy, method of intervention if performed, need for surgery, occurrence of rebleeding, discharge or exitus outcomes, reasons for exitus if occurred, and length of hospital stay were recorded. Charlson CCI and GBS were calculated. The CCI evaluates 22 comorbid conditions to predict the risk of mortality at one year. Each condition is scored from 1 to 6 based on its associated risk level. GBS is calculated using parameters that include blood urea level (mmol/L), hemoglobin level (g/dL), systolic blood pressure (mmHg) and other markers (pulse rate above 100, melena, syncope, presence of liver disease, and heart failure).

The patients were categorized according to hospitalization outcomes ending in mortality, and prognostic indicators of mortality were determined. The relationship between GBS and prognosis/mortality was statistically evaluated.

Statistical methods

All statistical analyzes were performed using IBM SPSS Statistics for Windows software (version 26; IBM Corp., Armonk, NY, USA). Descriptive statistics were reported as number and percentage for categorical variables, mean for numerical variables. The relationships between variables were explored using the Spearman correlation test. Comparison of numerical measurements between independent groups according to research groups was assessed using the Mann-Whitney U test. Comparison of rates between research groups for categorical variables was evaluated using the Chi-square test. ROC analysis was used to determine the sensitivity and specificity of GBS to predict endpoints. The Youden index was used to determine cutoff points. The predictive factors for the endpoints were investigated using logistic regression analysis. Statistical significance was established at $p<0.05$.

Ethical aspect of the study

The approval for the study was obtained from the Ethics Committee for Clinical Research of the Faculty of Medicine on September 13, 2023, under decision number 2023-120. The study was carried out according to the Helsinki Declaration and good clinical practices.

Table 1. Clinical characteristics of the study group*

Clinical charesteristics	n (%)
Age, mean	69.7±18
Sex, female	58 (41.4)
DOAC use	19 (13.6)
NSAID use	43 (30.7)
Kvit antagonist use	29 (20.7)
LMWH use	5 (3.6)
CCI, mean	5.5±3.5
Hospital admission	
ICU	67 (47.9)
Clinic	73 (52.1)
Melena	89 (63.6)
Syncope	12 (8.6)
Need for transfusion	86 (61.4)
Endoscopic intervention	20 (14.3)
Rebleeding	5 (3.6)
Exitus	21 (15.7)
Length of stay, days	5.1±6.3
GBS	9.5± 4.9

* DOAC – direct oral anticoagulant, ICU – intensive care unit, LMWH – low-molecular-weight heparin, NSAID – non-steroidal anti-inflammatory drugs

Results

Among 388 patients, 248 were excluded due to missing data, leaving a total of 140 patients included in the study. The mean age of the patients was 69.7±18 years. Of the patients, 41.4% (n=58) were women and 58.6% (n=82) were male. Demographic and clinical data of the patients are presented in Table 1.

Table 2. Etiologies of patients with upper gastrointestinal bleeding

Etiology	n=140	%
Peptic ulcer		
Bulbus	24	17.1%
Gastric	17	12.1%
Erosive gastritis	2	1.4%
Esophageal ulcer	2	1.4%
Mallory Weiss syndrome	7	5%
Esophagitis	21	15%
Premalign lesions	5	3.6%
Gastric cancer	11	7.9%
Esophageal varices	13	9.3%
Other	14	10%

Among the patients included in the study based on the etiology of GIS bleeding, 29.2% (n=41) had peptic ulcer disease. Bulbar ulcer was detected in 17.1% (n=24) of patients diagnosed with peptic ulcer. The distribution of patients according to the etiology of GIS bleeding is shown in Table 2.

Comparisons of patient groups based on mortality, need for blood transfusion, need for endoscopic intervention, and admission to wards or intensive care units are presented in Table 3.

The results of the correlation analyzes between continuous variables age, CCI, length of hospital stay, and GBS examined in the study group are shown in Table 4. There is a moderate positive correlation between age, CCI, length of hospital stay, and GBS.

Table 3. Comparisons of patient groups according to clinical outcomes*

	Exitus			Need for blood tranfusion			Need for intervention			Need for ICU		
	Yes (n=22)	No (n=118)	p	Yes (n=86)	No (n=54)	p	Yes (n=20)	No (n=120)	p	Clinic (n=73)	ICU (n=67)	p
Age (mean)	78.4	68	0.013	73	64.4	0.005	70.6	69.5	0.804	64.1	75.7	<0.001
Sex (male)	11	47	0.374	47	38	0.238	9	49	0.726	32	26	0.546
DOAC use	4	15	0.172	13	6	0.187	3	16	0.865	10	9	0.565
NSAID use	8	38	0.532	30	13	0.177	6	37	0.94	17	26	0.047
Kvit antagonist use	0	11	0.143	8	3	0.429	2	9	0.658	3	8	0.085
Antipletelet use	4	28	0.78	20	9	0.344	4	28	0.932	11	18	0.085
LMWH use	3	2	0.006	5	0	0.071	1	4	0.71	1	4	0.143
CCI (mean)	7.8	5.0	0.001	6.2	4.3	0.001	5.5	5.4	0.906	4.3	6.8	<0.001
Admission to clinic	2	71	<0.001	38	38	0.001	6	67	0.032			
Need for intervention	3	17	0.924	19	1	0.001				6	14	0.032
Need for transfusion	18	68	0.032				19	67	0.001	35	51	0.001
Exitus				18	4	0.032	3	19	0.928	2	20	<0.001
Rebleeding	1	4	0.789	5	0	0.071	2	3	0.094	2	3	0.58
Length of stay (days)	9.6	4.3	<0.001	6.3	3.2	0.005	5.7	5	0.626	3.6	6.7	0.003
GBS (mean)	11.9	9.1	0.015	11.9	5.7	<0.001	13	8.9	0.001	7.6	11.6	<0.001

* DOAC – direct oral anticoagulant, ICU – intensive care unit, LMWH – low-molecular-weight heparin, NSAID – non-steroidal anti-inflammatory drugs

Table 4. Correlations between Glasgow Blatchford score and continuous variables

	GBS (n=140)	
	r	p
Age	0.47	<0.001
CCI	0.518	<0.001
Length of stay	0.272	0.001

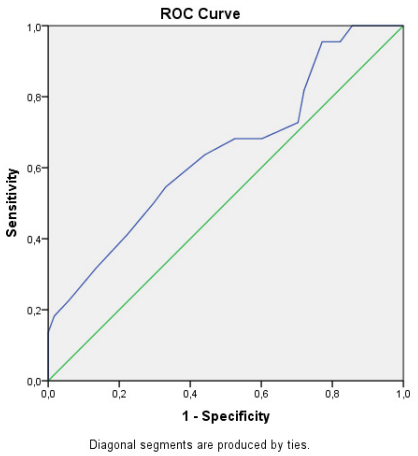
Logistic regression analyzes performed to predict mortality, need for blood transfusion, the need for endoscopic intervention, and admission to the wards or intensive care units are provided in Table 5. The parameters showing the differences between groups in the comparison table were included in the regression analysis.

Table 5. Logistic regression analysis to predict mortality and the need for blood transfusion, intervention, and intensive care unit

	OR	%95 CI	p
Mortality			
GBS	1.036	0.913-1.176	0.582
Age	1.02	0.976-1.066	0.389
LMWH use	0.145	0.020-1.027	0.053
CCI	1.203	1.008-1.437	0.046
Need for blood transfusion			
GBS	1.493	1.297-1.719	<0.001
Age	0.985	0.944-1.021	0.394
CCI	0.995	0.830-1.193	0.958
Need for intervention			
GBS	1.248	1.089-1.430	0.001
Need for ICU			
GBS	1.153	1.049-1.266	0.003
Age	0.956	0.956-1.016	0.354
NSAID use	1.636	0.733-3.653	0.239
CCI	0.909	0.782-1.056	0.212

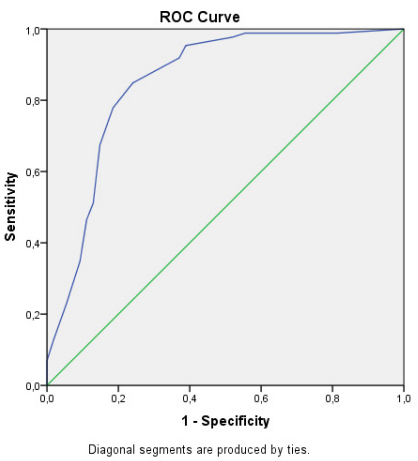
* CI – confidence interval, DOAC – direct oral anticoagulants, ICU – intensive care unit, LMWH – low molecular weight heparin, NSAID – non-steroidal anti-inflammatory drugs, OR – odds ratio

ROC curves for GBS predicting mortality, need for blood transfusion, need for endoscopic intervention, and admission to intensive care are presented in Figures 1–4, respectively. The area under the curve (AUC) for mortality was 0.64 (95% CI 0.513–0.775, p=0.032) with a sensitivity of 68.2% and specificity of 52.5% for the selected GBS threshold of 9.5. For the need for blood transfusion, the AUC was 0.853 (95% CI 0.782-0.924, p<0.001) with a sensitivity of 84.9% and a specificity of 75.5% for the selected GBS threshold of 8.5. For the need for endoscopic intervention, the AUC was 0.752 (95% CI 0.653-0.851, p<0.001) with a sensitivity of 90% and a specificity of 50.8% for the selected GBS threshold of 9.5. For admission to intensive care, the AUC was 0.729 (95% CI 0.646-0.812, p<0.001) with a sensitivity of 70.1% and specificity of 58.9% for the selected GBS threshold of 9.5.



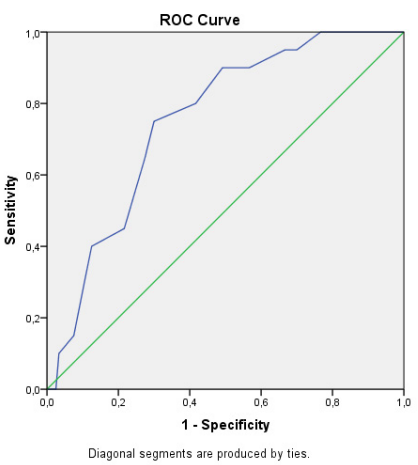
	AUC (%95)	Cut-off	p	Sensitivity (%)	Specificity (%)
GBS	0.644 (0.513–0.775)	9.5	0.032	68.2	52.5

Fig. 1. ROC curves for GBS predicting mortality



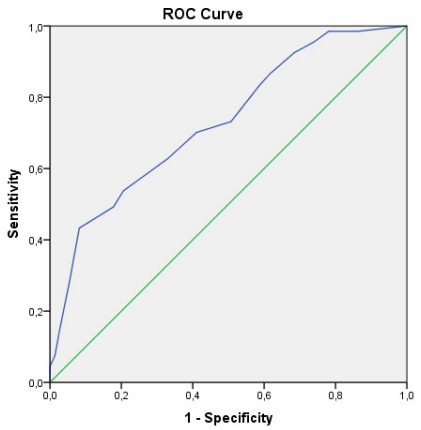
	AUC (%95)	Cut-off	p	Sensitivity (%)	Specificity (%)
GBS	0.853 (0.782–0.924)	8.5	<0.001	84.9	75.9

Fig. 2. ROC curves for the need for blood transfusion



	AUC (%95)	Cut-off	p	Sensitivity (%)	Specificity (%)
GBS	0.752 (0.653–0.851)	9.5	<0.001	90	50.8

Fig. 3. ROC curves for GBS need for endoscopic intervention



	AUC (%95)	Cut-off	p	Sensitivity (%)	Specificity (%)
GBS	0.729 (0.646–0.812)	9.5	<0.001	70.1	58.9

Fig. 4. ROC curves for GBS admission to intensive care

Discussion

Upper gastrointestinal bleeding (UGIB) represents a medical emergency with high hospitalization rates and a mortality risk of up to 10%.[6] Successful treatment of patients requires careful assessment of their risks. In this study, we investigated the performance of GBS in predicting mortality and clinical outcomes in patients with UGIB.

Firstly, in terms of patients, blood transfusion was performed in 86% of our patients, indicating a high rate of blood transfusion compared to the literature.[7-9] This high rate can be attributed to our hospital being a tertiary referral center, where complex cases are referred and patients presenting with severe comorbidities and more critical clinical conditions are admitted. In the literature, transfusions are reported in 33-53% of hospitalized patients due to UGIB internationally and as high as 75% in studies conducted in Turkey.[7-10] The latest recommendations from the European Society of Gastrointestinal Endoscopy (ESGE) and the International Consensus Group suggest targeting hemoglobin levels between 7 to 9 g/dL for transfusion, with higher targets considered for patients with significant comorbidities.[7] However, our study, like others, did not investigate the relationship between hemoglobin levels and the number of units transfused. Studies by Mokhtare et al. and Robertson et al. comparing GBS with other scoring systems found similar results.[8-9]

The second result was the rates of recurrent bleeding, whereas our study found a recurrence rate of 3.6%. Okutur et al. reported recurrent bleeding in 10% of cases, associated with higher average age, length of stay, and mortality rates.[10] Yavorsky et al. reported a recurrent bleeding rate of 7.1% in their retrospective study of 3294 patients.[11] Robertson et al.[9] also reported a recurrent bleeding rate of 9.7% in their study.

According to the Blatchford criteria, the mean GBS in our study was 9.5±4.9, which aligns with previous findings.[12,13] Advanced age and the presence of comorbid diseases increase mortality rates in GIB. Despite advances in diagnosis and treatment, a significant reduction in GIB mortality rates over the years has not been achieved.[12] Mortality rates vary depending on the cause, location, etiology of bleeding, and the presence of additional diseases. Mortality rates for GIB range from 8% to 20.3% in studies focusing on GIB and mortality.[12,13] In our study, the mortality rate was 15.7%, consistent with the literature.

As another outcome, the average length of hospital stay in our study was 5.1±6.3 days, which is similar to findings in other studies.[12-14] Uysal et al. reported endoscopic treatment in 38.1% of patients, 17.9% experiencing rebleeding.[14] Kim et al. reported re-bleeding in 12.7% and an endoscopic intervention need in 58.8% of their study population.[13] In our study, the rates of re-bleeding and endoscopic intervention were 20% each.

Endoscopy is crucial in identifying UGIB lesions due to its high sensitivity and specificity. Therapeutic endoscopy can achieve acute hemostasis and prevent rebleeding in most patients once a bleeding lesion is identified. In our study, 71.4% of the patients underwent endoscopy within the first 24 hours, consistent with the findings of the literature.[12-14]

With increasing age, the frequency of chronic diseases that predispose to bleeding and the use of medications that promote bleeding also increases. In our study, patients using low molecular weight heparin (LMWH) and direct oral anticoagulant (DOAC) had higher average GBS scores, which we attributed to accompanying comorbidities and age. We found that a higher GBS score predicts the need for transfusion and endoscopic intervention, but not mortality. Stanley et al. and Gökçek et al. reported that scoring systems predict mortality and the need for transfusions, while they do not have superiority over each other.[15,16]

In deceased patients, our study found higher ages, LMWH use, CCI, need for blood transfusion, length of stay, and GBS scores. Other studies have identified comorbid diseases as significant predictors of mortality in patients with nonvariceal upper gastrointestinal bleeding.[17,18] Kaplan et al. emphasized that advanced age and additional medical problems, even if asymptomatic, lead to worse outcomes and higher mortality rates.[18] A study in 3508 patients presenting to the emergency with UGIB reported a mortality rate, with more than half having one or more accompanying diseases at admission, and 83% of deaths being associated with one or more comorbid diseases.[19] Paksoy et al. found that 25.6% of patients had comorbid diseases, with 1.8% of deaths directly attributable to these conditions.[19]

Early and effective treatment aims to prevent patient mortality, accelerate recovery, and prevent compli-

cations. Identifying which patients require intervention (such as transfusion, endoscopic bleeding control) is crucial for prognosis. Patients at low risk for recurrent bleeding and death due to UGIB may still require transfusions. These patients should be hospitalized and not considered low-risk. In our study, we found that patients who underwent endoscopic intervention had a high need for blood transfusion and high GBS scores. In another study comparing risk scores, GBS was found to be the most successful score in predicting major transfusion needs and the need for endoscopic treatment.[18]

Various independent predictors have been identified in different studies that predict a poor prognosis and mortality in patients with GIB, including advanced age, male gender, presence of comorbidities, coagulopathy, replacement of blood products, redebleeding, high-risk lesions on endoscopy, and length of stay.[1,19] In our study, we focused on mortality, the need for blood transfusion, endoscopic intervention, and admission to the ICU as prognosis markers. We found a positive correlation between age, CCI, length of stay, and GBS.

Regression analyzes that evaluated parameters significantly associated with mortality among groups in our study found that each unit increase in CCI increased mortality by 1.2 times. Yurtsever et al. noted that high CCI affects mortality in logistic regression analysis.[20]

Most studies in the literature cover GBS and other scoring systems. Siebenhüer et al. found that GBS is the best system for predicting erythrocyte transfusion needs.[21] In our study, each increase in the GBS score was associated with a 1.4 times increase in the transfusion requirement.

In a study by Choi et al., it was observed that patients with a GBS of 10 or higher had a higher rate of admission to the emergency department ICU.[22] In a study by Taşldere et al, scores were compared, and a GBS of 11 or higher was found to predict admission to the ICU.[23] In our study, logistic regression analysis to predict admission to the ICU found GBS to be statistically significant.

This study had several limitations. First, this study was unicenter and retrospective. Second, the sample size was relatively small to obtain robust results. Third, our hospital is a tertiary referral center where complex cases are also referred, which may have introduced some bias in terms of patient selection.

Conclusion

This study has reaffirmed that GBS is a reliable predictive marker of the need for blood transfusion, endoscopic intervention, and intensive care management in upper gastrointestinal bleeding. Furthermore, CCI was identified as a significant predictor of mortality in this population of patients.

Declarations

Funding

This research did not receive external funding.

Author contributions

Conceptualization, F.İ. and T.D.; Methodology, H.K. and T.D.; Software, F.İ. and M.S.; Validation, T.D, H.K. and M.S.; Formal Analysis, T.D., H.K. and M.S.; Investigation, F.İ.; Resources, F.İ.; Data Curation, F.İ.; Writing – Original Draft Preparation, F.İ.and T.D.; Writing – Review & Editing, T.D., H.K. and M.S.; Visualization, T.D.; Supervision, T.D. and M.S.; Project Administration, T.D. and M.S.

Conflicts of interest

All authors declare that they have no conflicts of interest.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval

The ethics committee approval for the study was obtained from Hitit University Faculty of Medicine Clinical Research Ethics Committee (Date: 13.09.2023, No: 120).

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ORIGINAL PAPER

Optimizing rehabilitation strategies for elderly patients with femoral fracture – a prognostic perspective

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ABSTRACT

Introduction and aim. Femoral fractures in elderly people significantly impact their functional recovery and independence. Identifying reliable prognostic markers upon admission can facilitate optimal resource allocation and improve rehabilitation outcomes. This study aims to evaluate the predictive significance of pre-injury functional status, comorbid conditions, and cognitive function in determining independent living one year after injury.

Material and methods. This retrospective observational study involved 132 consecutive patients over the age of 60 years who had suffered femoral fractures and were admitted to a specialized geriatric orthopedic unit. Prior to hospitalization, all patients were capable of independent living. Upon admission, three key prognostic indicators were evaluated: (1) the level of pre-injury performance in activities of daily living (ADL); (2) the absence of comorbidities that could hinder recovery; and (3) cognitive function, measured by a Pfeiffer mental questionnaire score above 7. The relationship between these prognostic factors and the ability to live independently one year after injury was examined using odds ratios (OR) with 95% confidence intervals (CI).

Results. Among patients who possessed all three positive prognostic factors, 92% maintained independent living status after one year. In contrast, individuals with one, two, or three unfavorable predictors exhibited progressively lower rates of independent living, recorded at 96%, 82%, and 55%, respectively. The median duration of hospitalization within the first year varied across these groups, averaging 23, 74, 91, and 96 days, respectively. Furthermore, one-year mortality rates were found to be associated with the presence of comorbid conditions: 0% among those with only a femoral fracture, 14% in patients with one or two additional conditions, and 24% for those with three or more comorbidities.

Conclusion. Simple and robust admission predictors including pre-injury ADL performance, absence of significant comorbidities, and preserved cognitive function can effectively estimate long-term functional outcomes in patients with femoral fracture in elderly people. These findings support the optimization of rehabilitation resources to improve recovery and promote independent living.

Keywords. cognitive function, elderly rehabilitation, femoral fracture, independent living, prognostic factors

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Introduction

Femoral fractures are one of the common musculoskeletal injuries, especially in the elderly population. With the increase in life expectancy and the elderly population in various countries, the incidence of femoral fractures is also rising.[1,2] This injury can have a significant impact on the patient's quality of life, considering the importance of the femoral in supporting the body and aiding mobility.[3] Therefore, attention to the aspects of medical treatment, rehabilitation, and social support becomes increasingly important to ensure optimal recovery for the patient.[4,5]

In recent decades, various advances have been made in surgical procedures for femoral fractures, including internal fixation techniques, the use of more advanced implants, and joint replacement procedures for certain cases.[6,7] In addition, specialized rehabilitation programs have also been developed to help patients regain motor functions after surgery.[8] Social support, both from family and healthcare services, also plays an important role in patient recovery, especially in improving adherence to rehabilitation therapy and reducing the risk of postoperative complications.[9–11]

One of the crucial aspects in the management of femoral fractures is the assessment of prognosis from the moment the patient first enters the healthcare facility.[12] This assessment can help allocate resources more optimally, thus increasing the efficiency of the healthcare system.[13] Factors such as the patient's age, overall health condition, mental status, and ability to perform daily activities (Activities of Daily Living/ADL) before the injury can be important indicators to determine the most appropriate management strategy.[14–16]

Previous studies have identified various factors that can influence clinical outcomes in patients with femoral fractures, including patient characteristics and treatment approaches applied.[17] Analysis of these factors allows identification of key challenges in care and helps to formulate more effective health policies. However, there are still gaps in research on how the condition of patients before experiencing an injury can affect treatment outcomes and recovery. Therefore, this study focuses on evaluating the condition of patients before injury when they are first treated, to provide deeper insights into the factors that contribute to the final recovery outcomes of patients with femoral fractures.

Aim

This study aims to identify key prognostic factors that influence the likelihood of independent living one year after a femoral fracture in elderly patients. Specifically, it evaluates the impact of preinjury functional status, comorbid medical conditions, and cognitive function upon admission in predicting long-term recovery outcomes. By establishing reliable predictive markers, this research seeks to optimize rehabilitation resource allocation and improve post-fracture care strategies for older adults.

Material and methods

Study design

This study is a retrospective observational study that evaluates patients over 60 years of age who live independently and have been treated in a specialized orthopedic unit with 10 beds due to femoral fractures. This unit has a higher nurse-to-bed ratio compared to standard orthopedic wards (1.6 beds vs. 1.2 beds) to prevent patient isolation and enhance physical and mental activation. During the year 2023 to 2024, a total of 132 patients, consisting of 79 women and 53 men, were treated in this unit. Patients are treated until they show improvement or can be discharged home. All medications that are recognized to exert effects on the central nervous system must be avoided to the greatest extent possible. The patient is scheduled for surgery at the earliest opportunity; however, procedures during the night must be avoided. A sample size calculation was performed based on previous studies that assessed functional recovery in elderly patients with femoral fractures. Using a power of 80% and an effect size of 0.5, the required sample size was determined to be at least 120 participants, ensuring adequate statistical power to detect significant associations.

Inclusion criteria were: (1) age >60 years, (2) independent living before hospitalization, (3) no history of severe neurological or psychiatric disorders affecting cognition, and (4) ability to communicate and provide consent. Three prognostic factors were evaluated at admission: (1) preinjury activities of daily living (ADL) using the Katz Index, (2) absence of comorbid medical conditions that could impede recovery, and (3) cognitive function scores greater than 7 on the Short Portable Mental Status Questionnaire (SPMSQ).

Functional status was assessed using the Katz index of Independence in ADL. This index assesses six fundamental self-care tasks, including bathing, dressing, toileting, transferring, continence management, and eating. The scale categorizes individuals from A (completely independent) to G (entirely dependent), where a score of B or above signifies maintained functional independence. The Katz index has been widely validated, demonstrating strong inter-rater reliability and predictive validity in assessing functional decline in older adults. Cognitive function was evaluated using the Short Portable Mental Status Questionnaire (SPMSQ), a 10-item screening tool that assesses orientation, memory, and basic problem solving abilities. The total score ranges from 0 to 10, with 0–2 errors classified as normal cognition, 3–4 errors indicating mild cognitive impairment, 5–7 errors suggesting moderate cognitive impairment,

and 8 or more errors reflecting severe cognitive impairment. A score of >7 was used as the cutoff point, as previous research has shown that people with fewer errors tend to have better rehabilitation outcomes and greater independence after fracture.

Previous studies indicating that an ADL score of B or higher is associated with a significantly higher likelihood of independent living after rehabilitation, while an SPMSQ score of >7 has been associated with preserved cognitive function and better rehabilitation outcomes.[14,16,18] Medical conditions at the time of admission were classified according to whether additional health problems or injuries, apart from the fracture, which could complicate the rehabilitation process. These included conditions such as stroke, emphysema, uncontrolled diabetes, or fractures in other limbs.

Data collection

Data were collected from patient medical records, including general medical conditions, functional levels (ADL), and cognitive levels (SPMSQ). The Katz index and SPMSQ are standard assessment tools used in the orthopedic unit as part of routine admission procedures for elderly patients. These measures are incorporated into patient records to guide rehabilitation planning and predict long-term functional outcomes. Additionally, patients were advised to avoid medications known to affect the central nervous system (CNS) to the greatest extent feasible. This precaution was taken to minimize possible confounding effects on cognitive and functional evaluations, as CNS-active drugs, such as sedatives, antipsychotics, and certain analgesics, can alter cognitive function, alter alertness, and affect motor coordination. By limiting their use, cognitive function (SPMSQ) and activities of daily living (Katz index) more accurately reflected patients' baseline and recovery status rather than transient drug-induced effects. Furthermore, we recorded the functional status of the patients after discharge from the hospital, their living conditions, and whether the patients were readmitted to the hospital within one year after the injury.

Data analysis

The percentage of patients who maintained independent living at home one year after the fracture was analyzed in relation to different covariates using logistic regression. Covariates were gradually incorporated and the significance of each predictor was expressed as odds ratios with 95% confidence intervals. To identify factors that influence the total number of days of hospitalization within a year, an analysis of variance was performed. Before analysis, this variable underwent a logarithmic transformation to achieve a more normalized distribution.

Ethical approval

This study has received ethical approval from the Health Research Ethics Committee with number 368.6/II.3.AU/F/KEPK/II/2023. All respondents received complete information about the purpose and procedures, and their participation was voluntary. The researcher ensures the confidentiality of the respondents' personal data and that it is only used for the purposes of this study. Written consent was obtained from each respondent before data collection began.

Results

The study included 132 patients, consisting of 79 women (59.8%) and 53 men (40.2%). The mean age was 74.3 ± 6.8 years (range: 61–89 years). Most of the patients (68%) lived with family members, while 32% lived alone. The majority (81%) had at least one comorbid condition, with hypertension (47%) and diabetes mellitus (29%) being the most common. The educational levels varied, with 35% having only primary education, 40% completing secondary education, and 25% attaining higher education.

Table 1 shows the distribution of patients according to ADL levels, general medical conditions, and cognitive function. Three independent factors were identified that predicted the probability of independent living after one year (Table 2): an ADL level of at least B, the absence of comorbidities that could hinder rehabilitation, and an SPMSQ score greater than 7. When each predictor is simply categorized by its presence or absence, the predictive accuracy remains largely unaffected.

These three characteristics correspond to distinct subgroups with varying healthcare needs. Covariance analysis indicates that evaluating activities of daily living, overall health status, and cognitive function can help predict both the length of initial hospitalization and the total number of hospital days in the first year after the fracture. Table 3 illustrates the proportion of patients who continued to live in their own home one year post-fracture. Patients who passed away were categorized along with those who neither returned home upon discharge nor remained at home after one year. In particular, only 2% of the people who met all three positive criteria required long-term institutional care. To assess the predictive value of different factors, we examined variables such as age, sex, fracture type, social network size, frequency of social interactions (more than once a month), living alone, use of mobility aids, receipt of home care services, and the ability to perform essential daily activities like cooking, cleaning and shopping within a limited time frame. However, no other factors demonstrated stronger predictive power. Figure 1 illustrates the association between the number of favorable predictors and the total days inpatient during the first year following the fracture, showing both the median and percentile distributions.

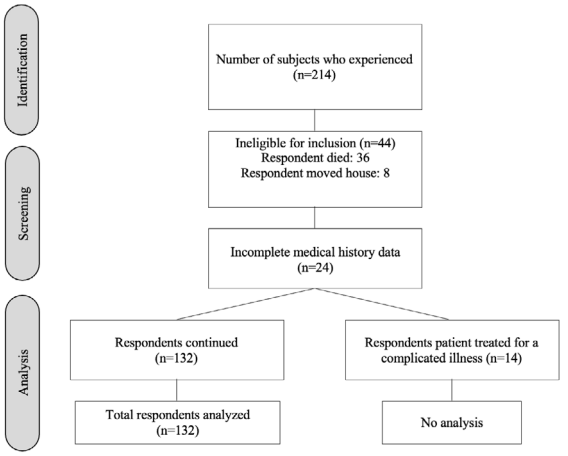

Fig. 1. Flow diagram for participant assignment in this study

Over the year, 30 individuals died, with ten fatalities occurring during the initial hospitalization. The predicted mortality upon admission was solely based on the number of preexisting medical conditions. Fractures alone (n=56) demonstrated a mortality rate of 0%. The death rate was 14% when one or two additional disorders were identified (n=125); however, with three or more additional diagnoses (n=51), it increased to 24% ($p<0.001$; chi-square analysis).

Table 1. Distribution of 132 patients according to pre-injury activities of daily living (ADL), general medical condition, and cognitive function[a]

	n (%)	Mean age (year; range)	Mean initial hospital stay (days, SD)	Mean hospital stay during the year (days, SD)	Discharge to own home (%)	At home after one year (%)
ADL function						
Good*	105 (79.5)	80.2 (60–84)	15 (12)	23 (34)	91	81
Dependent	37 (20.5)	84.1 (65–82)	20 (15)	73 (62)	52	33
Concomitant medical conditions**						
Yes	65 (49.2)	81.5 (60–84)	19 (15)	25 (27)	97	89
No	67 (50.8)	78.9 (60–82)	13 (8)	89 (112)	75	61
Cognitive function						
Lucid***	103 (78.1)	79.2 (60–82)	14 (9)	39 (52)	98	85
No lucid	29 (21.9)	81.6 (65–84)	21 (16)	92 (97)	52	43

[a] * – the patient was at least able to dress and undress independently (A or B on the Katz et al. (1963) graded scale),
** – impairing rehabilitation (Ceder et al. 1980),
*** – >7 points on the Pfeiffer (1975) ten-item questionnaire

This study explores the association between the number of favorable prognostic factors and the total number of hospitalization days among patients with femoral fractures, regardless of their specific diagnosis, within the first year after injury. The outlined sections represent the median values and encompass 50% of the data, corresponding to the interquartile range. The whiskers extend to the lower and upper limits of the box, adjusted by ±1.0 times the width of the box. Asterisks denote data points positioned beyond the box limits by ±1.5 times the width of the box, while circles indicate values exceeding the box limits by ±3.0 times the width of the box.

Table 2. Predictors for independent living one year after hip fracture

	Odd ratios	95% CI
ADL	3.5	1.3–9.1
General condition	2.9	1.3–6.1
Mental condition	2.4	1.9–4.9

Table 3. The outcome and use of health care resources during the year after fracture were related to the number of positive predictors

Positive predictors	n	Mean initial hospital stay (days, SD)	Mean hospital stay during the year (days, SD)	Discharge to own home (%)	At home after one year (%)
0	22	22 (15)	109 (96)	55	27
1	40	22 (17)	76 (91)	80	63
2	72	17 (11)	53 (74)	82	76
3	92	12 (7)	23 (23)	96	92

Discussion

Various indicators for prognosis after femoral fractures have been identified in research aimed at uncovering generally significant characteristics, including variables related to care. Research shows that overall health of the patient and the level of activity before fracture have a significant impact on the final outcome. According to Chow (2023), diagnosis, the number of activities of daily living (ADL), and the level of patient orientation were important factors for postoperative function. Many publications also identify age as a major predictor.[19]

In this study, separate inclusion of age did not improve the precision of the prediction for outcomes related to daily activities, overall health, and mental status.[20] This indicates that the three components used are sufficient to represent the biological age of the patients.[21] Surprisingly, the evaluation of ADL function and cognitive condition one week after surgery did not improve the prediction.[18,22] This reinforces findings in modern orthopedic care showing that show pre-fracture condition is a major factor in determining patient prognosis.[23]

Several studies have documented parameters related to mortality in the context of femoral fractures. Age and comorbidities are often identified as predictors of mortality, but several studies also show a relationship between mortality and walking ability.[24] Additionally, poor cognitive status has also been associated with an increased risk of mortality.[25,26]

Understanding these factors is crucial to optimal utilization of rehabilitation unit resources. Patients with

a very good prognosis do not require intensive care that is more needed by patients with a moderate prognosis.[27,28] In addition, understanding the predictive importance of these patient characteristics allows for more effective comparisons between different treatment modalities.[29] It is important to note that these findings currently lack sufficient precision to warrant the exclusion of patients with a poor prognosis from rehabilitation initiatives intended to enhance their functional recovery after fractures.

The three initial indicators associated with the need for hospitalization or institutional care after a femoral fracture do not show a significant correlation with one-year mortality.[30] This is consistent with the occurrence of other diagnoses related to fractures, and the management of these conditions imposes a significant strain on patients.[31,32] This pressure may become an unbearable burden when combined with other medical conditions, but it is often well tolerated by individuals who previously lived independently, regardless of their age.[33]

When the patient is admitted to the hospital, it is recommended to use the Katz ADL and Pfeiffer SPMSQ assessments together with standard medical history and physical examination documentation.[34] These assessment tools provide clear and reliable data that can be used to improve the allocation of available rehabilitation resources. Furthermore, this tool can also be beneficial in evaluating the effectiveness of various treatment methods, allowing a more structured and evidence-based approach in the treatment of patients with femoral fractures. The findings of this study emphasize the importance of early screening for functional and cognitive status prior to injury in elderly patients with femoral fractures. By integrating Katz's ADL and SPMSQ assessments into standard admission protocols, healthcare providers can improve rehabilitation planning and optimize resource allocation. These results advocate for structured rehabilitation strategies tailored to patient-specific prognostic factors, which may improve long-term independence outcomes. Although this study focuses on ADLs, comorbidities and cognition, other important factors such as social support, type of surgical intervention, and access to post-discharge rehabilitation have not been studied and can affect recovery. In addition, this study was conducted in a specialized orthopedic unit, which can limit generalization. Rehabilitation compliance was not systematically assessed, which could affect results. Future research should address these limitations by including a broader patient population and additional confounders.

Conclusion

This study emphasizes that the condition of the patient before experiencing a femoral fracture has a significant impact on the postoperative prognosis. Factors such as the level of activity before injury, overall health, and the patient's mental state have proven to be more relevant to determine rehabilitation outcomes compared to age individually. Initial evaluation using the Katz ADL scale and Pfeiffer SPMSQ provides accurate data and can help with care planning and more effective allocation of rehabilitation resources. Furthermore, although age and comorbidities are often associated with mortality, this study shows that walking ability and cognitive status are also factors that cannot be overlooked in predicting postoperative outcomes. The use of a more focused approach on patient characteristics allows for the optimization of more personalized care and rehabilitation.

Declarations

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Author contributions

Conceptualization, E.B.S., P.A.W.S. and E.S.; Methodology, E.B.S. and P.A.W.S.; Validation, P.A.W.S., E.S. and A.D.A.; Formal Analysis, P.A.W.S. and E.S.; Investigation, E.B.S.; Resources, E.B.S. and P.A.W.S.; Data Curation, P.A.W.S. and A.D.A.; Writing – Preparation of Original Draft, E.B.S. and P.A.W.S.; Writing – Review & Editing, P.A.W.S.; Visualization, P.A.W.S.; Supervision, E.B.S.

Conflicts of interest

All author declare have no conflict of interest.

Ethics approval

This study was approved by the local ethics committee (Health Research Ethics Committee in Universitas Muhammadiyah Gombong, date: February 11, 2023 decision number: 368.6/II.3.AU/F/KEPK/II/2023).

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ORIGINAL PAPER

Safety zone for surgical procedures for the prevention of neurovascular injury in minimally invasive total hip arthroplasty

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ABSTRACT

Introduction and aim. With the development of minimally invasive surgery (MIS), decreased incision size produces limited visualization leading to an increased risk of adjacent neurovascular structures during the procedure.
The aim was to identify the safest zones of surgical procedures for the prevention of major neurovascular structures around the acetabulum.

Material and methods. 84 cadaver pelvic specimens with 168 hips were used to analyze the anatomic relationship between the sciatic nerve and the piriformis muscle in the normal Korean population according to sex and height. We performed a qualitative and quantitative analysis of the anatomic relationship between the acetabulum and the sciatic nerve, based on the clockwise direction method, to identify the safety zones of the surgical procedure's proximity to the acetabulum.

Results. The prevalence of type I (normal type) was more than 79% and 88% in males and females, respectively. Distances A, B and E were 7.1±0.78 mm between the 3 and 4 o'clock position, 14.2±0.67 mm between the 8 and 9 o'clock position, 17.0±1.22 mm in the 9 o'clock position, respectively, on the left side, whereas 6.0±0.69 mm between the 8 and 9 o'clock position, 14.8±0.59mm in the 7 o'clock position, 17.9±1.08 mm in the 3 o'clock position on the right side. The proximity of the retractors to acetabulum should be placed with careful and proper retraction between the 3 and 5 o'clock position on the left side and between the 7 and 9 o'clock position on the right side, in which the placement in the 9 and 3 o'clock position should be cautious on the right and left side, respectively. The cautious use of electrocautery between the 3 and 5 o'clock position on the left side, and between the 7 and 9 o'clock position on the right side, is recommended. However, performing the electrocauterization in the 9 o'clock and 3 o'clock position respectively on the left and right side should be avoided. It is forbidden between the 10 and 12 on the left side and between 12 and 2 o'clock position on the right side. The absolute safety zones for the placement of the transacetabular screw were between 1 and 3, and between the position 5 and the 6 o'clock position on the left side and between the position 9 and 11, and between the 6 and 7 on the right side. Relative safe zones were between 12 and 1, and between the 6 and 7 o'clock position on the left and between the 11 and 12, and between the 5 and 6 o'clock positions on the right side. Screws with a length of <24mm could be safely inserted between the 3 and 5 o'clock position on the left side and between the 7 and 9 o'clock position on the right side. The risk zones were between 7 and 9, and between the 9 and 12 hours on the left side, and between 3 and 5, and between the 12 and 3 o'clock position on the right side. In cases with highly elevated trochanter major, the distances between the acetabulum and adjacent neurovascular structures changed and the risk of the SN increased.

Conclusion. These data could be helpful for arthroplasty surgeons in avoiding neurovascular injuries in MIS THA.

Keywords. femoral artery, minimally invasive total hip arthroplasty obturator nerve, piriformis muscle, sciatic nerve

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Introduction

Since the superiority of minimally invasive surgery over conventional surgery has been known as less damage and rapid functional recovery, it has been introduced to many orthopedic surgeries with the help of advanced surgical techniques and instruments.[1-6] Despite advancements in specialized instruments, limited incisions and a narrow surgical field can increase the risk of injury to adjacent neurovascular structures due to the potential for blind procedures, and the success of MIS-THA depends on arthroplasty surgeon's experience.[3,4,7]

The injury of the neurovascular structure close to the acetabulum is one of the major complications after hip surgery and total hip arthroplasty (THA).[7] The incidence of nerve injury in primary total hip arthroplasty ranges from 0 to 3 percent, in which the injury to the sciatic nerve (SN) is the most common.[8,9] Vascular injury during conventional THA is relatively rare (0.2-0.3%), but can result in considerable morbidity or even mortality.[10,11] It is reported that the incidence of neurovascular injury proximity to the acetabulum is higher in patients with hip dislocation and pelvic fracture, and superior gluteal neurovascular injury is relatively more common among them.[11-13]

There has been many literatures on the neurovascular injury around the acetabulum during surgery around the whole world.[12-16] Injury of the sciatic nerve injury is common in THA with a posterior approach, osteosynthesis of acetabular fracture and open reduction of hip dislocation, which could result in severe functional impairment of the hip and lower limb.[8,9] In THA, the most common identifiable cause of intraoperative SN injury is direct and indirect mechanical damage, including compression by malpositioned retractors, excessive/prolonged retraction, and inappropriate electrocauterization.[13] Furthermore, specific anatomic variations between the piriformis muscle (PM) and the SN have long been studied in cadaveric specimens, CT and MRI findings, all of which were focused on the prevention of the nerve injury during surgical procedure.[17-20] In early 1990s, Wasielewski et al. suggested the safe zone and depth of transacetabular screw fixation based on the quartering method of the acetabulum.[17] However, in MIS-THA with limited incision, the landmarks recommended by Wasielewski could not be identified due to small window and it has been an important issue especially for young arthroplasty surgeons with less experience. Aforementioned results have been limited to the sporadic identification of the anatomic relationship between the acetabulum and individual neurovascular structures including femoral artery (FA), SN and obturator nerve (ON), based on either quantitative or qualitative method, which did not include the practical mapping of safe zone for surgical procedure. There is a paucity of comprehensive literature that guides safe surgical procedures during THA.

Aim

Therefore, this study is designed to identify the anatomic relationship between the acetabulum and adjacent neurovascular structures and to determine the relative and absolute safety zone with the forbidden zone, quantitatively and qualitatively.

Material and methods

The anatomy of the acetabulum and adjacent neurovascular structures was examined in 168 hips derived from 84 formalin embalmed cadavers in our university's anatomy laboratory with an intact lower extremity for this study. Institutional Review/Ethics Board approval was obtained for the present study (2024-PRK-A10245, Ethics Assembly, Pyongyang University of Medical Sciences). The dissections were performed by the skilled anatomist (D.K.S.). Each cadaver was placed in a lateral position, and skin and the subcutaneous fat on the gluteus maximus were cut and completely removed to reveal the gluteus maximus. The gluteus maximus was dissected at its origin from the iliac crest. Its insertion in the iliotibial tract and the gluteal tuberosity was released to reflect the gluteus maximus inferiorly and medially. The gluteus medius was dissected from its insertion and laterally retracted to identify the course and the branches of the sciatic nerve. The connective tissue and fat were dissected to give a full demonstration of the anatomic course and branches of the SN in relation to PM. Cadavers with a history of surgery for hip osteoarthritis or intrapelvic viscera were excluded from this study, as surgery could have remarkably changed the relationship between the acetabulum and pelvic vessels remarkably. 48 male and 36 female cadavers, with an equal distribution of left and right lower extremities, were randomly selected from the available cadavers (Table 1). There was no significant difference in age between males and females ($p>0.05$).

Table 1. Characteristics of the cadaveric specimens

	Male (n=48)	Female (n=36)
Age (years)		
Mean±standard deviation	54.8±16.8	58.4±17.2
Min–Max	37–72	41–76
Height		
<155 cm	0 (0%)	9 (25%)
156–160 cm	5 (10.4%)	19 (52.8%)
161–165 cm	17 (35.4%)	7 (19.4%)
166–170 cm	19 (39.5%)	1 (2.8%)
>171 cm	7 (14.7%)	0 (0%)

Anatomical variations were analyzed according to the classification as follows:[18,19]

I – SN exits the pelvis undivided below the PM,

II – SN divides in the pelvis, the common peroneal nerve (CPN) passes through the bifid PM, and the tibial nerve(TN) lies below the PM,

III – SN divides in the pelvis, CPN courses over the PM, and TN lies below the PM,
IV – SN exits the pelvis undivided piercing the PM,
V – SN divides in the pelvis, CPN courses over the PM, and TN passes through the bifid PM,
VI – SN exits the pelvis undivided coursing over the PM,
VII – SN divides in the pelvis, both CPN and TN coursing separately below the PM.

For the identification of the anatomic relationship between the acetabulum and its adjacent neurovascular structures, such as SN, ON and FA, the intersection point (iliofemoral ligament attachment site) between the acetabular rim and the line connecting the anterior superior iliac spine and the anterior inferior iliac spine was used as 12 o'clock position to determine clockwise directions. Magnifying glass (×5) and the pin with ruler function were utilized to measure the distance measurement between the neurovascular structures and the acetabulum. The distance parameters used in the analysis of the anatomic relationship were as follows.
A – the shortest distance between the inferior margin and the inner surface of the acetabular floor at the site 24 mm below the greater sciatic foramen along SN,
B – the shortest distance between the inferior margin of the SN and the acetabular rim,
C – the shortest distance between the surface of the acetabular floor and the greater sciatic foramen,
D – the shortest distance between the FA and the acetabular rim,
E – the shortest distance between the ON and the acetabular rim,
X – the shortest distance between the SN and the trochanter major,
Y – the shortest distance between the SN and ischial tuberosity,
Z – the shortest distance between the SN and the sacral hiatus,
W – diameter of SN at the site 24 mm below the greater sciatic foramen along SN.

A pin with a ruler function was inserted vertically on the surface to measure the safe zone and depth of the transacetabular screw according to the clockwise direction method in the acetabulum of the cadaver. Intraoperative measurement was performed in 42 femoral neck nonunions with a highly elevated trochanter major, all who underwent THA with the posterolateral approach of the HU between November 2020 and June 2022. Post hoc power analysis was performed to assess the statistical power of the primary outcome measure. Analysis showed that with the current sample size of 42 patients, the study had adequate power (>80%) to detect clinically significant differences, with an effect size at a significance level of 0.05. There were 34 men (80.9%) and 8 women (19.1%) with a height of 169.3±13.2 cm and 158.8±13.2 cm, respectively, who had an elevated trochanter major with a mean height of 13.5±3.5 mm. Distances A, B, and X were measured during conventional primary THA according to clockwise direction in patients with femoral neck nonunion, based on which safe zone was determined. All distance values were presented as mean±standard deviation.

We retrospectively reviewed the operative note of 36 cases with transient sciatic nerve palsy after THA, based on the clockwise location and duration of retractor placement since we started the clinical measuring trial to determine the safe zone of surgical procedures during MIS-THA. All sciatic nerve palsy was recovered within 4 weeks. The duration of retractor placement was calculated as the time of acetabular preparation.

Results

Anatomic variations between SN and PM

In this study a total of 84 cadavers with 168 hips were examined. The normal type I variation in which the SN exits the pelvis as a single entity below the PM was the most common with a pooled prevalence of 83.3% followed by type II with 10.7%, type III with 2.4%, and type V with 1.8% (Table 2). The remaining types had a pooled prevalence of <1%. The abnormal type was observed in 20 hips (20.8%) of 12 male cadavers and in 8 hips (11.1%) of 6 female cadavers, demonstrating that its prevalence was lower in women than in men. Furthermore, abnormal type was observed in 18 (90%) and 7 (87.5%) left hips, respectively, in male and female cadavers, indicating that the left side was more prevalent (89.2% in total) in abnormal type. In male cadavers with height of >165cm, prevalence of abnormal type was 73.6% (56/76) and in female cadavers with >160cm it was 87.5% (7/8), indicating that the prevalence of abnormal type increased with height.

Table 2. Anatomical relationship between the sciatic nerve and the piriformis muscle*

Gender	n	Type I (cadavers/hips)	Abnormal type					
			Type II	Type III	Type IV	Type V	Type VI	Type VII
Male	48	40/76 (79.2%)	6/12 (12.5%)	2/3 (3.2%)	1/1 (1%)	1/2 (2.1%)	1/1 (1%)	1/1 (1%)
Female	36	34/64 (88.9%)	4/6 (8.3%)	1/1 (1.4%)	0/0 (0%)	1/1 (1.4%)	0/0 (0%)	0/0 (0%)
Overall	84	74/140 (83.3%)	10/18 (10.7%)	3/4 (2.4%)	1/1 (0.6%)	2/3 (1.8%)	1/1 (0.6%)	1/1 (0.6%)

* percentages in the parenthesis mean the proportion of individual variants among total number of hips

Anatomical relationship between the acetabulum and adjacent neurovascular structures (SN, ON, FA)

The acetabulum had anatomic relationship with sciatic nerve,, obturator nerve and femoral artery, as presented in Table 3. The other distance parameters measured in the female and male cadavers are shown in Table 4.

There was no significant difference in all distance parameters between the left and right sides. All parameters showed the tendency of increasing distances with height and being longer distances in males than in females.

Table 3. Anatomical relationship between the acetabulum and adjacent nerves (SN, ON)

Distance parameter	Distance			Position (clockwise direction)	
	Left (mm)	Right (mm)	p	Left	Right
A	7.1±0.78	7.0±0.69	0.86	3–4 o'clock	8–9 o'clock
B	14.2±0.67	14.8±0.59	0.81	5 o'clock	7 o'clock
E	17.0±1.22	17.9±1.08	0.78	9 o'clock	3 o'clock

Table 4. Anatomical relationship between the pelvis and the adjacent neurovascular structures (FA, SN)

Distance parameter	Left (mm)	Range (mm)	Right (mm)	Range (mm)	p
C	29.9±1.27	23.5–31.5	30±0.96	23.5–32	0.85
D	18.8±1.41	16–20.5	19.0±1.19	16.0–20.5	0.91
X	30.1±0.52	28–31	30.4±0.13	29.0–31	0.88
Y	18.1±0.99	16–19.5	18.2±0.87	16.0–19.5	0.94
Z	69.2±1.64	65–71	69.1±1.59	66.5–71	0.95
W	31.2±1.87	28.5–33.5	31.1±1.94	27.0–33	0.92

Electrocauterization safety zone and retractor placement in THA

On the basis of the aforementioned results, the safety zone was determined by clockwise method. When the iliofemoral ligament attachment site was determined as 12 o'clock position, the retractors should be careful to place the retractors between 3 and 5 o'clock position on the left side (L) and between 7 and 9 o'clock position on the right side (R) because there was SN and superior gluteal neurovascular bundle close to this position. Retractors should be placed carefully at the 9 o'clock position (L) and 3 o'clock position (R) to prevent the injury of FA and ON injury. If retractors are to be placed in this position, the use of electrocautery should be forbidden because of the potential injury owing to current conductivity. Electrocautery should not be used between 10 and 12 o'clock on the left side and between 12 and 2 o'clock position, because the femoral nerve (FN) passes close to this position.

Safety zone of screw fixation for acetabular cup in THA

Our recommended safety zone for screw fixation of the acetabular cup is summarized in Table 5. The absolute safety zone was between 1 and 3 (L) and between 9 and 11 o'clock on the right side (R) when the screw with length of >40 mm was inserted posterolaterally and superiorly. In the case of post-inferior screw insertion, the absolute safety zone was between 5 and 6 o'clock (L) and between 6 and 7 o'clock (R) while the safe length of screw was more than 35 mm. The relative safety zone was between 3 and 5 o'clock (L) and between 7 and 9 o'clock (R) when the screw with a length of <24 mm was inserted posterolaterally and superiorly. Based on safe depths, it was safe when the screw was inserted posterolaterally and superiorly between the 12 and 1 o'clock position (L) and between the 11 and 12 o'clock position (R), laterally and inferiorly between the 6 and 7 o'clock position (L) and between 5 and 6 o'clock position (R). Screw insertion was forbidden between the 7 and 9 o'clock position (L) and between the 3 and 5 o'clock position (R), to prevent the iliac and obturator to acetabular floor. It was also forbidden to insert the screw between 9 and 12 o'clock positions (L) and between 12 and 3 o'clock positions (R), due to the thin pelvic wall.

Table 5. Safety zone for transacetabular screw fixation

	Left	Right
Posterolateral and superior		
Relative safety zone	12–1 o'clock	11–12 o'clock
Absolute safety zone	1–3 o'clock	9–11 o'clock
Posterolateral and inferior(<24mm)		
Relative safety zone	3–5 o'clock	7–9 o'clock
Absolute safety zone	5–6 o'clock	6–7 o'clock
Forbidden zone	7–9 o'clock, 9–12 o'clock	12–3 o'clock, 3–5 o'clock

Anatomical relationship between SN and the acetabulum in femoral neck nonunion with a highly elevated trochanter major

The anatomical relationship was analyzed intraoperatively with regard to the anatomical relationship between SN and the acetabulum in 42 patients with THA with highly elevated trochanter major (Table 6). There were no significant differences in most distance parameters between cadavers and patients, however, a significant difference was observed in distance A, B, and X between them ($p<0.01$). Distance A was the shortest (>4 mm) between 2 and 3 o'clock positions (L) and between 9 and 10 o'clock positions (R). Distance B was the shortest (>9 mm) in the 4 and 8 o'clock position, respectively, on the left and right side. The distance between the SN and the trochanter major was more than 42mm. The three distance parameters were significantly longer than the cadaver results ($p<0.01$) and the direction of the shortest distance was different from the cadaver results.

Table 6. Anatomical relationship between the SN and the acetabulum

Distance parameter	Distance			Direction (clockwise method)	
	Left (mm)	Right (mm)	p	Left	Right
A	4.3±0.29	4.4±0.31	0.89	2–3 o'clock	9–10 o'clock
B	9.2±0.34	9.5±0.45	0.84	4 o'clock	8 o'clock
X	42.3±1.87	44.1±1.82	0.79	–	–

Relationship between the risk zone of retractor placement and sciatic nerve palsy

When the retractor was placed in the aforementioned risk zones during the acetabular preparation, the prevalence of transient SN palsy was 8.3%, 19.4%, 72.3%, respectively, when the retractor was placed for 25, 30, 35 minutes (Table 7). Most SN palsy occurred in the case with retractors placed in risk zones (63.9% vs 8.4%). The mean duration of retractor placement for transient SN palsy was 31.2±2.8 minutes.

Table 7. Sciatic nerve palsy in different risk zones of retractor placement with various duration of retraction*

Duration (min)	Left (n=19)			Right (n=17)			Overall
	1–3 o'clock	3–5 o'clock	5–7 o'clock	5–7 o'clock	7–9 o'clock	9–11 o'clock	
25	0	2 (5.5)	0	0	1 (2.8)	0	3 (8.3)
30	0	3 (8.3)	0	0	4 (11.1)	0	7 (19.4)
35	1 (2.8)	12 (33.3)	1(2.8)	1 (2.8)	11 (30.6)	0	26 (72.3)

* The numbers in parenthesis present the percentage of cases of SN palsy

Discussion

To reduce the intraoperative neurovascular injury during THA, many qualitative and quantitative analyses of the anatomic relationship around the hip have been performed with increasing attention to MIS-THA.[14,20,21] Especially, the anatomic relationship between SN and mini-incision posterolateral approach has been studied among several authors.[2,3,20-22] The sciatic nerve (SN), the largest nerve in the human body, is formed in the pelvis from the union of L4-S3 ventral nerve L4-S3 and it normally courses as a single trunk following its union and exits as the most lateral structure from the upper sciatic foramen below the piriform muscle (PM). Thereafter, it continues its course inferiorly and the tibial and common peroneal components of the SN typically bifurcate at the apex of the popliteal fossa.[23] Many studies have proved the existence of several anatomic variants of the relationship between SN and PM, by cadaver examination and imaging such as CT and MRI.[23,24] There are seven anatomic variants and type I, a normal type is a most common with the prevalence of 80 to 90% followed by type II with 10 to 15%, type III with 1 to 3% and type IVVI with 1% or less in cadaveric studies.[23,25,26] In addition, there is a specific type (type VII) that shows unusual anatomic split into the tibial nerve and peroneal nerve passing separately below the PM.[27] Our results suggested that type I had a prevalence of 83.3% and abnormal type had a pooled prevalence of 16.7% among the Korean population, which was comparable with the previous studies.[21-27] The prevalence of the abnormal type showed the tendency of being higher in male than in female and higher with height.

We evaluated the anatomic relationship between SN and the acetabulum, qualitatively with clockwise direction method and quantitatively with several distance parameters. The SN was located at the site about 6mm far away from the inner surface of the acetabular floor, between 3 and 4 o'clock position (L) and between 8 and 9 o'clock position (R). The mean value of the shortest distance between the SN and the acetabular rim was greater than 13mm at 5 (L) and 7 (R) o'clock position. The ON exits from the obturator foramen to pass anteroinferiorly above the transverse acetabular ligament, while the mean value of the shortest distance from the ON to the acetabular rim was approximately 16 mm at 9 (L) and 3(R) o'clock position. The shortest distance between ON and the acetabular rim was measured at 13 mm in a hip of a female cadaver, indicating that electrocauterization of the ligament of femoral head could cause sudden and unexpected internal rotation of patient's hip in THA with anterolateral approach due to current conductivity by the retractor placed in this position. Therefore, we recommend that the retractors should be placed carefully around this area, and the utilization of electrocautery on the acetabular rim should be limited to a defined distance. This result presented the difference from the other previous results because they use the MRI and CT slices to measure the distance between the anatomic structures; however, they showed the same tendency in measured values.[8,19]

The femoral artery (FA) begins at the common iliac artery which continues inferiorly into the femoral artery at the level of the inguinal ligament, giving deep branches around the acetabulum.[20] The deep femoral artery bifurcates into the lateral circumflex artery at the level of the obturator foramen, passing anteriorly to the femoral neck with the mean value of the shortest distance of 18 mm to the acetabulum. With its running parallel to the ON, FA was very close to the acetabulum in 9 (L) and 3(R) o'clock position. In 2 hips of 2 female cadavers, the shortest distance was 16mm, indicating that electrocauterization in the area less than 16mm away from the acetabular rim might induce ON injury and abnormal contraction of muscle innervated by ON.

The shortest distance between the SN and bony structures, including ischial tuberosity, trochanter major, and sacral hiatus were over 17 mm, 29 mm and 67 mm, respectively. The thickness of the SN was measured as approximately 31 mm in the lateral position which was different from the previous results due to the different preparation and measuring posture. Several authors measured the sciatic nerve by width and thickness, which could be varied with different embalming methods.[22] However, these distance parameters could be important in determining the site for safe placement of retractors in the trochanter major and around the acetabulum.[8]

From the aforementioned results, we determined the safe zone of electrocauterization and retractor placement by identifying the risk zone. Proper retraction should be

applied to the retractor placed between 3 and 5 o'clock position (L) and between 7 and 9 o'clock position (R) because the SN and the superior gluteal neurovascular bundle pass through this region. Prolonged and excessive retraction could cause nerve palsy in this region by compression-induced ischemia.[28] This suggests that if electrocauterization was applied near the retractor placed in this direction, current conduction might cause the indirect SN injury. Moderate retraction at 9 (L) and 3 (R) o'clock position could reduce the injury of FA and ON injury; however, excessive procedures could cause deep venous thrombosis and obturator nerve syndrome. The use of the electrocautery was forbidden between 10 and 12 o'clock position (L) and between 12 and 2 o'clock position (R) because the FN lies in this direction, which coincided with previous results.[19,28]

The mean value of the shortest distance between the greater sciatic foramen and the surface of the acetabular floor, reflecting the thickness of acetabular wall, was approximately 29 mm, which was larger in men than in female and with height. In a female cadaver with height of 142 cm, the shortest distance was 24 mm on the left side, demonstrating that the fixation screw with length of <24 mm could be absolutely safe in the acetabulum. Wasielewski et al. introduced the quartering method of the acetabulum for the determination of the safe zone of transacetabular screw fixation.[17] Our results suggested that screw fixation should be prohibited between 3 and 5 o'clock position (L) and between 7 and 9 o'clock position (R) due to the short distance to the SN. The absolute safety zone for screw fixation was between 1 and 3 o'clock position (L) and between 9 and 11 o'clock position (R) when a screw with the length of 40mm was inserted posterolaterally and superiorly, which was similar to the results (35 mm). The relative safety zone was between the 5 and the 6 o'clock position (L) and 6 and 7 o'clock position (R) when a screw with the length of <35 mm was inserted posteroinferiorly, comparable to the previous results.[17] The safe fixation of the screw with length of <24 mm was possible posterolaterally between the position 3 and 5 0'clock and between 7 and 9 o'clock position which was agreed with the fact that the shortest distance between greater sciatic foramen and the surface of the acetabular floor was 29 mm. Wasielewski et al. suggested that a screw with length of >25 mm could be fixed safely in this direction but this difference might come from the difference in skeletal phenotype between the European and Asian population.[17] In addition, posterolateral screw fixation was relatively safe between the position of 12 and 1 o'clock (L) and 11 and 12 o'clock position (R), respectively, superiorly and inferiorly. It might be explained by the fact that the safe direction of screw fixation was superior and inferiorly in posterolateral direction. An author reported that screw fixation should be avoided in this direction because this area had a thin wall (<25 mm), indicating the conflict with our results.[20] This conflict could come from variation in study subjects. Screw fixation was forbidden between the 7 and 9 o'clock position (L) and the 3 and 5 o'clock position (R) while it was avoided between the 9 and 12 o'clock position (L) and between the 12 and 3 o'clock position (R) because of thin wall and femoral neurovascular bundle close to it. Previous studies suggested the safety zone for transacetabular screw by mapping, but it was too complicated to understand and clinical application was limited.[17,20] In contrast, our recommended clockwise direction carries simplicity and practicality.

To identify the altered anatomic relationship between SN and adjacent structures, we measured three distance parameters during primary THA in patients with femoral neck nonunion. Our results demonstrated that among the distances from the SN to the inner surface of the acetabular floor, the acetabular rim and the trochanter major, two former were shorter and later was longer than the cadaveric results. The altered direction indicates that the surgeon should extrapolate the safe zone based on cadaveric results and care should be taken during surgical procedures such as retractor placement and electrocauterization.

For validation of the significance of the risk zones, we retrospectively reviewed a operative notes with the focus on the location and duration of retractors around the acetabulum during the acetabular preparation in THA. Our results suggest that SN was more vulnerable in the prolonged placement of the retractors in risk zones for more than 30 minutes, proving that our recommended zone for the safe procedure was practically significant.

However, the present study has certain limitations. The sample size was small and there may be a variation in the measurement of the cadaver specimens owing to the embalming process. Since cadaver samples are from a single institution, generalizability to other ethnic populations is limited. Despite this limitation, this study provides new information on the identification of safe zone of surgical procedures based on the qualitative and quantitative analysis of many anatomic parameters, which could contribute to the improvement of complications after MIS-THA with limited visualization, especially among unexperienced arthroplasty surgeons.

Conclusion

This study provides useful information to perform surgical procedures without causing neurovascular injury during MIS-THA, based on a comprehensive anatomic analysis of the acetabulum and the adjacent neurovascular structures.

Declarations

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Author contributions

Conceptualization, P.H-U. and R.K-C.; Methodology, P.H-U. and R.K-C.; Software, R.W-J.; Validation, P.H-U. and R.K-C.; Formal Analysis, C.T-H.; Data Curation, C.T-H.; Writing – Original Draft Preparation, P.H-U., P.C-J., C.T-H., R.W-J. and R.K-C.; Writing – Review & Editing, P.H-U.; Visualization, P.H-U. and R.K-C.; Supervision, P.C-J. and R.K-C.; Project Administration, P.H-U.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

Data available within the article or its supplementary materials.

Ethics approval

Institutional Review/Ethics Board approval was obtained for the present study (2024-PRK-A10245, Ethics Assembly, Pyongyang University of Medical Sciences).

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ORIGINAL PAPER

The detection of internal tandem duplication in the FMS-like tyrosine kinase 3 gene in a sample of Iraqi patients with acute myeloid leukemia

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ABSTRACT

Introduction and aim. Acute myeloid leukemia (AML) is a genetically heterogeneous malignancy significantly influenced by FMS-like tyrosine kinase 3 mutations. This study investigated the prevalence and clinical implications of internal tandem duplication (ITD) mutations in *FLT3* in patients with AML in Iraq.

Material and methods. The study involved blood samples collected from 50 newly diagnosed AML and 50 healthy subjects between April 2023 and January 2024 and used for conventional PCR. PCR products positive for the ITD mutation were subjected to fragment analysis using capillary electrophoresis (CF) to confirm ITD mutations.

Results. *FLT3-ITD* mutations were identified in 20% of patients with AML (10/50), with striking female predominance (90% of mutation-positive cases). Based on the FAB classification, M5 was the most prevalent FAB subtype among mutation carriers (40%), with no mutations detected in M7.

Conclusion. This study provides critical information on the prevalence and clinical implications of *FLT3-ITD* mutations in patients with AML in Iraqi, emphasizing the need for precise molecular diagnostic approaches. This finding revealed a distinct subset of patients with AML that harbor *FLT3-ITD* mutations, with a significant proportion exhibiting elevated allelic ratios, which reinforces their prognostic relevance.

Keywords. acute myeloid leukemia, capillary electrophoresis, FAB, *FLT3-ITD* mutation, sex disparity

Introduction

Acute myeloid leukemia (AML) is a hematologic tumor characterized by unrestricted proliferation of clonal myeloid progenitors in the bone marrow.[1,2] This arises from the accumulation of sequential mutations in hematopoietic stem cells, leading to impaired differentiation and excessive blast proliferation.[3] AML is the most common type of acute leukemia in adults and affects individuals of all ages.[4,5] In Iraq, leukemia is among the top ten malignancies in both sexes, accounting for 5.34% of all cancers (4.22 per 100,000 people) according to the Iraqi Cancer Registry.[6] The etiology of AML remains largely unknown, although various risk factors such as ionizing radiation, benzene exposure, smoking and prior cytotoxic chemotherapy have been implicated in its pathogenesis.[7] Fms-like tyrosine kinase 3 (*FLT3*) mutations are among the most frequent mutations, occurring in approximately 30% of cases. *FLT3* is a transmembrane receptor crucial for hematopoietic cell differentiation, survival, and proliferation through multiple signal transduction pathways.[8] The two primary *FLT3* mutations associated with AML are internal tandem dupli-

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cations (ITD) in the juxtamembrane domain (25%) and point mutations in the tyrosine kinase domain (TKD, 7–10%).[9,10] *FLT3-ITD* mutations lead to constitutive activation of downstream signaling pathways such as STAT5, RAS-MEK-MAPK, and PI3K-AKT, driving uncontrolled cell proliferation and inhibiting differentiation.[10] These mutations are associated with poor overall survival (OS) and high relapse rates following intensive therapy.[9]

Factors such as *ITD* length, allelic ratio, ITD site within *FLT3*, cytogenetic aberrations and genetic mutations, and overall patient variation in these areas suggest that *FLT3*-mutated AML is a highly ethnically heterogeneous disease with far-reaching prognostic implications.[11] Importantly, the allelic ratio of *FLT3-ITD* mutations has been identified as a critical prognostic factor influencing risk stratification and treatment decisions, particularly regarding the need for hematopoietic stem cell transplantation (HSCT).[12] Despite the well-established importance of *FLT3-ITD* mutations in the prognosis in Iraqi patients. Ethnic and genetic variabilities may influence the prevalence of mutation and its prognostic implications. Furthermore, standard diagnostic techniques such as gel electrophoresis may have limitations in detecting *FLT3-ITD* mutations and accurately determining the allelic ratio, which is essential for risk stratification. Fragment analysis offers greater sensitivity and accuracy in identifying *FLT3-ITD* mutations, allowing for a more precise assessment of their clinical impact.

This study aimed to evaluate the distribution of *FLT3-ITD* mutations in newly diagnosed Iraqi patients with AML and their association with sex and the French-American-British (FAB) subtype of AML. Furthermore, this study aimed to compare gel electrophoresis with fragment analysis to evaluate its effectiveness in *FLT3-ITD* detection and determine the allelic ratio.

Material and methods

The study included 50 patients (25 men and 25 women) with a new diagnosis of acute myeloid leukemia (AML) and 50 healthy controls matched for age and sex. Between April 2023 and January 2024, patients with AML were recruited from the Hematology Center at Medical City in Baghdad. All study participants provided their written informed consent and the study was approved by the institutional review board of the Hematology Center (approval number # 18982, 22 May 2023).

Control group subjects were randomly selected, ensuring gender distribution CLMSPAY distribution. The majority of controls were from the general population, and a small proportion were recruited from healthcare staff. Other exclusion criteria for AML patients were previous treatment (chemotherapy/radiotherapy) or a diagnosis of secondary AML. Controls were excluded if they had a history of blood disorders, malignancy, ongoing infections, use of immunosuppressive agents, autoimmune diseases or blood tests with abnormal results.

Blood samples (3 mL) from AML patients and controls were drawn into EDTA coated tubes and stored at -20°C for one week before processing. Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. DNA quality and concentration were determined using NanoDrop spectrophometry. Flow cytometry data was used to classify subclasses according to French-American-British (FAB) criteria from a review of clinical records of all patients with AML diagnosed at UTMB until October 2023.

FLT3-ITD detection

Exons 14–15 of *FLT3* were amplified using conventional PCR. The following primer sequences were used.

- Forward primer: 5'-GCAATTTAGGTATGAAAGCCAGC-3'
- Reverse primer: 5'-CTTTCAGCATTTTGACGGCAACC-3'.[13]

The initial PCR was performed in a 25 µL volume consisting of 12 µL of green master mix (Promega, Madison, USA), 6 µL of nuclease-free water, 1 µL of the forward primer, 1 µL of the reverse primer, and 5 µL of genomic DNA (25 ng). The PCR cycling conditions were as follows: an initial denaturation step at 94 °C for 3 minutes; 35 cycles of 94 °C for 30 seconds (denaturation), 56 °C for 1 minute (annealing), and 72 °C for 2 minutes (extension); followed by a final extension at 72 °C for 7 minutes. The PCR products were resolved on 3% agarose gel. The wild-type *FLT3* allele produced a 329 bp fragment, whereas any fragment larger than 329 bp was classified as a mutant (*FLT3-ITD* positive). Fragment analysis was performed on *FLT3-ITD* positive samples using a SeqStudio Genetic Analyzer (Applied Biosystems, MA, USA) with the LeukoStrat® *FLT3* Mutation Assay 2.0 (Invivoscribe, San Diego, CA, USA), according to the manufacturer's instructions. Genomic DNA from *FLT3-ITD* positive cases (as determined by gel electrophoresis) was re-amplified in a 50 µL PCR reaction containing 45 µL of *FLT3 ITD* master mix, 0.25 µl of Taq DNA polymerase (Promega, Madison, USA), and 5 µL of gDNA (5 ng). The PCR cycling conditions for this step were: initial denaturation: 95°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min; and final extension: 72°C for 60 min. For the fragment analysis, PCR products were mixed in a 96-well PCR plate with 9 µL of Hi-Di formamide (Applied Biosystems, CA, USA), 1 µL of GeneScan™ 600 LIZ ™ Size Standard v2.0 (Thermo Fisher Scientific, MA, USA), a fluorescently labeled marker for accurate sizing, and 0.5 µL of each amplicon. The sam-

ples were denatured at 95°C for 3 minutes, then chilled on ice for 5 min. The plate was subsequently loaded into the SeqStudio Genetic Analyzer, and the run was initiated according to the manufacturer's instructions. In this assay, any electrophoretic peak equal to or greater than 330 bp was considered positive for the *FLT3-ITD* mutations. Gene Marker™ Software v 3.0.1 (SoftGenetics, PA, USA) was used to evaluate fragment profiles by quantifying the size, quantity, and area under the curve (AUC) of each peak. The allelic ratio (AR) was determined by dividing the AUC of the mutant allele by that of the wild-type allele. In cases where multiple mutant alleles were detected, their AUCs were summed. The difference in the total number of base pairs between the mutant and wild-type alleles was used to determine the mutation size.

All positive samples were analyzed in duplicate to confirm the results, and negative control was included in each run to ensure the validity of the assay.

Statistical analysis

The Statistical Analysis System software (SAS Institute, Cary, NC, USA), version 9.4 was used for data analyses. For continuous variables, comparisons were performed by using Student t-test or the least significance difference (LSD) method. For categorical variables, the chi-square test was used. Pearson correlation coefficients were computed to assess relationships between variables. Statistical significance was determined by a probability value (p) less than 0.05.

Results

This study included 50 newly diagnosed AML patients and 50 healthy subjects. The AML patients had a median age of 43.5 years (range: 15–80 years), with a mean age of 43.06 years. In the control group, age ranged from 16 to 67 years, with a median age of 44 years and a mean age of 42.02 years. There were no significant differences in age between groups. *FLT3-ITD* mutations were detected in 10 of 50 AML patients (20%), while none of the healthy controls exhibited these mutations. In *FLT3-ITD*–positive patients, mutant clones were identified by the presence of one or two additional fragments, along with the wild-type fragment. The duplication region varied in size from 33 to 73 bp, as determined by agarose gel electrophoresis. Among the 10 patients with the ITD mutation, 9 (90%) had a single ITD (one mutated clone), and 1 (10%) had double ITDs (two mutated clones) and lane 4 (~380 bp and ~400 bp). None of the patients had more than two ITD mutations. In one patient (lane 9), there was very weak or absent amplification of the wild-type *FLT3* allele on agarose gel, with only a single band (~400 bp) corresponding to the mutant allele, as illustrated in Figure 1.

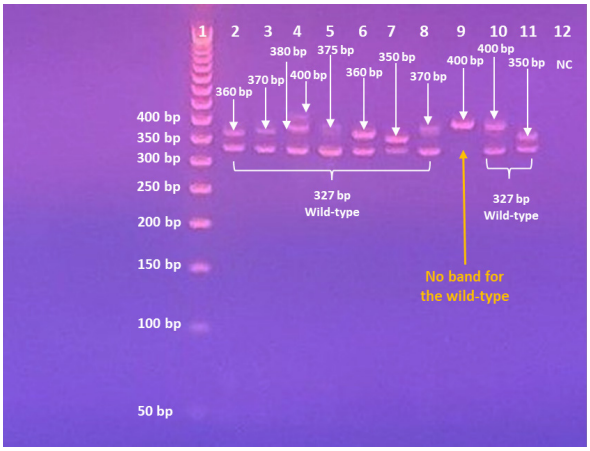

Fig. 1. Image of a 3% agarose gel stained with ethidium bromide displaying mutations in *FLT3-ITD*. Lane 12 served as a negative control. Lane 1 is a 50 bp DNA ladder; lanes 2–11 show patient samples that were positive for the *FLT3*-ITD mutation

Table 1. The nature of ITD in the *FLT 3* gene in Individuals with AML according to CE

	Size (bp)		
	Wild-type	Mutant -type	Inserted nucleotides
1	327	363	36
2	327	387	60
3	327	381	54
		408	81
4	327	390	63
5	327	363	36
6	327	353	26
7	327	378	51
8	327	391	64
9	327	390	63
10	327	347	20

Table 2. Comparison of the ITDs sizes in both agarose gel electrophoresis and CE methods

	ITD size via capillary electrophoresis (bp)	ITD size via agarose gel electrophoresis (bp)
1	363	~ 360
2	387	~ 370
3	381	~ 380
	408	~ 400
4	390	~ 375
5	363	~ 360
6	353	~ 350
7	378	~ 370
8	391	~ 400
9	390	~ 400
10	347	~ 350

Fragment analysis of *FLT3-ITD* revealed 11 distinct types, with one patient exhibiting double ITDs. The median ITD size was 50.3 bp, with sizes ranging from 21 bp to 81 bp, as determined by capillary electrophoresis (CE) (Table 1).

When comparing the allele sizes obtained via agarose and CE, the ITD sizes were nearly identical (Table 2).

Regarding the patient in lane 9 (Figure 1), no wild-type allele band was detected by gel electrophoresis in lane 9 (Figure 1); however, CE revealed the presence of both wild-type and mutant alleles (Figure 2). For *FLT3-ITD* positive patients, the mean age was 44.7 years, with ages ranging from 23 to 74 years and a median age of 44 years. There were no significant differences in age distribution among patients with these mutations.

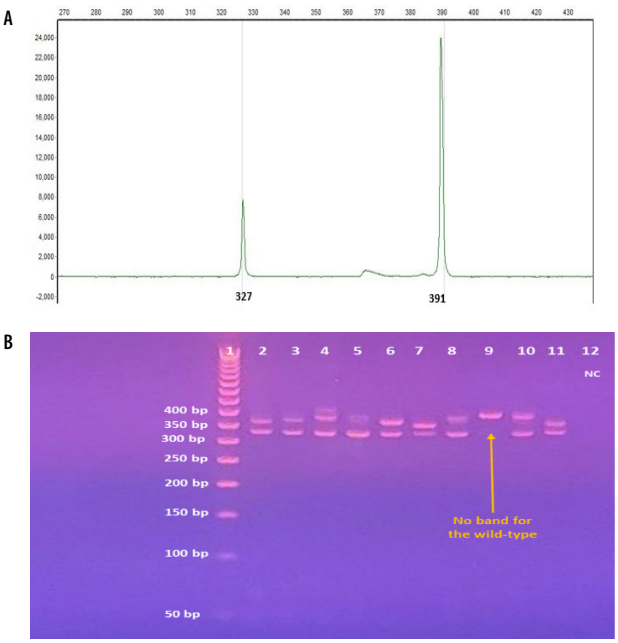

Fig. 2. A: Capillary electrophoresis for case number 8, B: Gel electrophoresis for case number 8 (lane 9)

AML patients without *FLT3-ITD* mutations were predominantly male. Conversely, in the group with *FLT3-ITD* mutations, females were predominant, with mutations detected in nine females (90%) and one male (10%), as shown in Table 3. The odds ratio (OR) was calculated to assess the association between sex and *FLT3* gene mutation status in AML patients. An OR greater than 1 indicates higher odds of the event (mutation) occurring in females compared to males, while an OR less than 1 suggests lower odds. In this study, the OR for the mutant *FLT3* type was 1.372, indicating that females had higher odds of carrying the FLT3 mutation compared to males. The associated p-value (p=0.01) suggests that this difference is statistically significant. Conversely, the OR for the wild-type *FLT3* gene was 0.317, indicating lower odds of females having the wild-type allele compared to males, although this was not statistically significant (p=0.206). These findings suggest a potential sex-related difference in the distribution of *FLT3* mutations among AML patients.

In the distribution of AML patients with and without the *FLT3-ITD* mutation across FAB subtypes, neither group included individuals with the M1 subtype and no *FLT3-ITD* mutations were observed in the M7 subtype. Among patients with wild-type *FLT3-ITD* (n=40), the most prevalent subtype was M5, accounting for 17 cases (42.5%), followed by M4 in 9 patients (22.5%). In contrast, in the *FLT3-ITD*–positive cohort (n=10), M5 remained the most common subtype (four patients, 40%), followed by M0 (two patients, 20%) (Table 4). The OR was calculated to assess the association between FLT3 mutation status and the different FAB subtypes of AML. The OR analysis revealed a significant association between *FLT3* mutations and FAB subtypes M5 and M4, indicating higher mutation frequency in these groups. In contrast, subtypes M2 and M3 showed lower, non-significant odds. OR values could not be determined for some subtypes (M0, M1, M6) due to limited sample size.

Table 3. Distribution of sample study according to ITD mutation in the *FLT 3* gene and sex

FLT 3 gene	Individuals with AML				
	Male	Female	χ2	Odds ratio (95% CI)	p
Wild Type	24 (96%)	16 (64%)	1.60	0.317 (0.22–0.78)	0.206
Mutant Type	1 (4%)	9 (36%)	6.40	1.372 (0.84–1.75)	0.01
Total	25 (100%)	25 (100%)	–	–	–

Table 4. Relationship between ITD mutation in the *FLT 3* gene and FAB classification

FAB n (100%)	Wild type	Mutant type	χ2	Odds ratio (95% CI)	p
M0	2 (5%)	2 (20 %)	0.00	–	>0.999
M1	0 (0%)	0 (0%)	0.00	–	<0.001
M2	3 (7.5%)	1 (10%)	1.00	0.294 (0.17–0.63)	0.317
M3	4 (10 (%)	1 (10%)	1.80	0.387 (0.22–0.93)	0.179
M4	9 (22.5%)	1 (10%)	6.40	1.372 (0.84–1.75)	0.01
M5	17 (42.5%)	4 (40%)	8.047	1.502 (0.92–2.26)	0.0046
M6	1 (2.5%)	1 (10%)	0.00	–	>0.999
M7	4 (10%)	0 (0%)	3.72	0.86 (0.47–1.59)	0.0419
Total	40 (100%)	10 (100%)	–	–	–

Table 5. Allelic ratio (AR) for the *FLT 3* – ITD mutations

FAB		Capillary band area		AR (%)
		Wild-type	Mutant-type	
1	M5	232121	108325	0.47
2	M2	82303	21559	0.26
3	M4	265237	100480	0.58
			54190	
4	M3	67911	3750	0.05
5	M5	171245	1128722	0.75
6	M5	190602	181357	0.95
7	M6	37533	12512	0.33
8	M0	52943	222877	4.2
9	M0	248057	162024	0.65
10	M5	11599	4123	0.36

Table 5 presents the allelic ratio (AR) for each patient with ITD mutation. The distribution was balanced, with 50% of the patients exhibiting a high AR and 50%

exhibiting a low AR. For patients with two mutant alleles, the band areas for each allele were summed.

Regarding the correlation of the hematological parameters between the patients harboring *FLT3*-ITD and other individuals with AML without mutation (wild-type) who were enrolled in this study, the mean counts of white blood cells and peripheral blood blast cell percentages in patients harboring the mutations of *FLT3*-ITD were insignificant compared to other patients without mutations (Table 6).

Table 6. Relationship between the *FLT 3* gene ITD mutation and hematological parameters

FLT 3 gene ITD mutation	Mean±SE	
	WBC count $\times 10^9$/L	Peripheral blood blast cell
Wild type	29.09±2.05	49.72±4.19
Mutant type	30.78±2.81	49.37±3.62
p	0.873	0.902

Discussion

FLT3 is a proto-oncogene that plays a critical role in hematopoietic cell survival, differentiation, and proliferation.[14] ITD mutations in *FLT3* occur in approximately one-third of patients with AML and are frequently associated with poor prognosis and diminished responses to chemotherapy.[15] In this study, the investigated the prevalence and characteristics of *FLT3-ITD* mutations in a cohort of 50 patients with AML in Iraq, using both gel electrophoresis and CE for mutation detection. Despite the clinical significance of these mutations, research on *FLT3* in Iraq is limited. no prior regional study has employed highly sensitive fragment analysis to detect *FLT3-ITD* mutations.

The median age of AML patients in this study was 43.5 years (range: 15–80 years), which aligns with the results of previous Iraqi studies. For example, Latif et al. reported a median age of 47 years (range: 14–86 years)[16], whereas Al-Shammari et al. found a median age of 46 years (range: 15–85 years).[17]

FLT3-ITD mutations were detected in 10 of 50 patients (20%), a prevalence consistent with regional reports (21.88% in one study, 20.15% in a Saudi Arabian study, and 20.4% in another).[18,19] However, lower frequencies have been reported, such as 16%, 17.4%, and 14.54%.[20–22] These discrepancies may be attributed to differences in the sample source (peripheral blood vs. bone marrow), nucleic acid type (DNA vs. RNA), and the assay used for amplification and detection.[13]

Since the detection of the *FLT3*-ITD mutation can facilitate the diagnosis, prognosis, and treatment of patients with AML, a pivotal component of current research is the comparative analysis of *FLT3-ITD* mutation detection using traditional gel electrophoresis versus CE.[23] While gel electrophoresis is widely utilized owing to its accessibility and cost-effectiveness, it relies on the visual interpretation of bands and is prone to errors, particularly when bands are faint or when the tumor cell content in a sample is low. Unlike traditional agarose gel electrophoresis, CE provides precise measurement of amplified DNA fragment sizes while eliminating distortions like band shifting and inconsistencies between different gels. Additionally, CE superior resolution enables distinguishing PCR products differing by as little as a single base pair. These advancements enhance analytical accuracy, proving particularly valuable for detecting tandemly duplicated regions, which may be nearly identical in length to non-duplicated (wild-type) alleles.[24]

The findings highlight these limitations. One patient was initially classified as homozygous (*FLT3-ITD/ITD*) by gel electrophoresis because of the apparent absence of a wild-type band, which was later correctly identified by CE as heterozygous (*FLT3-ITD*/WT), displaying two distinct peaks, one for the mutant allele and another for the wild-type allele.

It is crucial to differentiate between homozygous and heterozygous *FLT3-ITD* mutations because homozygosity is linked to a more aggressive progression of the disease, higher relapse rates, and worse prognosis than heterozygous or wild-type cases.[25] The *FLT3-ITD* allelic burden plays a pivotal role in disease outcomes, with patients lacking or having low levels of the wild-type allele exhibiting particularly poor survival rates.[26]

AML treatment achieves complete remission in 60–80% of patients with induction therapy, Over recent decades, advancements in targeted therapies and supportive care have significantly improved the 5-year survival rate for de novo AML, increasing from 13% to 55%.[27,28] Therefore, a highly accurate *FLT3-ITD* detection method is required for optimal risk stratification and treatment planning of AML.

When conducting gel electrophoresis, careful analysis of FLT3-ITD testing outcomes is crucial. This technique requires visual examination of band patterns to draw conclusions, and since human judgment is involved, there remains an inherent risk of inaccuracies in result interpretation. In present study, the wild-type band was not observed on gel electrophoresis but was clearly detected using CE. When the gel electrophoresis band is very faint, it is difficult to determine by visual inspection whether it represents a typical band for *FLT3*-ITD. CE provides higher resolution and consistent measurement of amplicon sizes, enabling the detection of subtle differences between mutant and wild-type alleles.[29] Therefore, it is essential to conduct fragment analysis during screening for *FLT3-ITD* to ensure accurate mutation detection and minimize misclassification. This issue is particularly pertinent in the Iraqi clinical context, where many laboratories continue to rely on PCR followed by gel electrophoresis for *FLT3-ITD* detection, primarily because of financial and logistical constraints. A study involving

25 Iraqi patients with acute lymphoblastic leukemia used conventional PCR techniques for *FLT3-ITD* detection, identifying mutations in 8% of cases. The study recommended the incorporation of advanced and potentially more sensitive methods, such as CE, to improve diagnostic accuracy.[30] Additionally, a comparative study found that CE detected *FLT3-ITD* mutations at lower allele ratios, reducing false negatives.[24] Given these advantages, integrating fragment analysis into routine AML diagnosis in Iraq is crucial for more precise risk stratification and treatment decisions.

Although fragment analysis has not yet been widely adopted in Iraq, it provides critical quantitative data on the mutant-to-wild-type AR, which has significant prognostic implications. High AR is associated with aggressive disease and poor outcomes. The European Leukemia Net (ELN 2017) set an AR cutoff of 0.5, but the revised ELN guidelines now classify all *FLT3-ITD* mutations as intermediate-risk, leaving the clinical significance of AR under debate.[31]

The current study found that 50% of *FLT3-ITD*-positive patients had high AR, consistent with Zhou et al., who reported 51.1% high AR cases, while Kivioja et al. found a high AR in only 36% of patients.[32,33]

Regarding AML subtypes (FAB classification), we found no *FLT3-ITD* mutations in M7 cases, whereas M5 exhibited the highest mutation frequency (40%). This aligns with Fahed et al. and regional studies by Hamed et al. and Sarojam et al., who reported *FLT3-ITD* prevalence rates of 50% and 25.4% in M5 cases, respectively.[20,21,34] The M2 subtype had the second highest mutation rate (20% and 23.9% in different studies), and these variations were potentially influenced by sample size and ethnic diversity.

The findings revealed a significantly higher frequency of *FLT3-ITD* mutations in females (90% of *FLT3-ITD* positive cases) than in males. This sex-related difference in incidence, molecular presentation, and outcomes is being increasingly recognized. Studies have shown that *FLT3-ITD* mutations are more prevalent in females with co-occurring mutations in NPM1 and DNMT3A, in contrast, males are more predisposed to possess supplementary mutations in splicing of RNA and methylation modifier genes. These molecular differences may contribute to variations in disease progression and treatment response.[35] The exact mechanisms underlying these sex-based disparities remain poorly understood, but may involve hormonal influences, environmental stressors, or socioeconomic factors. Further research is needed to elucidate the impact of sex on *FLT3-ITD* driven AML and develop sex-specific therapeutic strategies.

Study limitations

The research has several constraints, such as a comparatively limited participant pool and a single-site methodology, which could reduce the broader applicability of the outcomes. Although fragment analysis and allelic ratio assessment were performed for *FLT3-ITD* detection, sequencing of *FLT3-ITD* mutations was not conducted. Future studies should incorporate sequencing techniques to characterize ITD length, insertion sites, and sequence variations, which may have prognostic significance. Furthermore, collaborative research across multiple institutions involving expanded patient groups, along with combined genetic and RNA expression profiling, may enhance the understanding of both molecular mechanisms and clinical implications of FLT3-ITD mutations in Iraqi AML patients.

Conclusion

This study provides critical insights into the prevalence and clinical implications of *FLT3-ITD* mutations in AML patients in Iraqi, emphasizing the need for precise molecular diagnostic approaches. This finding revealed a distinct subset of AML patients harboring *FLT3-ITD* mutations, with a significant proportion exhibiting elevated allelic ratios, reinforcing their prognostic relevance. A striking sex-based disparity was observed as these mutations were predominantly detected in female patients, suggesting potential sex-specific molecular pathways in AML pathogenesis that merit further investigation. CE has been found to provide better resolution than conventional gel electrophoresis, especially in ambiguous cases, and to distinguish between heterozygous and homozygous mutations. This methodological advancement is critical for improving risk stratification and therapeutic considerations, including targeted therapies and transplantation eligibility. These findings were in line with mutation frequencies reported elsewhere, but highlight the need for standardized, high-sensitivity diagnostic protocols in clinical practice, which should help to reduce uncertainties in molecular diagnostics.

Declarations

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This study received no funding.

Author contributions

Conceptualization, S.A.L. and I.A.A.; Methodology, S.A.L.; Software, S.A.L.; Validation, S.A.L. and I.A.A.; Formal Analysis, S.A.L. and I.A.A..; Investigation, S.A.L. and I.A.A.. Resources, S.A.L.; Data Curation, S.A.L. and I.A.A.; Writing – Original Draft Preparation, S.A.L.; Writing – Review & Editing, S.A.L. and I.A.A.; Visualization, S.A.L.; Supervision, I.A.A.; Project Administration, I.A.A.; Funding Acquisition, S.A.L.

Conflicts of interest

The author declare that they have no competing interests.

Data availability

Relevant datasets from this investigation are available from the corresponding author upon substantiated request.

Ethics approval

The study protocol, along with the participant information sheet and consent form, was reviewed and approved by the Hematology Center of Medical City, Baghdad, Iran. Ethical approval was granted (document number: 18982) on May 22, 2023.

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ORIGINAL PAPER

Diuretic efficacy and phytochemical profiling of the 80% methanol extract of *Sida schimperiana* Hochst. ex A. Rich

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ABSTRACT

Introduction and aim. Diuretics are used for edema, heart failure, and hypertension, but may cause side effects and resistance. *Sida schimperiana* is used for urinary retention in Ethiopian folk medicine, although its diuretic effects are unverified. This study aims to evaluate the diuretic potential and phytochemical content of the 80% methanol extract of *S. schimperiana*.

Material and methods. Male rats were administered distilled water (2 mL/100 g), furosemide (10 mg/kg), or 80% methanol extract (100, 200, or 400 mg/kg). Diuretic effects were assessed by monitoring urination onset, urine volume, electrolyte levels, and pH. Phytochemical analysis was performed to explore underlying diuretic mechanisms.

Results. The 80% methanol extract demonstrated significant diuretic and natriuretic effects in a dose- and time-dependent manner, surpassing the negative control. At doses of 200 and 400 mg/kg, diuresis ($p<0.001$) and sodium and chloride excretion ($p<0.001$) were notably increased at all time points. The treated group also showed significant changes in urine pH. Phytochemical analysis identified alkaloids, flavonoids, polyphenols, and tannins, with high concentrations of flavonoids (187.3 mg quercetin equivalent/g) and polyphenols (128.4 mg quercetin equivalent/g).

Conclusion. This study affirmed the methanolic extract's diuretic potency, comparable to furosemide, emphasizing its prospective therapeutic value.

Keywords. diuretic activity, in vivo, phytochemicals, rats, *Sida schimperiana*

Introduction

Diuretics are drugs commonly prescribed for managing conditions such as edema, heart failure, and hypertension, they facilitate the excretion of sodium and water, thus mitigating excess extracellular fluid accumulation.[1-4]

Despite the significant therapeutic benefits offered by conventional agents, their use is frequently accompanied by a wide range of adverse effects, including electrolyte disturbances, ototoxicity, and renal dysfunction.[5] Among these, the most prominent issues are fluid and electrolyte balance disruptions, particularly hypokale-

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mia and volume depletion, which can severely compromise normal physiological functions. Such imbalances can give rise to serious complications, including hyperglycemia, cardiac arrhythmias, hyperlipidemia, and other potentially life-threatening conditions that may exacerbate underlying health problems.[5,6]

The most prominent are fluid and electrolyte balance perturbations, specifically hypokalemia and volume depletion, which can critically undermine physiological processes. These imbalances may lead to severe complications, such as hyperglycemia, cardiac arrhythmias, hyperlipidemia, and other potentially life-threatening conditions.[6] Additionally, diuretic therapy may induce metabolic disturbances, including acidosis or alkalosis, and escalate the overall cost of treatment.[7]

These limitations have prompted an increasing interest in natural products as alternative or complementary sources of diuretic agents, particularly those with fewer side effects and additional health benefits. Owing to the ease of access and affordability of herbal remedies, many people rely on them instead of modern medications, which are often expensive and difficult to obtain.[8]

Among these, *Sida schimperiana* Hochst. ex A. Rich. *(S. schimperiana)*, also known as "Chifrig" in Amharic in the genus Sida, belongs to the family Malvaceae and is commonly used as a diuretic for the treatment of urinary retention.[9-11] It is a perennial subshrub with woody, erect, or procumbent stems that can grow up to a height of about 90 centimeters and grows primarily in the seasonally dry tropical biome.[12,13]

Geographically, *S. schimperiana* is native to eastern Africa, specifically found in Eritrea, Ethiopia, Kenya, Tanzania, and Uganda. Additionally, it has been introduced to India.[14]

In Ethiopian traditional medicine, the leaves and stems of *S. schimperiana* are used orally for the treatment of gonorrhea, and urinary retention.[9] The roots treat abdominal pain and evil eye disease.[10] *S. schimperiana* has several pharmacologically proven activities. Its root extract has antifungal,[15] antibacterial, and immunomodulatory activities.[16] The crude root extract's phytochemical analysis has revealed a diverse array of bioactive compounds, including polyphenols, flavonoids, coumarins, and phytosteroids.[15]

Aim

The aim was to evaluate the diuretic potential and phytochemical contents of the 80% methanol extract of *S. schimperiana*.

Material and methods

Drugs, chemicals, and reagents

Distilled water and normal saline were obtained from EPHARM (Ethiopia). Acetic anhydride, atropine, chloroform, Folin-Ciocalteu reagent, gallic acid, glacial acetic acid, magnesium ribbon, methanol, phosphate buffer solution, quercetin, and tannic acid were purchased from Sigma-Aldrich (Germany). Aluminum chloride, ammonium hydroxide, bismuth nitrate, bromocresol green, dimethyl sulfoxide, ferric chloride, hydrochloric acid, iodine, isopropyl alcohol, lead acetate, mercuric chloride, potassium hydroxide, potassium iodide, sodium carbonate, sodium hydroxide, sodium nitrate, and sulfuric acid were purchased from Fisher Scientific (UK). Furosemide was obtained from Denk Pharma GmbH and Co. KG (Germany) and sodium pentobarbitone was purchased from Taj Pharmaceuticals Ltd. (India). All drugs, chemicals, and reagents used were of analytical grade.

Plant material collection and extraction

In September 2023, the stems of *S. schimperiana* were collected from the region surrounding Debre Markos. A taxonomist authenticated the botanical specimen and a voucher specimen (BGM04) was duly archived. Freshly collected stems were meticulously rinsed with tap water and subsequently air-dried in a shaded environment at ambient temperature. After that, the desiccated stems were ground into coarse powder. A 150-gram of the coarse powder was subjected to maceration in 1.5 liters of 80% methanol within a conical flask, and the mixture was agitated at 120 rpm for 72 hours at ambient temperature. The resultant extract underwent a sequential filtration process, initially utilizing muslin cloth, followed by filtration through Whatman® grade 1 filter paper. The residual marc was then subjected to two subsequent rounds of re-extraction, employing fresh solvent in identical volumes to those used in the initial extraction, as outlined previously. The fluid extract was concentrated using a rotary evaporator (Buchi, Rotavapor R-210/215, Switzerland) under reduced pressure at 40°C, and then dried in a lyophilizer (OPR-FDU-5012, Korea). The dried extract was 12.4% (w/w). It was subsequently stored in a deep freezer at -20°C until use.

Experimental animals

Sprague-Dawley rats (males for the main study and females for the acute toxicity study), aged 6–10 weeks and weighing 280–320 g, were used in the experiment. The rats were housed in polypropylene cages (6 rats per cage) under standard environmental conditions (25±2°C, 55±5% humidity, and a 12 h/12 h light/dark cycle). They had free access to tap water and laboratory pellets and were acclimatized to the laboratory conditions for one week before the experiment. Each rat was placed in a metabolic cage (Tecniplast, Italy) 24 hours before the experiment.

Acute oral toxicity test

An acute oral toxicity test for the 80% methanol extract of *S. schimperiana* was conducted following the guide-

lines of the Organization for Economic Co-operation and Development.[16] Rats were fasted overnight before and 4 hours after administration of the extract. Initially, a sighting study was performed to determine the starting dose, with one rat receiving 2000 mg/kg 80% methanol extract as a single dose via oral gavage. As no death was observed within a day, an additional four rats were administered the same dose. The rats were monitored continuously for 4 hours at 30-minute intervals and subsequently observed daily for 14 days to assess the general signs and symptoms of toxicity (diarrhea, weight loss, tremor, lethargy, and paralysis), food and water intake, and mortality. Three dose levels were selected for further testing: a middle dose, which was one-tenth of the maximum dose used in the acute toxicity study; a low dose, which was half of the middle dose; and a high dose, which was twice the middle dose.[17]

Experimental design

Rats were randomly assigned to five groups (two control and three test groups), comprising six animals per group. Negative controls were treated with the vehicle used for reconstitution (2 mL/100 g distilled water), positive controls were treated with the standard drug furosemide 10 mg/kg (F10), and the three test groups were treated with 100, 200, and 400 mg/kg doses of the 80% methanol extract of *S. schimperiana* as SSME100, SSME200, and SSME400, respectively. Each group received the assigned treatment via oral gavage. Doses were determined using data from an acute oral toxicity study. At the end of the experiment, all rats were euthanized by an intraperitoneal injection of sodium pentobarbital at a dose of 150 mg/kg.

Determination of diuretic activity

The diuretic activity was determined using the method described by Hailu and Engidawork.[18] Rats were fasted overnight with free access to water, and pretreated with normal saline at an oral dose of 15 mL/kg to impose uniform water and salt load. Rats were divided into groups and treated as described in the Experimental Design section. Immediately after the assigned treatment, the animals were placed in metabolic cages (a rat in a cage). Urine was collected and measured at the time of first urination, and 1, 2, 3, 4, and 5 h after dosing, and stored at –20°C for electrolyte analysis. No food or water was available at the time of the urine collection.

The following parameters were recorded for each rat: first urinary latency (time to the first urination); urine volume; urine concentration of Na^+, K^+, and Cl^-; and urine pH. To compare the effects of three different doses of the 80% methanol extract with each other and with the controls (positive and negative), parameters such as percent urinary excretion, diuretic action, and diuretic activity were also calculated using the following formulas:

$$Percent\ \ urinary\ \ excretion = \frac{Total\ \ urinary\ \ output}{Total\ \ liquid\ \ administered} \times 100,$$

$$diuretic\ \ action = \frac{Mean\ \ urinary\ \ excretion\ \ of\ \ treatment\ \ groups}{Mean\ \ urinary\ \ excretion\ \ of\ \ control\ \ group},$$

$$and\ \ diuretic\ \ activity = \frac{Diuretic\ \ action\ \ of\ \ test\ \ sample}{Diuretic\ \ action\ \ of\ \ standard\ \ drug},$$ [18]

Urinary electrolyte level determination

The 80% methanol extract of *S. schimperiana* and rat urine were analyzed for the levels of three electrolytes (Na^+, K^+, and Cl^-). The Na^+ and K^+ concentrations were determined using a high-precision flame photometer (FP8400 Flame Photometer, A. KRUSS OPTRONIC GmbH, Germany), and the Cl^- was determined using an ion-selective electrode analyzer (AVL 9181 Electrolyte Analyzer, Roche, Germany). Electrolyte ratios, including test/control, Na^+/K^+, and $Cl^-/[K^+ + Na^+]$, were calculated to assess saluretic, natriuretic, and carbonic anhydrase inhibitory activities, respectively. The urine pH was measured using a pH meter.[19]

Qualitative phytochemical screening

Qualitative phytochemical analysis of the 80% methanol extract of *S. schimperiana* was conducted to identify bioactive secondary metabolites with potential medicinal properties. The investigation targeted compounds such as alkaloids, cardiac glycosides, flavonoids, polyphenols, saponins, steroids, tannins, and terpenoids, employing established methods for detection.[20-24]

Alkaloid detection test

About 10 mg of the 80% methanol extract was dissolved in 2 mL of 5% hydrochloric acid and subsequently filtered. The resulting filtrate was divided into separate test tubes, with each aliquot treated with a few drops of one of the following reagents: Bouchardat, Dragendorff, Mayer, or Wagner. The formation of distinct precipitates; brown (Bouchardat), red-orange (Dragendorff), yellowish-white (Mayer), and red-brown (Wagner), provides conclusive evidence for the presence of alkaloids.

Anthraquinone glycoside detection test

Borntrager's assay: To 3 mL of the 80% methanol extract, 3 mL of chloroform was added, and the chloroform layer was meticulously separated. Subsequently, a 5% potassium hydroxide solution was introduced to the mixture. The emergence of a red coloration serves as a definitive indication of the presence of anthraquinone glycosides.

Ammonium hydroxide assay: A solution comprising 10 mg of the 80% methanol extract was meticulously dissolved in 10 mL of isopropyl alcohol. To this resultant mixture, a single drop of concentrated ammonium hydroxide was introduced. Following a two-minute incubation peri-

od, the emergence of a pink-red hue serves as an indicative marker for the presence of anthraquinone glycosides.

Cardiac glycoside detection test (Keller-Kiliani assay): An aliquot of 0.25 g of the 80% methanol extract was dissolved in distilled water. To 5 mL of the resulting 80% methanol extract solution, 2 mL of glacial acetic acid, fortified with a single drop of ferric chloride solution, was carefully added. The mixture was then underplayed with 1 mL of concentrated sulfuric acid. The development of a distinct brown ring at the interface of the two layers serves as a definitive indication of the presence of cardiac glycosides.

Flavonoid detection test

Alkaline reagent assay: A volume of 1 mL of the 80% methanol extract was subjected to treatment with a few drops of a 20% sodium hydroxide solution. The development of a vivid yellow coloration, which subsequently decolorizes upon the introduction of hydrochloric acid, serves as a qualitative indicator of the presence of flavonoids.

Shinoda assay: To 5 mL of the 80% methanol extract, fragments of magnesium ribbon were introduced, followed by the addition of concentrated hydrochloric acid. The emergence of a red-to-pink hue after a brief interval signifies the presence of flavonoids.

Polyphenol detection test

Lead acetate assay: A few drops of a 10% lead acetate solution were introduced into the 80% methanol extract. The formation of a white precipitate serves as a definitive indication of the presence of phenolic compounds.

Ferric chloride assay: To 2 mL of the filtered 80% methanol extract, four drops of a 10% ferric chloride solution were added. The manifestation of a blue-green coloration signifies the presence of phenolic compounds.

Saponin detection test (Frothing assay)

To 0.25 g of the 80% methanol extract, 20 mL of distilled water was added, followed by vigorous agitation for 15 minutes. The development of a stable and persistent froth is indicative of the presence of saponins.

Steroid detection test (Libermann-Buchard assay)

A volume of 1 mL of the 80% methanol extract was combined with 10 mL of chloroform and subsequently filtered. The filtrate was treated with a few drops of acetic anhydride, subjected to boiling, and then allowed to cool. Concentrated sulfuric acid was cautiously added to the mixture. The emergence of a distinct brown ring at the phase interface unequivocally confirms the presence of steroids.

Tannin detection test (Ferric chloride assay)

A quantity of 10 mg of the 80% methanol extract was dissolved in 2 mL of distilled water and subsequently filtered. To the resultant filtrate, a few drops of a 10% ferric chloride solution were introduced. The appearance of a blue or green coloration serves as a definitive indicator of the presence of tannins.

Terpenoid detection test (Salkowski assay)

To 10 mg of the 80% methanol extract, 2 mL of chloroform was introduced. To this mixture, 3 mL of concentrated sulfuric acid was cautiously added. The development of a reddish-brown coloration at the interface serves as a definitive indicator of the presence of terpenoids.

Quantitative estimation of phytochemical constituents

Phytochemicals detected through qualitative screening such as alkaloids, flavonoids, polyphenols, and tannins were subsequently quantified using meticulously validated standard methods outlined below.

Total alkaloid content determination

The total alkaloid concentration in the 80% methanol extract of *S. schimperiana* was quantified using a spectrophotometric method, relying on the interaction between alkaloids and bromocresol green (BCG).[25] One milligram of the plant extract was dissolved in dimethyl sulfoxide (DMSO). To this solution, 1 mL of 2N hydrochloric acid was added, and the resulting mixture was filtered. The pH of the filtrate was adjusted to neutral using 0.1 N sodium hydroxide. The solution was then transferred into a separatory funnel, and 5 mL of BCG solution, along with 5 mL of phosphate buffer (pH 4.7), was introduced. The mixture was vigorously shaken with chloroform in volumes of 1, 2, 3, and 4 mL, then diluted to a total volume of 10 mL with additional chloroform. Similarly, standard solutions of atropine (12.5, 25, 50, 100, 200, and 400 µg/mL) were prepared by combining accurately measured atropine aliquots with 5 mL of BCG solution and 5 mL of phosphate buffer (pH 4.7), followed by shaking with chloroform in volumes of 1, 2, 3, and 4 mL. These solutions were collected in a 10 mL volumetric flask and diluted with chloroform to a final volume of 10 mL. The absorbance of both the atropine standards and the test solutions was measured against a reagent blank at 470 nm using a UV-visible spectrophotometer (SHIMADZU UV 1800, Kyoto, Japan). To ensure accuracy, all procedures were conducted on the same day and repeated in triplicate. The total alkaloid content was expressed as milligrams of atropine equivalents per gram of dry plant extract (AE/g).

Total flavonoid content determination

The total flavonoid content in the 80% methanol extract of *S. schimperiana* was assessed using the aluminum chloride colorimetric method.[26] A series of quercetin standards in methanol were prepared at con-

centrations of 12.5, 50, 100, 200, and 400 µg/mL. To each standard, 250 µL of the solution was combined with 125 µL of water and 75 µL of sodium nitrate solution. The mixtures were kept in the dark at 25°C for 6 minutes. Afterward, 150 µL of aluminum chloride was added, and the samples were left in the dark for 5 hours. Subsequently, 500 µL of sodium hydroxide and 275 µL of water were added to each solution. The absorbance was recorded at 510 nm using a UV-visible spectrophotometer, and a standard curve correlating concentration to absorbance was constructed. The test sample was treated in the same manner and analyzed. All experiments were performed in triplicate. The total flavonoid content was expressed as milligrams of quercetin equivalents per gram of dry plant extract (QE/g).

Total phenolic content determination

The total phenolic content of the 80% methanolic extract of *S. schimperiana* was quantified using the Folin-Ciocalteu reagent assay.[27,28] Serial dilutions of methanolic gallic acid solutions at concentrations of 12.5, 25, 50, 100, 200, and 400 µg/mL were prepared, and 50 µL of each solution was combined with 100 µL of 10% Folin-Ciocalteu reagent and 100 µL of 7% sodium carbonate. The reaction mixture was incubated at ambient temperature for 30 minutes, after which the absorbance was measured at 765 nm using a UV-visible spectrophotometer, with a reagent blank serving as the reference. A calibration curve was generated by plotting absorbance values against gallic acid concentrations. The test sample was prepared and analyzed following the same procedure. All measurements were conducted in triplicate on the same day. The total phenolic content was reported as milligrams of gallic acid equivalents per gram of dry plant extract (mg GAE/g).

Total tannin content determination

The total tannin content of the 80% methanolic extract of *S. schimperiana* was determined using the method described by Chandran and Indira.[29] To 0.1 mL of the sample extract, 0.5 mL of Folin-Ciocalteu reagent, and 1 mL of 35% sodium carbonate solution were added. The resulting mixture was diluted to a final volume of 10 mL with distilled water, thoroughly mixed, and allowed to stand at room temperature for 30 minutes. In parallel, a series of tannic acid reference standards at concentrations of 12.5, 25, 50, 100, 200, and 400 µg/mL were prepared and processed similarly. The absorbance of both the test samples and the standard solutions was recorded at 700 nm using a UV-visible spectrophotometer, with a reagent blank serving as the baseline reference. All analyses were performed in triplicate on the same day. The total tannin content was expressed as milligrams of tannic acid equivalents per gram of dry plant extract (mg TAE/g).

Statistical analysis

The study findings were expressed as mean±standard error of the mean (S.E.M). Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc multiple comparison test to discern specific group differences using SPSS version 25 (IBM, Armonk, NY, USA). Dose-dependent effects were examined using linear regression analysis. Statistical significance was determined using a threshold p-value of less than 0.05.

Ethical consideration

Animal handling was conducted following the guidelines set forth by the National Institutes of Health for the Care and Use of Laboratory Animals, as well as with the standards for international animal care and welfare.[30,31] The study received formal approval from the Research Ethics Committee of the Department of Pharmacy, College of Medicine and Health Sciences at Debre Markos University, as documented in the ethical approval letter with reference number 1921/23.

Results

Acute oral toxicity test

The acute oral toxicity test of the 80% methanol extract of *S. schimperiana* demonstrated that it did not induce gross behavioral changes or mortality either within 24 hours or over the course of 14 days. Consequently, the median lethal oral dose of the 80% methanol extract was estimated to exceed 2000 mg/kg in rats.

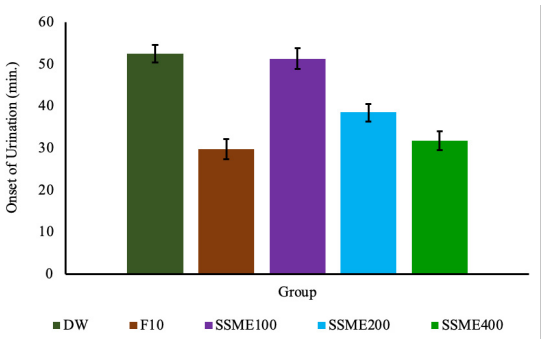

Fig. 1. Effect of 80% methanol extract on the onset of urination in rats (DW – distilled water; F10 – furosemide 10 mg/kg; SSME100 – *S. schimperiana* methanol extract 100 mg/kg; SSME200 – *S. schimperiana* methanol extract 200 mg/kg; SSME400 – *S. schimperiana* methanol extract 400 mg/kg)

Determination of diuretic activity

Effect on the onset of urination

The effect of the 80% methanol extract of *S. schimperiana* on the onset of urination is shown in Figure 1. Rats in the treatment group that received 100 mg/kg of the extract did not exhibit a significant difference in the onset of urination compared to the negative con-

trol. However, rats treated with 200 mg/kg and 400 mg/kg of the extract showed a significantly faster onset of urination, with times of 38.51±1.94 and 31.68±2.26 minutes, respectively ($p<0.001$), compared to the negative control (52.46±2.02 minutes).

Effect on urine volume

The effect of the 80% methanol extract of *S. schimperiana* on urine volume in rats is presented in Table 1. The extract-induced diuresis appeared to be dose- and time-dependent ($r^2=0.901$; $p<0.001$). Although rats treated with SSME100 showed a significant increase in urine output ($p<0.01$) after the fifth hour, no detectable change in urine volume was observed during the first four hours post-dosing, compared to the negative control. In contrast, rats treated with SSME200 and SSME400 demonstrated a significant increase in urine volume at all time points, with maximum diuresis of 111% ($p<0.001$) and 124% ($p<0.001$), respectively. Additionally, the diuretic effects of SSME200 and SSME400 were comparable to those of F10. Among the three doses, SSME200 and SSME400 produced significantly greater diuresis than SSME100 at all time points. Specifically, SSME200 and SSME400 exhibited urinary excretion rates of 103% and 110%, respectively, and diuretic actions of 2.11 and 2.24 (Table 1).

Effect on cumulative urine output

The cumulative urine output of rats administered the 80% methanol extract of *S. schimperiana* is illustrated in Figure 2. SSME100 showed no statistically significant change in cumulative urine output during the first 4 hours; however, it induced a notable increase ($p<0.01$) by the end of the fifth hour compared to the negative control. Both SSME200 and SSME400 demonstrated urine outputs comparable to that of F10. Furthermore, SSME400 resulted in a cumulative urine output that exceeded that of the standard at both the third and fourth hours (Fig. 2).

Table 1. Effect of 80% methanol extract of *S. schimperiana* on urine volume in rats*

Group	Volume of urine (mL)					% Urinary excretion	Diuretic action	Diuretic activity
	1 h	2 h	3 h	4 h	5 h			
DW	1.62 ±0.18	2.40 ±0.21	2.75 ±0.08	3.04 ±0.11	3.22 ±0.20	48	1.00	
F10	3.62 ±0.30 a###	4.72 ±0.23 a###	5.73 ±0.40 a###	6.82 ±0.29 a###	7.27 ±0.36 a###	112	2.26	1.00
SSME100	1.89 ±0.17 b###,c##,d###	2.46 ±0.19 b###,c###,d###	3.18 ±0.22 b###,c###,d###	3.46 ±0.20 b###,c###,d###	3.95 ±0.09 a##,b###, c###,d###	63	1.23	0.54
SSME200	2.86 ±0.26 a##	3.87 ±0.23 a###	5.32 ±0.29 a###	6.64 ±0.25 a###	6.80 ±0.10 a###	103	2.11	0.93
SSME400	3.08 ±0.24 a###	4.08 ±0.27 a###	6.14 ±0.42 a###	6.96 ±0.34 a###	7.21 ±0.28 a###	110	2.24	0.99

* each value represents the mean±S.E.M, n=6, [a] – against negative control (DW), [b] – against standard (F10), [c] – against 200 mg/kg, [d] – against 400 mg/kg, [#] – $p<0.05$, [##] – $p<0.01$, [###] – $p<0.001$, DW – distilled water, F10 – furosemide 10 mg/kg, SSME100 – *S. schimperiana* methanol extract 100 mg/kg, SSME200 – *S. schimperiana* methanol extract 200 mg/kg, SSME400 – *S. schimperiana* methanol extract 400 mg/kg

Effect on urinary electrolyte excretion

The electrolyte contents (Na^+, K^+, and Cl^-) in the collected urine samples were quantified and are presented in Table 2. SSME100 did not significantly increase the excretion of any of the three electrolytes compared to the negative

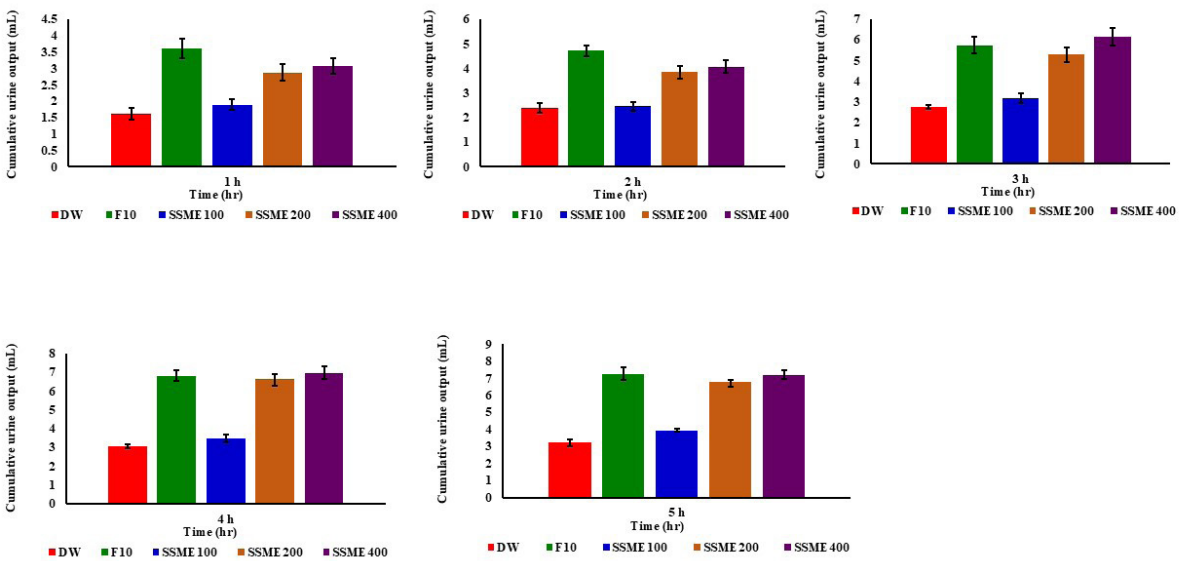

Fig. 2. Effect of 80% methanol extract on cumulative urine output in rats (DW – distilled water; F10 – furosemide 10 mg/kg; SSME100 – *S. schimperiana* methanol extract 100 mg/kg; SSME200 – *S. schimperiana* methanol extract 200 mg/kg; SSME400 – *S. schimperiana* methanol extract 400 mg/kg)

control. In contrast, SSME200 and SSME400 significantly enhanced the excretion of Na^+ and Cl^- ($p<0.001$), as well as K^+ ($p<0.01$), when compared to the negative control. Specifically, SSME200 increased Na^+ and Cl^- excretion by 131% and 44%, respectively, while SSME400 increased Na^+ and Cl^- excretion by 184% and 52%, respectively, relative to the negative control (Table 2). No significant differences were observed in Na^+ and Cl^- excretion between F10 and the two doses of the 80% methanol extract. However, F10 significantly outperformed both SSME200 and SSME400 in K^+ excretion, with a 209.2% increase ($p<0.001$) compared to the negative control, which was significantly greater than the K^+ excretion induced by SSME200 (63%, $p<0.01$) and SSME400 (85%, $p<0.01$).

Table 2. Effect of 80% methanol extract of *S. schimperiana* on urinary electrolyte excretion in rats*

Group	Urinary electrolyte concentration (mmol/L)			Saluretic index			Na^+/K^+	Na^+/Cl^-	$Cl^-/(Na^+ + K^+)$
	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-			
DW	53.81 ± 3.74	38.56 ± 2.89	76.87 ± 4.08	1.00	1.00	1.00	1.39	0.70	0.83
F10	156.34 ± 4.38 $^{a\#\#}$	119.21 ± 4.03 $^{a\#\#}$	138.07 ± 5.42 $^{a\#\#}$	2.91	3.09	1.79	1.31	1.13	0.50
SSME100	68.88 ± 2.78 $^{b\#\#\#,c\#\#\#,d\#\#\#}$	41.64 ± 2.08 $^{b\#\#\#,c\#\#\#,d\#\#\#}$	89.94 ± 3.16 $^{b\#\#\#,d\#\#\#}$	1.28	1.08	1.17	1.65	0.77	0.81
SSME200	124.30 ± 4.93 $^{a\#\#\#}$	62.85 ± 3.75 $^{a\#\#,b\#\#}$	110.69 ± 4.31 $^{a\#\#\#}$	2.31	1.63	1.44	1.98	1.12	0.59
SSME400	152.68 ± 3.66 $^{a\#\#\#}$	71.35 ± 3.24 $^{a\#\#,b\#\#}$	116.49 ± 5.86 $^{a\#\#\#}$	2.84	1.85	1.52	2.14	1.31	0.52

* each value represents the mean±S.E.M, n=6, ^{a} – against negative control (DW), ^{b} – against standard (F10), ^{c} – against 200 mg/kg, ^{d} – against 400 mg/kg, $^\#$ – $p<0.05$, $^{\#\#}$ – $p<0.01$, $^{\#\#\#}$ – $p<0.001$, DW – distilled water, F10 – furosemide 10 mg/kg, SSME100 – *S. schimperiana* methanol extract 100 mg/kg, SSME200 – *S. schimperiana* methanol extract 200 mg/kg, SSME400 – *S. schimperiana* methanol extract 400 mg/kg

The indices for saluretic, natriuretic (aldosterone secretory), thiazide secretory, and carbonic anhydrase inhibitory activities of the 80% methanol extract of *S. schimperiana* at three different doses are summarized in Table 2. SSME400 exhibited saluretic indices of 2.84 for Na^+, 1.85 for K^+, and 1.52 for Cl^-, while F10's indices were 2.91, 3.09, and 1.79 for these three ions, respectively. The natriuretic indices (Na^+/K^+) for SSME100 (1.65), SSME200 (1.98), and SSME400 (2.14) were all higher than that of the standard drug (1.31). The carbonic anhydrase inhibitory activity indices, represented by the $[Cl^-/(Na^+ + K^+)]$ ratios, were 0.52 for SSME200 and 0.59 for SSME400 (Table 2).

Electrolyte content of the 80% methanol extract

To preclude potential interference, the 80% methanol extract of *S. schimperiana* was rigorously analyzed for the presence of three specific electrolytes: Na^+, K^+, and Cl^-. The results revealed that none of these ions were detectable across any of the concentrations tested.

Effect on urine pH

The pH of the urine samples was measured, yielding varying results (Fig. 3). Both SSME200 and SSME400 generated relatively alkaline urine, with pH values of 8.54±0.32 and 8.82±0.37, respectively. F10 also resulted in slightly basic urine, with a pH of 8.37±0.21 (Figure 3).

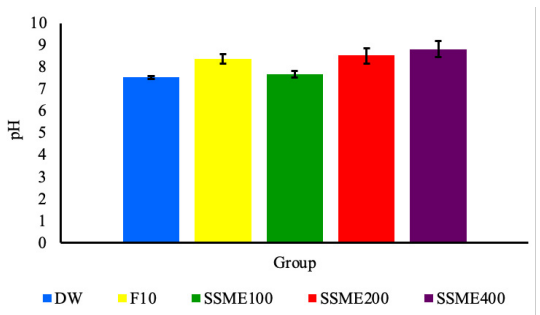

Fig. 3. Effect of 80% methanol extract on urine pH of rats (DW – distilled water; F10 – furosemide 10 mg/kg; SSME100 – *S. schimperiana* methanol extract 100 mg/kg; SSME200 – *S. schimperiana* methanol extract 200 mg/kg; SSME400 – *S. schimperiana* methanol extract 400 mg/kg)

Table 3. Qualitative phytochemical screening of the 80% methanol extract of *S. schimperiana*

Phytochemical	Test method	Observation	Result
Alkaloids	Bouchardat	brown precipitate was formed	the presence of alkaloids confirmed
	Dragendorff	a red-orange precipitate was formed	the presence of alkaloids confirmed
	Mayer	a yellowish-white precipitate was formed	the presence of alkaloids confirmed
	Wagner	a red-brown precipitate was formed	the presence of alkaloids confirmed
Anthraquinone Glycosides	Borntrager's test	the red color did not occur	absence of anthraquinone glycosides
	Ammonium hydroxide test	the pink-red color was not formed	absence of anthraquinone glycosides
Cardiac Glycosides	Keller-Kiliani test	no formation of brown-ring between layers	absence of cardiac glycosides
Flavonoids	Alkaline reagent test	yellow fluorescence was present	presence of flavonoids
	Shinoda test	the red-pink color was formed	presence of flavonoids
Polyphenols	Lead acetate test	a bulky white precipitate was formed	presence of polyphenols
	Ferric chloride test	the blue-green color was formed	presence of polyphenols
Saponins	Frothing test	a stable persistent froth was not formed	absence of saponins
Steroids	Libermann-Buchard test	no formation of a brown ring at the interface	absence of steroids
Tannins	Ferric chloride test	the blue-purple or green precipitate was formed	presence of tannins
Terpenoids	Salkowski test	a reddish-brown precipitate was not formed	absence of terpenoids

Qualitative phytochemical screening

The preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, polyphenols, and tannins in the 80% methanolic extract of *S. schimperiana* (Table 3).

Quantitative estimation of phytochemical constituents

The analysis of phytochemical constituents demonstrated a marked increase in the total concentrations of flavonoids and polyphenols, with values quantified at 187.3 mg of GAE/g and 128.4 mg of QE/g, respectively (Table 4).

Table 4. Quantitative phytochemical content estimation of the 80% methanol extract of *S. schimperiana**

Parameter	Content (mg/g dry weight of plant extract)
Total alkaloid content (mg AE/g dry weight of plant extract)	61.8
Total flavonoid content (mg QE/g dry weight of plant extract)	187.3
Total polyphenol content (mg GAE/g dry weight of plant extract)	128.4
Total tannin content (mg TAE/g dry weight of plant extract)	22.5

* AE – atropine equivalent, QE – quercetin equivalent, GAE – gallic acid equivalent, TAE – tannic acid equivalent

Discussion

An ethnopharmacological survey conducted in Northwestern Ethiopia highlighted the traditional use of *S. schimperiana* stem powder for treating urinary retention.[9] This study investigates the plant's diuretic potential and analyzes the phytochemicals found in the 80% methanol extract. The results reveal that the 80% methanol extract of *S. schimperiana* (SSME) enhanced diuresis in a dose- and time-dependent manner (r^2=0.901, p<0.001). While the 100 mg/kg dose (SSME100) did not induce significant diuresis, both the 200 mg/kg (SSME200) and 400 mg/kg (SSME400) doses substantially increased urine volume at all time points, with maximum diuresis of 111% (p<0.001) and 124% (p<0.001), respectively. These findings suggest that 100 mg/kg is below the minimum effective dose, while 200 mg/kg and 400 mg/kg are sufficient to induce a diuretic effect. Notably, the diuretic action of SSME400 was comparable to the standard, with ratios of 2.24, 0.99, 2.26, and 1.00, respectively.

The onset of urinary latency was significantly delayed in rats administered SSME100 (51.29±2.51 min, p<0.001). In contrast, both SSME200 and SSME400 caused only a modest delay in the onset of diuresis, with latencies of 38.51±1.94 min and 31.68±2.26 min, respectively (p<0.001), compared to F10, which exhibited a latency of 29.72±2.37 min (Fig. 1). The rapid onset of diuresis observed with the higher doses is likely attributable to their action on the ion co-transport mechanism, similar to the mode of action of loop diuretics.[32]

Treatment with SSME100 did not significantly affect the excretion of any of the three electrolytes compared to the negative control. However, SSME200 and SSME400 treatments notably increased the excretion of Na^+, Cl^- (p<0.001), and K^+ (p<0.01) relative to the negative control. Specifically, Na^+ and Cl^- excretions were elevated by 131% and 44% with SSME200, and 184% and 52% with SSME400, respectively. No significant difference in Na^+ and Cl^- excretion was observed between F10 and the two doses of the 80% methanol extract. In contrast, F10 induced a 209.2% increase in K^+ excretion (p<0.001) compared to the negative control, a result that was significantly higher than the K^+ loss observed with SSME200 (63%, p<0.01) and SSME400 (85%, p<0.01).

The extract significantly enhanced the excretion of three urinary electrolytes (Na^+, K^+, and Cl^-) in a dose-dependent manner. The lowest dose did not significantly affect the excretion of any of these electrolytes, while the intermediate and highest doses notably increased Na^+ and Cl^- excretion compared to the standard. However, K^+ excretion was lower than that observed with the standard drug. The natriuretic and carbonic anhydrase inhibitory activities can be quantified via the aldosterone secretory index, employing the Na^+/K^+ ratio to measure natriuresis and the $[Cl^-/(Na^+ + K^+)]$ ratio to evaluate carbonic anhydrase inhibition.[19,33]

The natriuretic indices of the extract were found to be higher than those of the standard across all measures: 1.65 for SSME100, 1.98 for SSME200, and 2.14 for SSME400, compared to 1.31 for F10. Similarly, at the highest dose, the extract exhibited carbonic anhydrase activity comparable to that of F10, as determined by the $Cl^-/[Na^+ + K^+]$ ratio. Ratios between 1.0 and 0.8 indicate no inhibition of carbonic anhydrase, while a reduction in this ratio suggests enzyme-inhibitory activity.[19,33] As shown in Table 2, the values of 0.52 for SSME200 and 0.59 for SSME400 indicate significant carbonic anhydrase inhibition, which is further supported by the observed increase in urinary pH (Fig. 3); a characteristic effect of carbonic anhydrase inhibitors.[5]

The plant extract exhibited a potassium-sparing effect while promoting the excretion of sodium and chloride ions, presenting a notable advantage over conventional pharmacological agents. This is especially significant given that a primary adverse effect associated with loop and thiazide diuretics is hypokalemia, a condition that often requires oral potassium supplementation or the use of potassium-sparing diuretics to mitigate potassium loss in urine.[5,34,35]

Collectively, these results suggest that the potent natriuretic, potassium-sparing, and carbonic anhydrase-inhibitory effects of the extract may be attributed to its secondary metabolites, as has been observed in several other plants.[18,36,37] While the precise bioactive constituents responsible for the diuretic and natriuretic properties of *S. schimperiana* remain unidentified, qual-

itative phytochemical assays have identified the presence of alkaloids, flavonoids, polyphenols, and tannins (Table 3). These phytoconstituents may exert their effects either individually or synergistically, contributing to the observed physiological outcomes.

Existing literature supports the notion that these phytochemicals possess diuretic and natriuretic properties through various biochemical mechanisms. For example, alkaloids inhibit carbonic anhydrase activity, promote renal perfusion through vasodilation, and may increase sodium excretion while conserving calcium.[38-40]

Flavonoids induce diuresis by enhancing renal perfusion, inhibiting carbonic anhydrase, blocking the sodium-potassium-chloride cotransporter, and modulating Na^+/K^+-ATPase activity. They also inhibit angiotensin-converting enzyme (ACE) and act as A1 adenosine receptor antagonists, promoting sodium excretion and diuresis through reduced sodium reabsorption and afferent arteriole dilation.[41-44]

Similarly, polyphenols promote diuresis by modulating ion channels and pumps involved in sodium and chloride reabsorption, inhibiting carbonic anhydrase, and regulating ACE and Na^+/K^+-ATPase activity.[43-46]

Flavonoids exhibit dual roles, providing both diuretic and potassium-sparing effects. The minimal impact of the plant extract on potassium excretion supports its potassium-sparing potential. Meanwhile, tannins contribute to hypotensive effects by promoting the excretion of water and electrolytes.[6] Overall, empirical evidence suggests that *S. schimperiana* exhibits diuretic activity through multiple mechanisms, which can be attributed to its diverse phytochemical profile.

Above all, this study presents the first comprehensive, dose-dependent assessment of the diuretic activity of 80% methanol extract from *S. schimperiana*, revealing its efficacy to be comparable to that of the standard diuretic, furosemide, at higher doses. Furthermore, the study provides a systematic exploration of the plant's phytochemical composition, establishing a connection between these compounds and the observed pharmacological effects. This suggests that *S. schimperiana* operates through multiple mechanisms of action. Notably, the findings underscore the potential of *S. schimperiana* as a natural diuretic, particularly for the management of fluid retention disorders. Its potassium-sparing properties offer promise as a safer alternative, with the potential for synergistic effects when combined with conventional diuretics.

Study limitations

Although the study offers compelling evidence for the diuretic potential and phytochemical composition of 80% methanol extract of *S. schimperiana*, it is not without limitations. First, the specific bioactive compounds responsible for the observed effects remain unidentified. While preliminary phytochemical analysis indicates the presence of several bioactive classes, advanced techniques such as high-performance liquid chromatography-mass spectrometry or nuclear magnetic resonance spectroscopy are necessary to isolate and characterize these compounds. Second, as a preclinical study, the findings are based solely on a rat model, which, despite its relevance, may not fully replicate human physiology. To confirm the extract's safety, efficacy, and therapeutic potential in humans, further isolation and characterization of bioactive compounds, along with clinical trials, are indispensable. It remains difficult to extrapolate findings from animal models to humans in the absence of such studies.

Moreover, the long-term effects of *S. schimperiana* methanolic extract were not investigated. Prolonged administration may lead to cumulative toxicity or adverse outcomes, particularly electrolyte imbalances. The study also lacked pharmacokinetic profiling, including absorption, distribution, metabolism, and excretion, which are essential for determining bioavailability and the therapeutic window. Finally, potential interactions between the extract and conventional diuretics or other medications were not evaluated, potentially limiting its clinical utility.

Conclusion

The 80% methanolic extract of *S. schimperiana* exhibits significant dose-dependent diuretic activity with confirmed safety at tested doses. Its efficacy, comparable to furosemide, is linked to its rich phytochemical profile. Further studies are needed to isolate bioactive compounds, clarify mechanisms, and validate clinical efficacy and safety.

Declarations

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This study did not receive any funding.

Author contributions

Conceptualization, B.G.M. and T.A.T.; Methodology, B.G.M.; Software, B.G.M.; Validation, D.A.G., A.D.T., G.N.A., G.T.B., and Y.Y.L.; Formal Analysis, B.G.M.; Investigation, B.G.M. and A.A.; Resources, Y.W.E.; Data Curation, M.T.K.; Writing – Original Draft Preparation, B.G.M.; Writing – Review & Editing, B.G.M.; Visualization, D.A.G.; Supervision, B.G.M.; Project Administration, B.G.M.; Funding Acquisition.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

All relevant data and materials are available from the corresponding author upon reasonable request.

Ethics approval

The study received formal approval from the Research Ethics Committee of the Department of Pharmacy, College of Medicine and Health Sciences at Debre Markos University, as documented in the ethical approval letter with reference number 1921/23.

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ORIGINAL PAPER

An overview of the diagnostic and prognostic values of biochemical markers in patients with COVID-19

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ABSTRACT

Introduction and aim. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a global pandemic that disrupts both public health operations and financial structures throughout the world. This article aims to evaluate the relationship between the severity of coronavirus disease 2019 (COVID-19) and its alterations in specific biomarkers such as D-dimer and C-reactive protein (CRP) together with alanine transaminase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH).

Material and methods. Participants in this study were split into two groups consisting of 200 COVID-19 patients and 200 healthy controls ranging from 18 to 80 years old. Polymerase chain reaction and chest radiograph examinations were used to officially verify the participant's diagnosis. This study used Mann-Whitney U tests combined with logistic regression and receiver operating characteristic (ROC) curves to identify and determine their value as diagnostic tools and prognostic indicators.

Results. D-dimer and CRP along with ALT, AST, and LDH demonstrated significant and elevated levels in COVID-19 infected participants when analyzed against control participants ($p<0.0001$). D-dimer emerged as a diagnostic biomarker according to ROC analysis with an AUC value of 0.96 and a p-value<0.001 which signified its quality for the evaluation of the severity of the disease. The additional biomarkers AST and LDH received AUC scores of 0.79 and 0.76, respectively,,with ALT reaching an AUC value of 0.74.

Conclusion. The combination of the biochemical markers D-dimer, AST, and LDH significantly improves risk assessment while enhancing predictions about disease outcomes. These biomarkers provide vital data for early disease detection in combination with disease progression through tracking patient outcomes and therapeutic planning assessments.

Keywords. COVID-19 biomarkers, D-dimer, lactate dehydrogenase, liver enzymes

Introduction

New unparalleled difficulties for both public healthcare organizations and healthcare delivery systems arose due to the worldwide spread of coronavirus disease 2019 (COVID-19) from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Precise disease identification in combination with effective disease management practices is desireable for healthcare success. Biochemical markers serve as an important diagnostic tool for identifying COVID-19 and the ability to forecast its disease path.[1]

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Therefore, rapid diagnostic methods combined with risk assessment capabilities and smart use of critical healthcare resources emerged as essential requirements during the current pandemic. Through biochemical assessments, practitioners achieve early diagnosis and identify disease severity which directs medical choices which begin at hospital entry and cover the entire treatment period.[2] Moreover, COVID-19 produces complex medical manifestations involving systemic responses between inflammatory pathways and immunological processes together with coagulation abnormalities. When COVID-19 progresses to life-threatening levels, it creates dangerous acute respiratory distress syndrome while simultaneously damaging heart systems and liver and kidney impairment across the body.[3,4]

Researchers in the medical field have discovered essential biochemical indicators that prove valuable in differentiating COVID-19 from comparable viral conditions.[5] Biochemical indicators in COVID-19 assessment include the standard inflammatory measures C-reactive protein (CRP), interleukin-6 (IL-6), and procalcitonin together with coagulation factors including D-dimer and fibrinogen. The risk of thromboembolic events, along with mortality rates and severe disease outcomes, such as multiple organ failure (MOF) and disseminated intravascular coagulation (DIC), increases with high levels of these biochemical markers.[6,7]

This study combines biomarkers alongside medical observations and imaging scans to develop a better understanding of disease assessment. Through daily marker monitoring, clinicians obtain useful disease information which helps them enhance care for COVID-19 patients.

In addition, the findings from this research established new information about how biochemical markers serve as diagnostic tools together with prognostic indicators for patients particularly considering D-dimer, aspartate aminotransferase (AST) and lactate dehydrogenase (LDH).

The study develops biomarker diagnostics for COVID-19 while providing clinically applicable statistical evidence confirming how D-dimer, AST, and LDH function in predicting disease severity. The research data shows potential to shape clinical guidelines through D-dimer promotion as front-running severity indicator and offer encouragement in gender-based disease susceptibility research.

Previous studies

Previous studies showed the relation between biochemical markers and COVID-19 disease severity.[7-9] Studies on these biomarkers showed the essential value for disease diagnosis and clinical evaluation alongside patient outcomes. Table 1 shows key studies that have investigated the role of D-dimer, CRP, alanine transaminase (ALT), AST, and LDH levels in patients with COVID-19.

Table 1. Previous related studies

Sample size	Key findings
179 patients	The research showed that severe COVID-19 cases had elevated levels of D-dimer and CRP that proved useful for predicting disease severity.[10]
2912 patients	Research findings showed that COVID-19 patients with higher levels of AST and ALT exhibited liver problems that indicated poorer disease outcomes.[11]
123 patients	LDH demonstrated excellent potential to evaluate tissue damage and predicted a poor outcome during COVID-19 patient treatment.[12]
100 patients	D-dimer levels combined with CRP and LDH levels displayed strong correlations with mortality in COVID-19 patients admitted to hospital care.[13]
5971	Studies revealed that measuring AST ALT and LDH levels together provides a diagnostic tool for assessing the severe progression of COVID-19 disease.[14]

The findings of previous studies establish the core knowledge necessary to understand the decisive effect of diverse blood biomarkers during COVID-19 clinical care.[19] This investigation expands upon earlier studies through detailed disease severity correlations, while utilizing expanded sample diversity to enhance both generalizations and information about SARS-CoV-2 pathophysiology.

Aim

This article aims to evaluate the relationship between the severity of COVID-19 and its alterations in specific biomarkers such as D-dimer and CRP together with ALT, AST and LDH.

Material and methods

Study sample

This study used a case-control approach within the AL Fayhaa Teaching Hospital in Basrah, southern Iraq. Data collection started on April 30 until October 1, 2020. 400 participants enrolled in the study, including 200 patients confirmed having COVID-19 and 200 as healthy controls. A power analysis from previous studies showed that 200 participants in each group would be needed using a significance threshold of 0.05 and 80% power.[15,16] This study increased the original participant target of 200 per group to prevent measurement problems and participant dropouts and maintain reliable data detection between patient groups. Official approval was obtained to conduct this investigation from the University of Basrah-Al-Zahraa College of Medicine (no. 4/35 on 1-04-2020), and the Basrah Health Directorate. Verbal consent was obtained from all patients who participated.

The medical diagnosis of patients with COVID-19 occurred through polymerase chain reaction (PCR) tests, while their symptoms correlated with typical COVID-19. The study exclusively examined viral impacts by including healthy asymptomatic controls who had no previous SARS-CoV-2 infection or vaccination, while also absent any other respiratory diseases.

Inclusion criteria and exclusion

The inclusion criteria for the COVID-19 case group: Age ≥ 18 years) Adults only), positive RT-PCR test for COVID-19 from a nasopharyngeal swab with other specific lab tests and clinical investigations, onset of symptoms within the past 14 days, hospitalized patients, and no prior history of COVID-19 infection or other chronic diseases before the study period.

Exclusion criteria: pediatric patients (<18 years), negative COVID-19 test and unconfirmed or suspected cases, individuals with chronic diseases, immunosuppressive conditions or drugs, smoking and other abnormalities are excluded and those with incomplete medical investigations or missing data.

Control subjects were randomly from the general population which were healthy participants without a history of COVID-19 infection or chronic diseases, and matched the COVID-19 case group by age, sex, and location. The samples were collected in the fasted state and all samples processed in the same manner.

Samples were ran at one time. In this research that involved standard good laboratory practice of biochemical analysis (biomarkers in COVID-19 patients), the running of samples was performed at one time to ensure reliability of results.

All laboratory instruments and procedures were calibrated according to the manufacturer's guidelines prior to analysis. In addition, internal quality control samples were run regularly with each run to ensure the precision and accuracy of the tests.

Patients and controls

Additionally, COVID-19 patients were divided into categories according to their disease. In the first category, patients with mild COVID-19 developed fever alongside cough and fatigue without imaging findings of pneumonia. The second was moderate cases with patients who demonstrated pneumonia-related respiratory symptoms alongside imaging proof of lung infection while staying off supplemental oxygen and who matched the moderate COVID-19 classifications of the CDC. Finally, severe cases in which patients showed at least one of several possible criteria that included over 30-breaths-per-minute respiratory rate of more than 30 breaths per minute combined with a resting oxygen saturation below 93% or significant lung involvement above 50% within 24-48 hours, sometimes developing respiratory failure or septic shock complications.

All participants provided blood samples which allowed periodic evaluation of D-dimer, CRP, ALT, AST, and LDH through automated measurements with Roche Diagnostics' Integra 400 PLUS analyzer during three days. The basis for AST and ALT principles is IFCC, which does not require pyridoxal phosphate activation. In contrast, the turbidimetric immunoassay (TIA) is the basis for the CRP assay.

Statistical analysis

A Shapiro-Wilk test was performed to verify whether the biomarker data sets of all measurements had normal distribution patterns. Additionally, this study used the Mann-Whitney U test for biomarker evaluation between COVID-19 patients and healthy control participants and between all participants by sex. Furthermore, a Kruskal-Wallis test analyzed biomarker levels between patients in mild-to-moderate-to-severity and severe severity groups was conducted. In addition, logistic regression analysis evaluated the predictive power of biomarkers for COVID-19 severity after adjustments for participant age demographic data of the age of the participants and premorbid conditions. The chance of disease worsening for each increase in biomarker intensities was estimated using odds ratios together with their associated 95% confidence intervals (95% CI). Receiver operating characteristic (ROC) curves: Areas under curve (AUC) analysis determined the diagnostic discrimination power of each biomarker by generating assessment scores to distinguish severe from nonsevere cases of COVID-19.

IBM SPSS Statistics 26 (Armonk, NY, USA) served as the platform to perform all statistical computations. The cut-off point for statistical significance was $p<0.05$ for this study. The detailed method revealed subtle relationships between marker levels and disease severity to deliver authoritative clinical conclusions.

Results

Demographics and clinical classification

The research enrolled an equal number of 400 participants, comprising 200 patients suffering from COVID-19 and 200 individuals without the virus. Table 2 presents the demographic distribution. The 49–63-year-old age group comprised the study's largest segment at 45% of COVID-19 patients and 33.5% of controls. Men measured more frequently than women in the study population (53.5% compared to 46.5%).

Table 2. Demographics of Patients and Controls

Variable	COVID-19 patients (n=200)	Healthy controls (n=200)	Mean difference (95% CI)
Age (years)	54.72±12.83	46.93±16.28	7.79
Gender			
Male (%)	107 (53.5%)	146 (73%)	–
Female (%)	93 (46.5%)	54 (27%)	–

This study classified the severity of COVID-19 infection into three categories according to clinical definitions. Patient data by severity level appears in (Table 3).

Table 3. Severity classification of COVID-19 patients

Severity level	Number of patients (n=200)	Percentage (%)
Mild	65	32.5%
Moderate	70	35%
Severe	65	32.5%

Biochemical markers in patients and controls

A comparison of biochemical marker levels is presented in Table 4 between COVID-19 patients and control groups. The patient group showed a significant elevation of all biomarkers ($p<0.001$).

Table 4. Biomarkers in COVID-19 patients vs. controls

Biomarker	COVID-19 patients (Mean±SD)	Controls (Mean±SD)	p
D-dimer (ng/mL)	286.08±90.12	114.93±40.15	<0.001
CRP (mg/dL)	300.03±110.07	100.97±35.25	<0.001
ALT (IU/L)	267.18±75.36	133.83±45.40	<0.001
AST (IU/L)	270.82±80.90	130.18±40.75	<0.001
LDH (IU/L)	294.89±95.32	106.11±35.15	<0.001

Gender-based differences in biomarker levels

Biomarkers reveal gender-specific variations according to data presented in (Table 5). Given the analysis results, males showed elevated D-dimer and LDH concentrations compared to females ($p<0.05$). CRP evaluation along with the analysis of ALT and AST showed no substantial differences between assessment points.

Table 5. Biomarker levels by gender

Biomarker	Male (Mean±SD)	Female (Mean±SD)	p
D-dimer (ng/mL)	108.68	91.09	0.032
CRP (mg/dL)	102.23	98.51	0.649
ALT (IU/L)	107.00	93.03	0.089
AST (IU/L)	106.74	93.32	0.102
LDH (IU/L)	109.05	90.66	0.025

Biomarker trends with disease severity

The results in Table 6 show biomarkers trending upward along with the severity of the progression of COVID-19 disease. The D-dimer, along with AST, presented the most prominent association between these biomarkers and the deterioration of COVID-19 disease.

Table 6. Biomarker levels by disease severity

Biomarker	Mild (Mean rank)	Moderate (Mean rnk)	Severe (Mean rank)	p
D-dimer (ng/mL)	68.68	108.68	125.90	<0.001
CRP (mg/dL)	60.78	77.92	91.46	0.02
ALT (IU/L)	76.72	83.89	100.35	0.04
AST (IU/L)	65.85	87.72	106.58	<0.001
LDH (IU/L)	73.18	88.92	98.83	0.01

The logistic regression model evaluated the predictive characteristics of COVID-19 severity associated with five biomarkers, including D-dimer, CRP, ALT, AST, and LDH. Results are presented in (Table 7). Adjusting for age, gender, and preexisting conditions produced odds ratios with corresponding 95% CI.

Table 7. Logistic regression analysis for biomarkers and COVID-19 severity

Biomarker	Odds ratio	95% CI	p
D-dimer (ng/mL)	1.50	1.20–1.80	<0.001
CRP (mg/dL)	1.30	1.10–1.60	<0.001
ALT (IU/L)	1.20	1.00–1.50	0.02
AST (IU/L)	1.40	1.10–1.70	<0.001
LDH (IU/L)	1.70	1.30–2.00	<0.001

The D-dimer along with LDH and AST shows the most robust indicator potential for the of COVID-19, of the severity assessment but CRP and ALT help support supplemental diagnostic information. When used together during clinical evaluations, these metrics help medical staff uncover high-risk patients before it becomes too late for intervention. A ROC curve analysis was performed to determine the discriminative power of biomarkers for identifying COVID-19 severity levels. Table 8 shows pictorially the AUC values from the biomarker analysis in Figure 1.

Table 8. ROC analysis for biomarker predictive performance

Biomarker	AUC	95% CI	p	Interpretation
D-dimer	0.96	0.90–1.00	<0.001	Excellent
CRP	0.28	0.15–0.42	0.02	Poor
ALT	0.74	0.61–0.86	<0.001	Fair to Good
AST	0.79	0.67–0.89	<0.001	Good
LDH	0.76	0.64–0.88	<0.001	Good

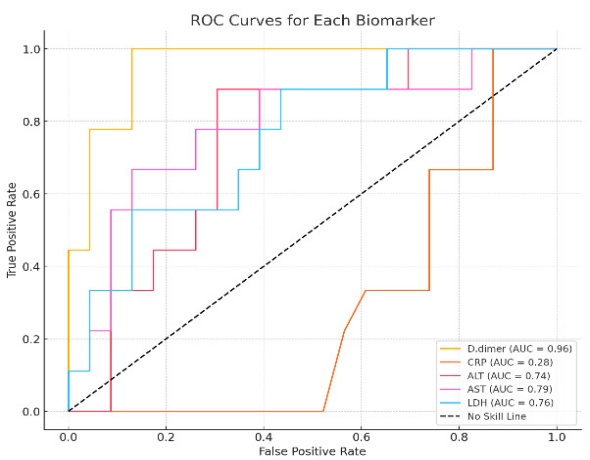

Fig. 1. ROC curves

Each biomarker in Figure 1 presents a visual representation of the sensitivity versus specificity trade-offs it offers. The steep D-dimer curve shows improved diagnostic capabilities with its position in the top left corner of Figure 1. The ROC curve analysis evaluates biomark-

ers for their ability to detect severe from nonsevere cases of COVID-19. Subsequent data analysis presents information from ROC visualization using extracted AUC measures.

Discussion

The results demonstrate that the D-dimer, together with CRP, ALT, AST, and LDH, serves as important molecular markers to assess the severity of the COVID-19 disease, but D-dimer shows better diagnostic potential for this purpose. This study demonstrates that the D-dimer exhibits a very high diagnostic accuracy (AUC=0.96, $p<0.001$) indicating its potential to detect coagulation abnormalities and disease severity among COVID-19 patients due to its proven association with thrombotic conditions and unfavorable outcomes. Previous studies confirm that higher D-dimer measurements signify higher probabilities of deep vein thrombosis along with pulmonary embolism and death among patients with severe illness who need intensive care hospitalization.[17,18] Our study results demonstrate a strong predictive value which supports current literature on the administration of rapid anticoagulation therapy administration in patients with COVID-19. Monitoring D-dimer levels as a routine medical practice allows clinicians to make better decisions about thromboprophylaxis against anticoagulant medication administration for patients with severe COVID-19 who face high mortality risk.[18]

The evaluation of the severity of COVID-19 benefits from AST and LDH since they add valuable predictive diagnostic power. Biomarkers exhibited substantial diagnostic power (AUC=0.79 and 0.76 respectively) confirmed using statistical tests ($p<0.001$) and have shown regular correlation with inflammatory tissue damage and organ dysfunction. AST levels increase when patients with COVID-19 develop hepatic and myocardial injuries leading to more severe disease progression.[19] LDH functions as a surrogate marker of cell damage and disease severity because observed increases become associated with lung injuries and multi-organ failure.[20] Therefore, research findings on LDH predictive capability establish its value as a biomarker for disease tracking alongside medical prognosis assessment. Healthcare providers use standard clinical laboratory results for AST and LDH to obtain additional information regarding the progression of COVID-19 disease when lung infections and swelling of body tissue occur in patients.

The AUC outcome for CRP was 0.28 along with $p=0.02$ which indicated insufficient capacity for distinguishing COVID-19 among patients. The CRP results of the study failed to validate the previous claims made by Pourfathi and Kadlecek[20] about its usefulness in signaling the inflammatory response and disease progression. CRP functions as a nonspecific indicator of inflammation that does not specifically reflect the severity levels specifically. The diagnostic potential of CRP testing for COVID-19 detection depends on various environmental factors because these levels rise during numerous infectious and inflammatory diseases, yet in their own right they provide limited clinical value. Researchers must investigate if using combinations of CRP with inflammatory markers IL-6 and ferritin improves predictive accuracy levels.

The study findings showed that D-dimer and LDH levels differed according to sex, indicating that patients of each sex experience dissimilar immune responses to COVID-19 infection. The literature shows that hormones and genetics lead to males producing greater inflammatory responses and demonstrating increased thrombotic risks compared to females.[21] Studies indicate that female patients experience reduced severity of COVID-19 outcomes because estrogen provides protection, so females typically show lower concentrations of D-dimer and LDH compared to male patients.[22]

Biomarker concentration levels show a direct connection to disease severity in this research, demonstrating their value for clinical practice in determining patient risk and predicting health outcomes and planning treatment approaches. Medical professionals can use regularly performed laboratory tests to detect D-dimer together with AST and LDH values that enable them to start early treatments effectively. Our study includes specific restrictions that must be noted. Robust statistics from our work exist, yet larger studies must analyze various patient groups uniformly to prove these findings.

Study limitations and future directions

This research produces valuable findings, yet it also contains some performance limitations. Sample measurement cannot adequately capture diverse types adequately but larger-scale confirmation studies are needed. This research omitted analysis of confounding variables, including pre-existing cardiovascular diseases and liver disorders, which might affect biomarker values. The use of a cross-sectional design restricts the assessment of biomarker dynamics that unfolds throughout time. Future research should investigate the biological mechanisms that produce elevated biomarkers and then use this knowledge to create treatment algorithms based on these biomarkers.

Conclusion

Biochemical biomarkers that include D-dimer as well as AST ALT LDH and CRP, provide significant support for COVID-19 recognition and disease severity ratings. The combination of these markers with clinical symptoms and imaging data improves both diagnosis precision and helps producers better understand risk levels. Clinical studies validate the diagnostic superi-

ority of D-dimer over other biomarkers due to its high diagnostic performance rate (AUC=0.96, p<0.001) that supports its use as a central measure of hypercoagulability and severe pandemic health outcomes. AST and LDH tests showed strong diagnostic values (AUC=0.79 and 0.76) when combined with evidence of systemic inflammation and tissue damage. The results showed that CRP provided minimal diagnostic benefit (AUC = 0.28, p = 0.02) to distinguish severe cases which suggests its use should support diagnostic procedures instead of serving as the primary assessment tool for COVID-19. Tracking biomarker levels during disease progression demonstrates their effectiveness as tools to assess both the patient's condition and determine appropriate timely interventions. Energy metabolism measurements including D-dimer and LDH show different patterns between sexes, thus supporting the utilization of custom COVID-19 management methodologies.

Declarations

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Author contributions

Conceptualization, Z.A.A. and A.N.M.; Methodology, W.N.H.; Software, Z.A.A.; Validation, I.M.A-B., A.N.M. and H.J.; Formal Analysis, A.N.M.; Investigation, Z.A.A.; Resources, H.J.; Data Curation, I.M.A-B.; Writing – Original Draft Preparation, A.N.M.; Writing – Review & Editing, Z.A.A.; Visualization, I.M.A-B.; Supervision, Z.A.A.; Project Administration, H.J.; Funding Acquisition, W.N.H.

Conflicts of interest

All authors declare that there is no conflict of interest.

Data availability

Data related to the study are available upon request and are therefore not publicly available. The University of Basra - Al-Zahra College of Medicine does not object to the authors making the data available upon reasonable request.

Ethics approval

Official approval was granted to conduct this research from the University of Basrah-Al-Zahraa College of Medicine, (no. 4/35 on dated 1-04-2020) and the Basrah Health Directorate.

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ORIGINAL PAPER

Hormonal profiles and metabolic changes in women diagnosed with concomitant Hashimoto's thyroiditis and polycystic ovary syndrome via sonography

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ABSTRACT

Introduction and aim. Women with both Hashimoto's thyroiditis (HT) and polycystic ovary syndrome (PCOS) often experience hormonal imbalances and metabolic changes. We investigated the correlation between sonographic changes and hormonal abnormalities in women with Hashimoto's thyroiditis and PCOS.

Material and methods. A case-control study including 150 women with PCOS and Hashimoto's thyroiditis, and 50 healthy women as a control group, was conducted at Al-Habobbi Teaching Hospital from 7/1/2023 to 7/10/2024. Lipid, blood sugar, luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, testosterone, and thyroid hormones were assessed, the groups had similar mean ages and smoking rates.

Results. When the case group was compared with the control group, significant hormonal and metabolic differences were observed. Specifically, levels of LH were significantly higher in the case group (14.68±1.21 vs. 3.31±1.03 mIU/mL, p=0.001), as were levels of FSH (14.85±1.07 vs. 5.26±0.51 mIU/mL, p<0.001), prolactin (28.90±1.34 vs. 7.02±1.16 ng/dL, p<0.001), and testosterone (57.71±2.61 vs. 12.41±2.27 ng/dL, p<0.001). In terms of lipid profile, the case group showed elevated total cholesterol (229.93±14.61 vs. 134.51±9.38 mg/dL, p<0.001), triglycerides (287.78±41.43 vs. 128.04±10.20 mg/dL, p<0.001), low-density lipoprotein (LDL) (136.98±20.02 vs. 58.67±11.45 mg/dL, p<0.001), and very low-density lipoprotein (VLDL) (57.55±8.28 vs. 25.60±2.04 mg/dL, p<0.001), while levels of high-density lipoprotein (HDL) were significantly lower (35.39±3.54 vs. 50.23±4.55 mg/dL, p<0.001). Regarding thyroid function, thyroxine (T4) levels were significantly reduced in the case group (9.80±0.77 vs. 15.02±1.25, p<0.001), while thyroid-stimulating hormone levels were elevated (6.25±1.10 vs. 2.17±0.74 µIU/mL, p<0.001).

Conclusion. These findings suggest a potential complex interaction between the thyroid and reproductive glands, which may influence the pathogenesis and metabolic effects of these endocrine disorders. However, the individual and combined effects require further detailed investigation.

Keyword. Hashimoto's thyroiditis, polycystic ovarian syndrome, sonographic changes

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most prevalent endocrine and metabolic disorders among women of reproductive age, with a global prevalence ranging between 3% and 15%. This complex disorder affects not only reproductive function but is also strongly associated with metabolic and mental health issues, which may collectively compromise overall health and well-being.[1,2] PCOS is a heterogeneous disorder that varies based on factors such as age, genetics, race, and environmental influences. Despite this variability, PCOS is classified into four phenotypes, which are distinguished by three key factors such as polycystic ovary morphology, ovulatory dysfunction, and elevated androgen levels.[3] Thyroid disorders are also common and often overlapped with PCOS, aside from reproductive dysfunction. Women with PCOS are at an increased risk of thyroid disease such as sub-clinical hypothyroidism and autoimmune thyroiditis (AIT). Researchers have found that women with PCOS are significantly more likely to have hypothyroidism, with a prevalence of 11% to 14%, compared to only 1% to 2% among women without the disorder. Both conditions commonly share metabolic abnormalities such as insulin resistance, dyslipidemia, and obesity.[4,5] The most common autoimmune thyroid disorder is HT, first reported by Hakaru Hashimoto in 1912. However its ability to cause inflammation was not fully realized until the 1950s. Epidemiologic data suggests that the most common cause of hypothyroidism is HT.[6] The disease is characterized by lymphocytic infiltration of the thyroid gland and may present in various forms, including painless (silent) thyroiditis and subclinical or overt hypothyroidism. HT could be secondary to immune-modulating medical therapies like interferon-alpha or monoclonal antibodies against Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).[7,8] Abnormal thyroid function is a common hormonal imbalance in both PCOS and HT. Hypothyroidism is often caused by an autoimmune response in which the immune system produces antibodies against thyroid proteins such as thyroid peroxidase (TPO) and thyroglobulin (TG). This autoimmune activity may interfere with estrogen metabolism. Elevated estrogen levels are associated with enhanced humoral immunity, while androgen and progesterone levels are generally reduced in women with PCOS.[9,10] The metabolic disturbances observed in both conditions such as insulin resistance, dyslipidemia, and weight gain are significantly more pronounced in women with concurrent PCOS and hypothyroidism.[11] Dysfunction of thyroid has been linked with a more severe metabolic profile in the population of PCOS. Patients with either condition have higher triglycerides or fasting insulin or homeostasis model assessment of insulin resistance (HOMA-IR) than subjects with PCOS alone. Obese women with HT and longstanding PCOS show significantly greater body mass index (BMI), fasting blood glucose and cholesterol levels than controls or those with HT alone.[12,13] Moreover, the coexistence of PCOS and hypothyroidism has been associated with more severe metabolic and hormonal disturbances. Studies have shown that correcting thyroid hormone levels in hypothyroid patients leads to significant improvements in metabolic health. These findings support the growing body of evidence linking thyroid dysfunction with the metabolic abnormalities commonly observed in patients with PCOS.[14,15]

Aim

The aim of this study is to investigate the correlation between sonographic changes and hormonal abnormalities in women with both HT and PCOS. The study will analyze sonographic changes in the ovaries and thyroid, correlating these with hormone levels such as thyroid hormones and androgens.

Material and methods

This case-control study included 150 women diagnosed with both HT and PCOS. Diagnosis of PCOS was confirmed by physicians using the Rotterdam criteria, which require at least two of the following: irregular ovulation, clinical or biochemical signs of hyperandrogenism, and polycystic ovarian morphology on ultrasound. Participants in the case group were consecutively recruited as they presented and met the inclusion criteria. The control group consisted of 50 age-matched healthy women who were randomly selected from individuals attending routine check-ups. The health status of the control participants was verified through clinical evaluation, detailed medical history, and laboratory screening to exclude thyroid or reproductive disorders. These sample sizes of cases and controls were selected to achieve adequate statistical power and robust comparisons, given the estimated prevalence of PCOS and HT. This 3:1 ratio optimized data collection from cases while preserving sufficient controls. The age range (18–45 years) was chosen to encompass the reproductive years, guiding enrollment based on study endpoints and supported the feasibility and objectives of the study. All information was recorded by participants and transcribed. The study was conducted at Al-Haboubi Teaching Hospital for the period between 1/7/2023 to 10/7/2024. Women with hyperthyroidism, those on treatment, and patients who had had their thyroid removed were excluded. 5 mL was collected from each participant and placed in a gel tube and left for 15 minutes at room temperature until clotting. Serum was separated using a centrifuge at 3500 rpm for 15 minutes. The blood serum was isolated and kept at a temperature of minus 20°C until use. Lipid profile levels (total cholesterol, low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and

high-density lipoprotein (HDL)) and fasting blood sugar (FBS) were estimated using colorimetric methods using a spectrophotometer (Biolabo, Firance). The levels of triiodothyronine (T3), thyroxine (T4), thyroid-stimulating hormone (TSH), thyroid peroxidase antibodies (TPOAb), luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, and testosterone were estimated using the Cobas E411 device (Roche, German). Thyroid hormone levels were measured in enzyme-linked immunosorbent assay (Biotechne, USA). Additionally, all assays were validated in terms of their sensitivity (limit of detection), specificity, and variability (coefficient of variation, CV%), which were closely monitored to ensure the reliability of the results. These measures guarantee the robustness of the findings and enhance the overall credibility of the study.

Statistical analysis

Statistical analysis was conducted using SPSS version 26 (IBM, Armonk, NY, USA). Data were expressed as frequencies, percentages, and means±SD. Independent and dependent t-tests were applied for normally distributed variables, while Mann-Whitney U, Wilcoxon, and Chi-square tests were used for non-normal data. A p-value <0.05 was considered statistically significant.

Ethical approval

Prior to sample collection, all participants were thoroughly informed about the study's objectives and procedures. Written informed consent was obtained from each participant, confirming their voluntary participation. The study was approved by the Committee on Publication Ethics at the Thi-Qar Health Directorate (Al Habbobi Teaching Hospital) and Ninevah Health Directorate (Al Batool Teaching Hospital) under approval form No. 3997, dated January 7, 2023.

Results

Socio-demographic characteristics and clinical profiles of patients with concomitant HT and PCOS

The study revealed significant demographic and clinical differences between the control and case groups. While there was no significant difference in mean age (years) (p=0.92) or smoking status (p=0.77), BMI was notably higher in the case group (32.72±2.95 vs. 23.63±1.80 kg/m^2, p<0.001). Physical activity was lower in the case group (2.1±1.1 vs. 3.5±1.2 hours/week, p=0.02), and a higher proportion of the case group had a family disease history (40% vs. 10%, p<0.001). Differences in education (secondary education: 30% vs. 50%, p=0.05), marital status (unmarried: 60% vs. 40%, p=0.03), and socioeconomic status (low class: 50% vs. 20%, p<0.001) were also significant, as detailed in Table 1.

Table 1. A comparative analysis of demographic, lifestyle, and clinical parameters

Parameters	Control group (n=50) Mean±SD	Case group (n=150) Mean±SD	p
Age (years)	28.98±7.10	29.09±7.35	0.92
BMI (kg/m^2)	23.63±1.80	32.72±2.95	<0.001
Smoking status (%)	15% (7)	18% (27)	0.77
Physical activity (hours/week)	3.5±1.2	2.1±1.1	0.02
Family history (%)	10% (5)	40% (60)	<0.001
Education level	High school: 50% (25)	High school: 30% (45)	0.05
	College: 30% (15)	College: 40% (60)	
	University: 20% (10)	University: 30% (45)	
Marital status (%)	Single: 40% (20)	Single: 60% (90)	0.03
	Married: 60% (30)	Married: 40% (60)	
Socioeconomic status	Low: 50% (25)	Low: 20% (30)	<0.001
	Medium: 40% (20)	Medium: 50% (75)	
	High: 10% (5)	High: 30% (45)	

Ultrasound findings

Transvaginal ultrasound imaging revealed characteristic features in patients presenting with ovarian and thyroid abnormalities. As shown in Figure 1, both ovaries appeared bilaterally enlarged with borderline dimensions. Multiple small follicles, each numbering more than ten, were observed to be arranged peripherally a typical finding consistent with polycystic ovary morphology. In Figure 2, a significantly enlarged right ovary was noted, exhibiting a peripheral distribution of follicles and a centrally located echogenic stroma, further supporting the diagnosis of ovarian dysfunction. Regarding thyroid abnormalities, Figure 3 demonstrates a longitudinal scan of a female patient diagnosed with nodular Hashimoto's thyroiditis. The image reveals a solid, hypoechoic, homogeneous nodule with poorly defined margins, while the background thyroid parenchyma appears normal. In contrast, Figure 4 displays a transverse scan from another patient with the same diagnosis, showing a solid, hyperechoic, homogeneous nodule with sharply defined margins and a thin hypoechoic halo. The background parenchyma in this case exhibits a micronodular pattern, which is commonly associated with chronic autoimmune thyroiditis.

Lipid profile alterations in patients with concomitant HT and PCOS

The study demonstrated significant differences in lipid profiles between the case and control groups. The case group had higher mean total cholesterol (229.93±14.61 vs. 134.51±9.38 mg/dL, p<0.001), triglycerides

(287.78±41.43 vs. 128.04±10.20 mg/dL, p<0.001), LDL (136.98±20.02 vs. 58.67±11.45 mg/dL, p<0.001), and VLDL (57.55±8.28 vs. 25.60±2.04 mg/dL, p<0.001). In contrast, HDL levels were lower in the case group (35.39±3.54 vs. 50.23±4.55 mg/dL, p<0.001). These findings highlight a significant impact of disease state on lipid profiles, as shown in Table 2.

Table 2. A comparative study of serum lipid levels between control and case groups

Parameters	Control group (n=50) Mean±SD	Case group (n=150) Mean±SD	p
TC (mg/dL)	134.51±9.38	229.93±14.61	<0.001
TG (mg/dL)	128.04±10.20	287.78±41.43	<0.001
HDL (mg/dL)	50.23±4.55	35.39±3.54	<0.001
LDL (mg/dL)	58.67±11.45	136.98±20.02	<0.001
VLDL (mg/dL)	25.60±2.04	57.55±8.28	<0.001

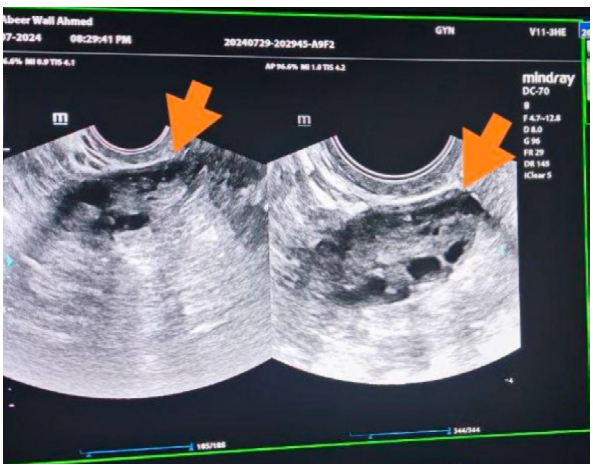

Fig. 1. Transvaginal ultrasound image shows bilaterally enlarged ovaries with a borderline size, multiple small follicles, more than 10 in number, are located peripherally

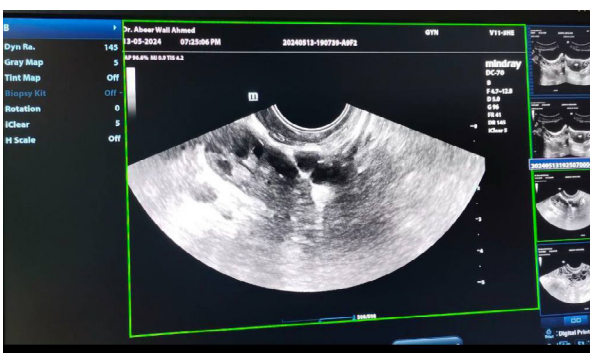

Fig. 2. Transvaginal ultrasound reveals a significantly enlarged right ovary, characterized by peripherally distributed follicles and a centrally located echogenic stroma

Hormonal profile differences in patients with HT and PCOS

The study revealed significant hormonal differences between the case and control groups. The case group had a significantly higher mean level of LH (14.68±1.21 vs. 3.31±1.03 mIU/mL, p=0.001), FSH (14.85±1.07 vs. 5.26±0.51 mIU/mL), prolactin (28.90±1.34 vs. 7.02±1.16 ng/dL, p<0.001), and testosterone (57.71±2.61 vs. 12.41±2.27 ng/dL, p<0.001). These findings indicate notable changes in circulating hormones, demonstrating the differential effects of disease on hormone levels, as shown in Table 3.

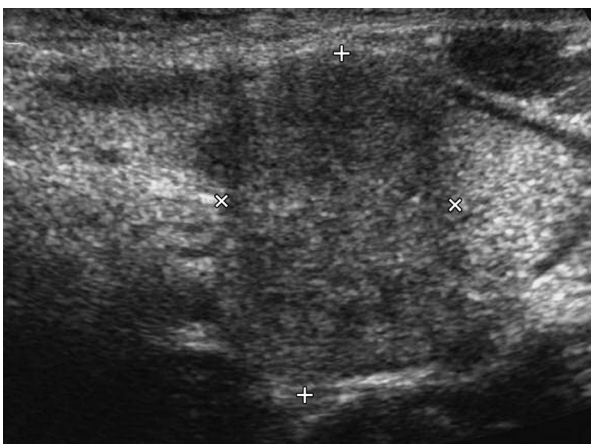

Fig. 3. Woman with nodular HT, longitudinal scan shows solid hypoechoic homogeneous poorly marginated nodule (cursors), background parenchyma is normal

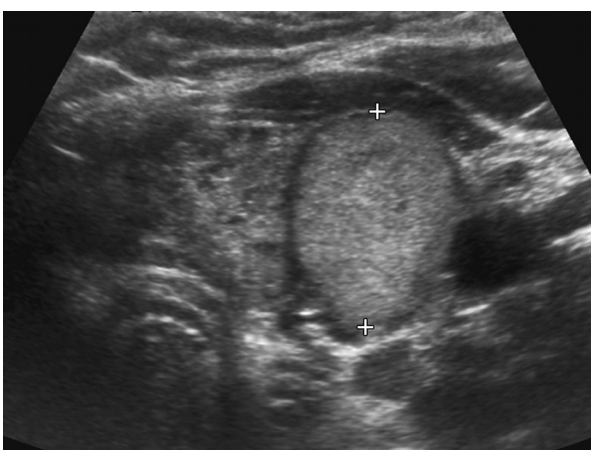

Fig. 4. Woman with nodular HT, transverse scan shows solid hyperechoic homogeneous sharply marginated nodule (cursors) with thin hypoechoic halo, background parenchyma is micronodular

Table 3. A comparative analysis of LH, FSH, prolactin, and testosterone levels

Parameters	Control group (n=50) Mean±SD	Case group (n=150) Mean±SD	p
LH (mIU/mL)	3.31±1.03	14.68±1.21	<0.001
FSH (mIU/mL)	5.26±0.51	14.85±1.07	<0.001
Prolactin (ng/mL)	7.02±1.16	28.90±1.34	<0.001
Testosterone (ng/dL)	12.41±2.27	57.71±2.61	<0.001

Comparative study of glycemic and thyroid function parameters in patients with HT and PCOS

The study showed significant differences in thyroid function between the case and control groups. While FBS and T3 levels were similar between the groups (p=0.53 and p=0.52, respectively), T4 levels were significantly lower in the case group (9.80±0.77 vs. 15.02±1.25 μg/dL, p<0.001) and TSH levels were significantly higher (6.25±1.10 vs. 2.17±0.74 μIU/mL, p<0.001). These findings highlight the significant impact of disease status on thyroid function, as shown in Table 4.

Table 4. Analysis of fasting blood sugar and thyroid hormone levels between control and case groups

Parameters	Control group (n=50) Mean±SD	Case group (n=150) Mean±SD	p
FBS (mg/dL)	95.54±8.81	96.46±9.05	0.53
T3 (ng/dL)	4.46±0.54	4.52±0.55	0.52
T4 (μg/dL)	15.02±1.25	9.80±0.77	<0.001
TSH (μIU/mL)	2.17±0.74	6.25±1.10	<0.001

Correlation analysis of age, BMI, lipid profile, hormonal levels, and glycemic parameters in patients with HT and PCOS

The Table 5 shows correlations between key clinical parameters using Spearman's test. Significant relationships were found between some key variables. For example, LDL, VLDL, and TG levels showed strong negative correlations with each other (p<0.01), indicating an interdependence between these lipids. A significant positive correlation was also observed between TSH, T4, and T3 (p<0.01), indicating a hormonal interaction between these substances that play a role in thyroid regulation. On the other hand, testosterone showed a moderate correlation with BMI (p<0.05), reflecting its potential effect on lipids or body weight. FBS levels also showed relatively weak correlations with most other parameters. An asterisk (*) indicates statistical significance, with one asterisk meaning p<0.05 and two asterisks meaning p<0.01, reflecting different levels of statistical significance of these relationships.

Discussion

Thyroiditis is a diverse disorder that impacts the thyroid gland and has multiple underlying causes. HT, or chronic lymphocytic thyroiditis, is an AIT that frequently occurs in young women and is a prevalent form of thyroid inflammation. HT can occur simultaneously with clinical hypothyroidism, normal thyroid function, or hyperthyroidism.[16] Significant differences between the control and case groups have been observed at the level of various patient demographic, lifestyle, and clinical parameters, as described below. Age (p=0.92) is matched between groups, removing a confounder of age found in studies including, which used age-matched cohorts to minimize bias.[17] The much higher BMI in the case group (32.72±2.95 vs. 23.63±1.80 kg/m^2, p<0.001) emphasizes the contribution of obesity in making people more susceptible to the disease, like, which elevated BMI as a predictor of metabolic disorders; however, Serin et al. pointed out that only calculating fat mass or BMI is not enough and should be placed in the context of body composition and factors reflecting metabolic activity with the call for a better metabolic fingerprint.[18,19] Given that there was no substantial difference in smoking status (p=0.77), we agree with Arduc et al., who described the variability in smoking exposure impact across cultural contexts in contrast to the findings of Ho et al., who highlighted smoking as a major risk factor.[20,21] The reduction in physical activity in the case group (2.1±1.1 hours/week vs. 3.5±1.2 hours/week, p=0.02) is concordant with, making associations between sedentary behavior and chronic disease risk; however, argued that differences in dietary intake may play a more significant role.[22] The crystal-clear family history (40% vs. 10%, p < 0.001) risk positioned the case group up significantly which underpins genetic susceptibility as supported by,

Table 5. Evaluating interrelationships among key clinical variables[a]

Parameters	BMI	TC	TG	HDL	LDL	VLDL	LH	FSH	Pro.	Test.	FBS	T3	T4	TSH
BMI	1.000	-0.070	0.018	0.065	-0.070	0.018	-0.147	0.015	0.002	0.190*	0.000	0.005	-0.013	0.053
TC	-0.070	1.000	-0.287**	-0.281**	0.898**	-0.287**	0.028	-0.033	0.106	0.009	0.035	-0.070	0.152	-0.194*
TG	0.018	-0.287**	1.000	0.128	-0.646**	0.287**	0.073	0.094	-0.140	0.089	0.000	0.046	-0.320**	0.270**
HDL	0.065	-0.281**	0.128	1.000	-0.435**	0.128	-0.040	-0.083	-0.115	-0.040	0.128	-0.057	-0.040	0.248**
LDL	-0.070	0.898**	-0.646**	-0.435**	1.000	-0.646**	-0.003	-0.048	0.156	-0.023	0.036	-0.060	0.251**	-0.297**
VLDL	0.018	-0.287**	0.287**	0.128	-0.646**	1.000	0.073	0.094	-0.140	0.089	0.000	0.046	-0.320**	0.270**
LH	-0.147	0.028	0.073	-0.040	-0.003	0.073	1.000	0.094	0.070	0.089	-0.062	-0.070	0.046	0.270**
FSH	0.015	-0.033	0.094	-0.083	-0.048	0.094	0.094	1.000	0.070	0.094	-0.014	-0.044	-0.017	0.047
Pro.	0.002	0.106	-0.140	-0.115	0.156	-0.140	0.070	0.070	1.000	-0.041	-0.093	0.069	-0.035	-0.096
Test.	0.190*	0.009	0.089	-0.040	-0.023	0.089	0.089	0.094	-0.041	1.000	0.278	0.119	-0.138	0.038
FBS	0.000	0.035	0.000	0.128	0.036	0.000	-0.062	-0.014	-0.093	0.278	1.000	0.026	0.063	-0.088
T3	0.005	-0.070	0.046	-0.057	-0.060	0.046	-0.070	-0.044	0.069	0.119	0.026	1.000	-0.091	-0.006
T4	-0.013	0.152	-0.320**	-0.040	0.251**	-0.320**	0.046	-0.017	-0.035	-0.138	0.063	-0.091	1.000	-0.156
TSH	0.053	-0.194*	0.270**	0.248**	-0.297**	0.270**	0.270**	0.047	-0.096	0.038	-0.088	-0.006	-0.156	1.000

[a] * – p<0.05, ** – p<0.01

who highlighted heredity as risk factor, while Mukherjee et al. that environmental changes can reduce genetic susceptibility.[23,24] Differences in education level with a moderate link (p=0.05) suggest that higher education facilitates better health management. The greater proportion of singles within the case group (60% vs. 40%, p=0.03) highlights a possible association with stress and limited social support. Additionally, the elevated socioeconomic status in the case group (p<0.001) challenges traditional assumptions and indicates region-specific factors or sample-related biases. These findings suggest multifactorial relationships genetic, lifestyle, and socioeconomic that require further investigation in regional and cultural contexts, as these parameters collectively influence human health and protection against various pathologies.[25,26] The values in table 2 demonstrate a statistically significant difference in serum lipids observed in the case group versus controls, with total cholesterol, triglycerides, LDL, and VLDL being higher and HDL being lower in the case group, all with p<0.001. These findings strongly point towards dyslipidemia in the case group, an established determinant of cardiovascular and metabolic disorders. In the case group, the total cholesterol and LDL were higher (229.93±14.61 and 136.98±20.02 mg/dL, respectively), a finding in agreement with Cai et al., emphasized the importance of hypercholesterolemia for atherosclerosis risk.[27] Contrarily, Zhao et al., These findings suggest that the majority of lipid dysregulations are a function of abnormal diets, and not due to genetic or pathophysiological predispositions.[28] The high levels of triglycerides (287.78±41.43 mg/dL) and VLDL (57.55±8.28 mg/dL) are similar with.[29] Establishing connections with each of these parameters with insulin resistance and metabolic syndrome. However, Arduc et al. indicated that such elevations could be related to genetic factors, especially in populations with a history of dyslipidemia.[30] Reduced HDL levels (35.39±3.54 in the case group vs. 50.23±4.55 mg/dL in controls) were in accordance with Fan et al. and noted that HDL provided a protective effect and the absence of it also increased cardiovascular risk. Such dietary interventions have been shown to increase HDL levels, and so even though HDL levels are affected by risk factors at their origins as mentioned, this leaves open the possibility for corrective action to be taken to address the imbalance.[31] As proposed by Palomba et al. (2023), the dyslipidemic profile in the case group can be attributed on a scientific basis to chronic inflammation, oxidative stress and changes in lipid metabolism pathway.[32] Studie like Batóg et al., also support this interpretation, which stated that systemic inflammation directly disrupts lipid metabolism by reducing the production of HDL and increasing the production of LDL and VLDL. This highlights the need for timely interventions such as lifestyle changes, medications to manage lipids to reduce the risk of chronic diseases resulting from poor lipid profiles. Future work should investigate the genetic and molecular pathways associated with myogenic lipids in finer detail to better align therapeutic targeting approaches.[33] The case group has higher total cholesterol, triglycerides, LDL, and VLDL levels, and lower HDL levels, which means more significant atherosclerosis risk and cardiovascular diseases are mediated. Management of dyslipidemia involves lifestyle modifications (diet, exercise) and medicinal therapy (statins or fibrates) to correct lipid abnormality to normal. "Indeed, the low HDL levels most specifically demand treatment programs designed to raise HDL, such as weight loss and aerobic exercise. Dysfunction of thyroid hormones aggravates aberrations of lipid metabolism mediated by the way of decreased hepatic lipase activity and disrupted cholesterol transport.[31,32] Levothyroxine therapy for correcting hypothyroidism may also improve the lipid profile. Elevated androgen levels contribute to increased insulin resistance, which exacerbates dyslipidemia. Both androgen excess and lipid disturbances can be managed with insulin-sensitizing agents, such as metformin. Addressing the complex interaction between hormonal and lipid abnormalities requires a multidisciplinary approach involving endocrinologists, gynecologists, and dietitians. Early diagnosis and targeted treatment are essential to prevent long-term complications, such as type 2 diabetes, cardiovascular diseases, and infertility.[32,33] The key is to monitor thyroid function and lipid profiles regularly, which allows for adjustment of therapy to promote optimal patient outcomes. Tailored therapy, according to the severity of hormonal and metabolic derangements, may result in increased effectiveness and less adverse effects. Chronic low-grade inflammation, pathological in PCOS and thyroid disorders for example, may also play a role in modulating metabolic profiles. Anti-inflammatory agents (e.g., omega-3 fatty acids or antioxidants) may supplement standard therapies.[33] Table 3 shows the differences in hormonal levels between control and case groups, noting that levels of LH, FSH, prolactin and testosterone were higher in the case group with p<0.001. Strong evidence suggest disruption of hypothalamic-pituitary-gonadal axis which may be associated with bas pathology or some physiological conditions. The significantly high LH and FSH levels of case group are similar to Lee et al., who noted similar hormonal spikes in diseases such as PCOS or gonadal dysfunction. Heightened gonadotropins might represent compensatory mechanisms to correct disrupted gonadal feedback or increased androgen synthesis.[34] However, Batóg et al., further proposed that such elevations could be attributed to hypothalamic hyperactivity or pituitary adenomas, warranting additional clinical evaluation.[35] Prolactin were significantly higher in the case group in agreement with Davoudi et al., associated hyperprolactinemia with

stress or the pituitary disorders. Elevated prolactin can inhibit gonadotropin secretion leading to a feedback dysregulation worsening hormone dysregulation.[36] Contrastingly, Elnour et al., postulated that transient elevation in prolactin levels could also stem from medication or physiological stress, necessitating distinction from pathological causes.[37] The excessively high testosterone levels are indicative of hyperandrogenism, the classic sign of a disease such PCOS or adrenal hyperplasia. This observation is in agreement with what was observed by Ma et al., who identified androgen excess as a key driver of metabolic and reproductive abnormalities.[38] However, Kim et al. indicated that elevations in testosterone could be derived from exogenous supplementation or tumors, thus requiring more extensive diagnostic workup.[39] These hormonal derangements might be indicative of systemic inflammation, oxidative stress, or genetic mutations that influence endocrine pathways, according to Abdul-Ameer. Finally, lifestyle factors with obesity and insulin resistance, which commonly come along with dysregulated testosterone and prolactin levels.[40] Data in Table 4 demonstrated significant variance in T4 levels of the control and case group however, FBS and T3 levels showed non-significant variance. The similar results were observed for FBS and T3, indicating that these parameters are not significantly affected by the condition of interest, consistent with Elslimani et al., who stated that increased T3 in the presence of normal FBS could reflect early thyroid dysfunction or non-metabolic conditions.[41] However, Novais et al. suggested that glucose metabolism changes may happen in later stages, and longitudinal studies are needed to confirm.[42] On the contrary, T4 levels in the case group were markedly lower ($p<0.001$), while TSH levels were higher ($p<0.001$). This pattern is consistent with primary hypothyroidism, as outlined by Gaberšček et al. further recognized lower T4 and higher TSH as key evidence of dysfunction of thyroid gland.[43] Du et al. also note that elevated TSH reflects compensatory activity in the pituitary gland in response to insufficient thyroid hormone production. Elevated TSH and reduced T4 levels indicate subclinical or overt hypothyroidism, likely linked to Hashimoto's thyroiditis. Thyroid hormone replacement therapy with levothyroxine is essential to restore euthyroid status, improve metabolic functions, and mitigate long-term cardiovascular risks.[44] Extreme dysfunction of the thyroid gland may be due to autoimmune processes, chronic inflammation or iodine deficiency.[45] also found that hypethyroidization of the hypothalamic-pituitary-thyroid axis due to systemic stress might also be involved. In contrast to Shanmugham et al., observing small reductions in T4 without elevated TSH, highlight the heterogeneous nature of thyroid dysfunction, potentially influenced by genetic and environmental factors.[46] The elevation of both LH and testosterone in the case group indicates hyperandrogenism, a hallmark of PCOS, strongly linked with menstrual irregularities, infertility and hirsutism. The treatment performed is going to lower amount of androgen in the body and this consist of combined oral contraceptive or anti-androgens like spironolactone. Elevated FSH levels and alterations in the LH:FSH ratios might decrease ovarian folliculogenesis. Ovulation induction agents such as clomiphene citrate or letrozole may be used to restore ovulatory cycles. Hyperprolactinemia was also noticed in the case group which can be a sign of pituitary invasion or an alteration of the endocrine system was related to stress. In order to normalize the serum prolactin levels and concomitant symptoms such as galactorrhea and infertility, pituitary function tests and dopaminergic medications such as cabergoline should be discussed.[47]

Study limitations

Although a single-center design is superior for research focused on clinical needs, the paradigm could be stretched to multicenter operations to advance generalizability of findings. A lack of demographic characteristics could impact results, and controlling for them would make stronger conclusions, as seen from differences between the case and control groups. Likewise, greater diversity would enhance the applicability of the study to different ethnic backgrounds, allowing for more global conclusions. Implementing a longitudinal design that considers key lifestyle aspects will advance the awareness of the interrelationship of these conditions and the potential implications they will have throughout lives, laying foundations for further deep and actionable research.

Conclusion

In summary, the results show significant differences regarding the hormonal and lipid profiles between HT and PCOS women and healthy control group. The evidence indicates a potential bidirectional relationship between the thyroid and reproductive gland that may contribute toward the pathogenesis and metabolic consequences of these endocrine disorders. Nonetheless, more extensive exploration of their isolated and collective roles is warranted. The absence of separate subgroup analysis in the present study hampers the possibility of exploring the effects of PCOS or HT in isolation or in combination. Future studies will need to revisit the design to allow subgroup comparisons and potentially to elucidate the relationship between these two endocrine disorders.

Declarations

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Author contributions

Conceptualization, O.A. and A.W.; Methodology, O.A.; Software, O.A.; Validation, H.A., S.A. and Z.Z.; Formal Analysis, A.W.; Investigation, O.A.; Resources, H.A.; Data Curation, S.A.; Writing – Original Draft Preparation, O.A.; Writing – Review & Editing, A.W.; Visualization, S.A.; Supervision, H.A.; Project Administration, O.A.; Funding Acquisition, A.W.

Conflicts of interest

The authors declare that they have no competing interests.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval

Ethical approval was obtained from the Ethics Committee at the Thi-Qar Health Directorate, Al Habbobi Teaching Hospital, and Ninevah Health Directorate, Al Batool Teaching Hospital (Approval No. 3997, dated January 7, 2023).

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ORIGINAL PAPER

A cross-sectional study of psychosocial variables associated with medication burden among type 2 diabetes mellitus with multiple comorbidities in geriatric patients

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ABSTRACT

Introduction and aim. As the population ages, the management of type 2 diabetes mellitus in older adults with multiple comorbidities becomes more complex. Geriatric patients often face a medication burden, impacting their quality of life and adherence. Psychosocial variables such as depression, anxiety, social support, and health literacy may influence how patients cope with their medications. This study explores the relationship between these variables and the burden in geriatric patients with type 2 diabetes and comorbidities, offering insight into improving care and outcomes.

Material and methods. The cross-sectional survey was carried out from April to September 2024. 250 patients were included. The demographics of the participants, the burden of the disease, polypharmacy, the burden of the medication, and the psychosocial variables were evaluated. Univariate and multivariate linear regression analyzes assessed the variables associated with the burden of medications.

Results. There was a positive correlation between the belief in medication, depression, disease burden, number of medications, number of comorbidities, and medication burden ($p<0.05$). Knowledge about medications was not significantly correlated with the burden ($p>0.05$).

Conclusion. Low self-efficacy, depression, polypharmacy, high disease burden, and decreased medication satisfaction can all contribute to medication burden. Comprehending these factors helps identify patients with geriatric diabetes and enables personalized treatment to ease their burden.

Keywords. geriatric patients, medication burden, multiple comorbidities, psychosocial variables, type 2 diabetes mellitus

Introduction

The global prevalence of type 2 diabetes is increasing, with 25.2 million adults affected by impaired glucose tolerance (IGT) today expected to rise to 35.7 million by 2045. India is second after China, with 77 million people living with diabetes, particularly older adults over 60, due to aging, urbanization, poor lifestyle choices and lack of physical activity. To maintain this glycemic target and prevent long-term problems, type 2 diabetes frequently requires a gradual increase in pharmaceutical therapy in addition to lifestyle modifications.[1] The geriatric cohort, often burdened with multiple comorbidities such as hypertension, cardiovascular disease, osteoarthritis, and dyslipidemia, faces unique challeng-

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es in the management of type 2 diabetes mellitus, all of which put diabetes patients at high risk of polypharmacy. According to a national population-based survey, more than 80% of older adults in India were prescribed at least one drug and over one-third were taking at least five prescription drugs at the same time.[2]

Polypharmacotherapy and high pill load are caused by microvascular and macrovascular problems related to diabetes, as well as the risk of linked comorbidities such as hypertension and hyperlipidemia in many patients with type 2 diabetes. These challenges are not only medical but also psychosocial, and the complexities of managing multiple chronic diseases frequently leading to medication burden that significantly impacts quality of life.[3]

Geriatric patients with type 2 diabetes with multiple comorbidities are particularly vulnerable to the negative impacts of medication burden, which may manifest itself as increased difficulty in adhering to treatment, a higher risk of drug interactions, and the potential for adverse drug reactions. The psychosocial aspects of managing chronic diseases, such as mental health problems, social support, cognitive function, and health literacy, can significantly influence how elderly patients perceive and manage their medication regimens.[4] These psychosocial variables can either exacerbate or alleviate the challenges associated with medication burden.

Medication adherence is complicated by aging-related physiological changes, such as altered drug metabolism and declining cognitive functions, which make it difficult for older adults to comply with multidose schedules. Different physical conditions, such as arthritis or a lack of financial resources, can also contribute to the difficulty of drug intake. Research conducted in Spain indicates that the more chronic diseases accumulated by older people, the more medicine-related problems appeared: drug interactions and inappropriate prescriptions.[5]

The medication burden is the time and effort required to manage chronic diseases, which is especially high in older patients with multiple comorbid conditions. A significant part of this is the medication burden, which is the workload and burden associated with medication use.[5] Maidment et al. defined five types of medication burden: ambiguity (unclear medication management), concealment (lack of information sharing), unfamiliarity (inconsistent care providers), fragmentation (difficulty navigating fragmented healthcare services), and exclusion (lack of participation in decision making). These factors significantly affect the ability of older adults to manage their health effectively.[6]

Overuse of medications can result in poor social functioning, noncompliance, suboptimal treatment outcomes, and a lower quality of life.[7] The burden depends on both the individual's ability to manage the burden and the workload associated with taking medications. According to the cumulative complexity model, an adaptive balance between burden and capacity is necessary for the successful self-management of chronic disease.[8]

Managing pharmaceutical loads and their impact on health results from internal resources of the patient (functional and cognitive abilities) combined with external support, including the healthcare system and the family.[9] The term "medication self-management capacity" refers to the skills and knowledge needed to correctly follow a prescribed regimen, including the filling, organizing, taking and monitoring medications. Demographic factors, including age, education level, and financial background, influence these abilities, while some patient attitudes and external support could further facilitate or hinder medication management. "Understanding the competence of patients in medication self-management is relevant because difficulties faced by patients with respect to the control of their prescription indicate areas of intervention".[10]

Attributes are factors directly affecting patient satisfaction of the patient on medication, which makes their satisfaction dependent on treatment expectations, side effects, ease of use, and efficiency. Patients report discontent primarily due to a lack of perceived benefits, side effects, or inconvenience. Negative perceptions about medication often evolve from dissatisfaction. Patients dissatisfied with their treatment are less effective at managing side effects and are even less likely to incorporate medications into their daily lives.[11]

Most research on the burden is qualitative, focusing on drug use, with some studies examining the impact of clinical and demographic factors. Univariate analyses often produce incomplete data due to the interdependence of these factors. Limited literature exists on psychosocial influences that contribute to medication burden among patients with type 2 diabetes mellitus (T2DM). Previous studies observe that healthcare providers are prone to overestimate patients' ability to handle their medication and management issues; therefore, knowledge of psychological factors associated with medication burden becomes imperative for implementing suitable treatment plans for improved compliance and self-management.[12]

However, older adults with T2DM and multimorbidity are most susceptible to medication burden and low self-management. Therefore, understanding these psychosocial variables is paramount in patient-centered care strategies to promote adherence, reduce complications, and improve quality of life.

Aim

Given the increasing burden of T2DM globally and, particularly, in India, this study aims to evaluate psychosocial variables related to the medication burden among elderly T2DM patients with comorbidities.

Material and methods

Study design and participants

Considering that geriatric patients may face challenges in terms of mobility, cognitive function, and participation in long-term studies, we conducted a cross-sectional study at the tertiary healthcare center from April to September 2024. The single proportion sample size formula was used; therefore, the proportion was taken as 50%, with a margin of error of 6% and a confidence interval of 95%. $n=z^2 p(1-p)/w^2$, where n=sample size, p=proportion (50%), z=1.96 confidence level, w=margin error (6%) and $n=1.96^2(0.5(1-0.5)/(0.06)^2=267.89$. Therefore, n=268. In that 12 patients were not interested in participating in the study. And 6 patients had missing data, and their responses were unclear. Hence, 18 patients were excluded. And based on the G* power analysis with a medium effect size (Cohen's d=0.5), assuming 80% power and a 0.05 alpha level, the sample size of 250 patients is sufficient to detect the observed effects. Finally, a total of 250 patients were recruited in this study.

Inclusion and exclusion criteria

Patients with type 2 diabetes mellitus 60 years or above with multiple comorbidities, prescribed with one or more medications and able to self-administer medications were included in the study, and the exclusion criteria were having type 1 or gestational diabetes, cognitive impairment, severe mental illness, deafness and not willing to participate in the study.

Data collection tools

Demographic characteristics

The first section assesses demographic characteristics, including age, sex, marital status, education level, monthly income and type of multimorbidity.

Medication knowledge

The second section of the survey assesses the patient's knowledge of medications. Medication knowledge was assessed using the Patient's Perceived Knowledge in Medication Use Questionnaire (PKMUQ).[13] The assessment includes five items that assess the knowledge of medication use and drug interactions. The survey uses a 5-point Likert scale, with 1 representing severe disagreement and 5 representing strong agreement. The scores for each item are added together to create a total score that ranges from 5 to 25. A higher score denotes greater awareness of medications. Cronbach's alpha for the scale in the current study was 0.738.

Medication beliefs

The Beliefs About Medication Questionnaire (BMQ), which included 18 items, was used to assess medication beliefs.[14] The BMQ consists of two sections with two subscales each. (1) BMQ-Specific assesses beliefs about the necessity and concerns (5 items), while (2) BMQ-General assesses beliefs about pharmaceutical damage and overuse by physicians (4 items). The items were evaluated on a 5-point Likert scale, with 1 indicating strong disagreement and 5 indicating strong agreement. A higher overall score suggests greater trust in the relevant notions of each subscale. The current study had a Cronbach's alpha of 0.702 for the BMQ.

Treatment satisfaction

The Treatment Satisfaction Questionnaire Version II (TSQM-II) was used to assess medication treatment satisfaction.[15,16] Four dimensions are included in the 11-item survey: global satisfaction, side effects, effectiveness, and convenience. A five- or seven-point Likert scale is used to rate each item. The scores of the components that make up each dimension are added together to determine the composite score, which is then converted into a number between 0 and 100. This study evaluated participants' satisfaction with pharmacological treatments using the global satisfaction score. A higher score denotes greater contentment with the use of medications. The scale's Cronbach's alpha in the current study was 0.643.

Depression

The nine-item Patient Health Questionnaire (PHQ) was used to measure depression.[17] The purpose of this scale is to collect information about a person's depressive episodes during the last two weeks. The Likert scale has four points (0 being not at all and 3 being almost daily). A higher score denotes a stronger depression; the total score ranges from 0 to 27. In the current investigation, the scale's Cronbach's alpha was 0.759.

Polypharmacy and disease burden

Furthermore, participants were questioned about the quantity of medications they were currently using. If an elderly person took five or more medications, it was considered that they had polypharmacy. The Cumulative Illness Rating Scale-Geriatric (CIRS-G) was used to evaluate the burden of disease in 14 bodily systems in an individual.[18-20] The severity of each condition and its effects on the individual are scored on this scale in addition to chronic disorders. Each body system is assessed on a 5-point Likert scale, with 0 denoting no problem and 4 denoting extremely severe. The sum of each system's severity scores yields the overall score. The burden of the disease is higher when the total score (which can vary from 0 to 56) is higher. Salvi et al. devised a set of guidelines that were followed to score the scale. Elderly individuals have validated this scale.

Medication burden

The third section of the survey assesses the burden of medication. The first four items assessed the burden of medication. The 15-item self-reported scale assesses the behavioral and emotional burden associated with treatment procedures. The tool assesses an individual's perceived burden from pharmaceutical use, lifestyle modifications, and treatment-related social and economic consequences.[21,22] On the Likert scale, 0 represents "not a problem" and 10 represents "large problem". The greater hardship experienced is indicated by a higher score. The scale employed in this study had a Cronbach's alpha of 0.865. The first four items assess drug burden, including discomfort, daily medication schedules, reminders, and necessary precautions. The pharmaceutical burden was calculated by adding their scores on four questions, resulting in a total score ranging from 0 to 40.

Data collection procedure

The entire survey took around 15 to 20 minutes to complete. Data collection for this study was divided into three phases. In phase 1, the patient's demographic and clinical characteristics were gathered from records or through face-to-face interaction with the patient. Phase 2, the patient's knowledge (PKMUQ), belief (BMQ), treatment satisfaction (TSQM-II), depression (PHQ), disease burden (CIRS-G), polypharmacy (number of medications) and phase 3, medication burden (TBQ) these were obtained from face-to-face interviews using questionnaires. Each participant gave their informed consent before the study.

Data analysis

The characteristics were summarized using means and standard deviations for continuous data and frequencies and percentages for categorical data. The student's t-test, one-way ANOVA, and Pearson's 2 test assessed medication burden across demographics and clinical features. Pearson correlation identified correlations between variables, while univariate and multivariate linear regression models identified variables linked to medication burden. Variables with $p<0.05$ in the univariate analysis were included in multivariate models and multicollinearity was assessed using the Pearson's correlation coefficient. Additional analysis considered psychosocial factors and significant variables, with IBM SPSS statistics (Armonk, NY, USA) version 25.0 used for all analyses. A $p<0.05$ was considered significant.

Ethical considerations

The institutional ethics committee approved an ethical authorization for the study (approval number 1056/2024). The ethical criteria of the national and/or institutional research committee, the Declaration of Helsinki of 1964 and its subsequent modifications or comparable standards were adhered to by every procedure used in this study involving human subjects.

Table 1. Sociodemographic and clinical characteristics among 250 type 2 diabetes mellitus patients

Characteristics	n (%)	Medication burden, mean (SD)	t	p
Age (years)				
60–70	158 (63.2)	8.19 (9.46)	0.40	0.852
>70	92 (36.8)	7.32 (8.21)		
Gender				
Male	112 (44.8)	6.67 (7.09)	-0.20	0.537
Female	138 (55.2)	7.44 (8.87)		
Education level				
Literate	154 (61.6)	6.34 (8.06)	-1.05	0.449
Illiterate	96 (38.4)	7.90 (9.85)		
Marital status				
Married	178 (71.2)	6.83 (9.50)	-1.32	0.248
Single/Widow/Divorces	72 (28.8)	7.16 (8.44)		
Monthly income (Rupees)				
≤10000	198 (79.2)	7.48 (11.14)	-0.09	0.654
>10000	52 (20.8)	6.68 (8.64)		
HbA1c (%)				
<7	60 (24)	5.6(1.21)	2.86	<0.001
≥7	190 (76)	9.7 (0.39)		
Hypertension				
Yes	187 (74.8)	7.59 (8.62)	1.09	0.131
No	63 (25.2)	8.84 (9.42)		
Lipid disorder				
Yes	62 (24.8)	8.53 (10.67)	-1.28	0.007
No	188 (75.2)	4.58 (6.48)		
Coronary heart disease				
Yes	122 (48.8)	6.46 (8.58)	-0.54	0.279
No	128 (51.2)	7.47 (9.44)		
Glaucoma/cataract				
Yes	54 (21.6)	9.87 (10.34)	-0.67	0.020
No	196 (78.4)	5.97 (6.78)		
Kidney problems				
Yes	95 (38)	10.67(9.84)	0.20	0.045
No	155 (62)	4.33 (5.90)		
Any chronic painful condition				
Yes	181 (72.4)	10.45 (10.98)	2.07	0.009
No	69 (27.6)	5.72 (7.24)		
Polypharmacy				
Yes	98 (39.2)	11.46 (10.89)	0.001	0.001
No	152 (60.8)	4.52 (6.34)		

Results

This analysis includes 250 T2DM patients. Sociodemographic and clinical characteristics are shown in Table 1. Most of the participants were in the age group – 60–70 years (63.2%); gender – female (55.2%); educational level – literate (61.6%); marital status – married (76%). Chronic illnesses with >30% prevalence included hypertension (74.8%), lipid disorders (24.8%), coronary heart disease (48.8%), glaucoma/cataracts (21.6%), kidney problems (38%), and chronic painful conditions (72.4%). The average number of prescription drugs was 3.78 (SD: 2.48). More than one-third (39.2%) of the subjects. Individuals with chronic painful conditions, poly-

pharmacy and kidney problems had considerably high medication burdens (all p<0.05).

Relationships between continuous variables and medication burden

The associations between the number of chronic illnesses, the burden of disease, the psychosocial variables and medication burden are shown in Table 2. The findings demonstrated a statistically significant negative relationship between medication knowledge, social support, self-efficacy, and treatment satisfaction (r ranged from -0.12 to -0.96, p<0.05). Additionally, there was a positive correlation between medication belief, depression, disease burden, number of medications, number of comorbidities, and medication burden (r ranged from 0.01 to 0.57, p<0.05).

Table 2. Association between the number of chronic diseases, disease burden, psychosocial variables, and medication burden

Variable	Mean (SD)	Correlation with medication burden	
		r	p
Medication Knowledge	9.74 (1.83)	-0.12	0.023
Medication beliefs			
i. Necessity of medication	12.17 (2.35)	0.01	0.064
ii. Concerns about medication	19.28 (5.01)	0.02	0.016
iii. Overuse of medication	14.34 (3.15)	0.01	0.006
iv. Harm of medication	10.06 (4.92)	0.45	0.001
Medication social support	1.37 (0.47)	-0.26	0.046
Medication self-efficacy	22.64 (3.05)	-0.48	0.009
Treatment satisfaction	72.37 (8.39)	-0.96	<0.001
Depression	3.05 (2.65)	0.20	<0.001
Disease burden	7.34 (1.53)	0.57	0.01
Number of medications	4.70 (1.65)	0.18	0.004
Number of comorbidities	3.90 (1.73)	0.52	<0.001
Medication burden	8.18 (5.38)	1.4	–

Univariate linear regression analysis for the burden of medication

A univariate linear regression analysis (Table 3) revealed a statistically significant correlation between mediation burden and age (β=0.08, p=0.008), monthly income (β=0.07, p=0.11), HbA1c (β=-0.01, p=0.005), coronary heart disease (β=-0.23, p=0.029), glaucoma/cataract (β=0.19, p=0.002), kidney problems (β=0.12, p=0.03), polypharmacy (β=0.51, p=0.004), medication knowledge (β=-0.17, p=0.002), medication belief [necessity of medication (β=-0.12, p<0.001); concerns about medication (β=0.45, p<0.001); overuse of medication (β=0.06, p<0.001) and harm of medication (β=0.34, p=0.007)], medication self-efficacy (β=-0.28, p=0.011), treatment satisfaction (β=-0.63, p=0.062), depression (β=0.47, p <0.001), disease burden (β=0.42, p<0.001), and number of comorbidities (β=0.31, p<0.001).

Table 3. Variables associated with medication burden using univariate linear regression analysis

Variables	Unstandardized coefficients		Standardised coefficients	t	p	95% CI
	B	Standard error	Beta			
Age	0.12	0.06	0.08	-0.13	0.008	-1.352 to 1.142
Gender	0.56	1.17	0.05	1.68	0.539	-0.115 to 1.277
Educational level	-0.46	1.42	-0.09	-1.46	0.308	-3.667 to 1.359
Marital status	1.29	1.84	-0.05	-0.03	0.689	-1.756 to 1.358
Monthly income	-0.78	1.47	0.07	-0.32	0.011	-2.014 to 3.270
HbA1c	-0.27	1.96	-0.01	1.02	0.005	4.926 to 6.016
Hypertension	-2.5	1.65	-0.02	0.47	0.824	-2.361 to 3.552
Lipid disorder	-1.86	1.59	-0.06	-0.83	0.625	-1.045 to 2.464
Coronary heart disease	2.43	1.47	-0.23	-0.74	0.029	-2.048 to 4.846
Glaucoma/Cataract	2.05	1.35	0.19	1.06	0.002	-0.541 to 1.237
Kidney problems	1.92	1.08	0.12	-0.12	0.03	-1.686 to 2.571
Any chronic painful condition	3.97	1.36	0.51	2.86	0.004	2.110 to 4.096
Polypharmacy	2.45	1.25	-0.17	1.45	0.002	5.017 to 1.146
Medication knowledge	-1.56	0.19	0.03	-0.54	0.014	-1.680 to 2.502
Medication belief						
i. Necessity of medication	1.42	0.20	-0.12	1.86	<0.001	-0.131 to 1.288
ii. Concerns about medication	0.12	0.64	0.45	-0.19	<0.001	-0.214 to 1.702
iii. Overuse of medication	-1.58	0.34	0.06	-0.46	<0.001	-1.078 to 3.781
iv. Harm of medication	0.37	0.11	0.34	1.41	0.007	3.140 to 0.055
Medication social support	-1.10	0.22	-0.22	0.08	0.721	-0.788 to 1.003
Medication self-efficacy	-0.18	0.33	-0.28	1.05	0.011	-1.155 to 1.990
Treatment satisfaction	3.79	0.20	-0.63	-0.07	0.062	-1.085 to 1.588
Depression	3.03	0.76	0.47	2.48	<0.001	5.217 to 6.305
Disease burden	1.00	0.14	0.42	5.53	<0.001	7.952 to 10.544
Number of comorbidities	6.02	0.32	0.31	4.45	<0.001	6.271 to 8.246

Multivariate linear regression analysis for the burden

In Table 4, the results of the multivariate analysis also showed that age (β=0.16, p=0.001), monthly income (β=0.45, p=0.003), HbA1c (β=0.13, p=0.041), any chronic pain condition (β=0.15, p<0.001), polypharmacy (β=0.34, p=0.001), medication [necessity of medication (β= -0.09, p<0.001); concerns about medication (β= -0.32, p=0.007); overuse of medication (β=0.14, p=0.016) and harm of medication (β=0.03, p<0.001)], medication self-efficacy (β=0.54, p≤0.001), treatment satisfaction (β= -0.13, p<0.001), depression (β=0.02, p≤0.001), and disease burden (β=0.12, p=0.04) have statistically significant associations with medication burden. Medication knowledge and social support are not significantly associated with medication burden (β=0.04, p=0.524). Variables like glaucoma/cataract and the number of comorbidities also show no significant impact on medication burden in this analysis. In general, 36.8% of the variation was explained by this model [F (11, 410) = 19.24, p <0.001]. VIF values <5 for all variables indicate the absence of multicollinearity.

Table 4. Variables associated with medication burden using multiple linear regression analysis

Variables	Unstandardized coefficients		Standardized coefficients Beta	t	p	95% CI
	B	Standard error				
Age	1.20	0.03	0.16	-0.43	0.001	-1.685 to 1.906
Monthly income	0.92	0.17	0.45	-1.34	0.003	-1.467 to 2.291
HbA1c	-1.58	0.52	0.13	3.47	0.041	-2.441 to 5.070
Glaucoma/Cataract	-0.08	1.01	-0.23	-0.12	0.20	-1.550 to 2.456
Kidney problems	1.87	0.12	0.14	-1.67	0.02	-3.125 to 4.380
Any chronic painful condition	1.22	0.46	0.15	-2.69	<0.001	5.463 to 8.431
Polypharmacy	2.38	1.98	0.34	1.57	0.001	-2.464 to 3.012
Medication knowledge	0.13	1.06	0.04	-1.01	0.524	-1.464 to 2.464
Medication belief						
i. Necessity of medication	0.48	0.16	-0.09	-0.73	<0.001	-7.993 to 4.552
ii. Concerns about medication	-0.10	0.05	-0.32	-0.504	0.007	-15.632 to 8.024
iii. Overuse of medication	1.35	0.78	0.14	-0.18	0.016	-5.135 to 9.465
iv. Harm of medication	-0.17	0.41	0.03	-1.14	<0.001	-1.664 to 2.707
Medication social support	0.67	0.06	-0.26	-0.79	0.489	-5.210 to 2.348
Medication self-efficacy	0.15	0.24	0.54	-1.09	<0.001	-4.777 to 2.146
Treatment satisfaction	-0.84	0.13	-0.13	-1.35	<0.001	-9.134 to 11.235
Depression	1.16	0.43	0.02	2.45	<0.001	-1.054 to 2.684
Disease burden	0.91	0.29	0.12	-1.92	0.04	10.362 to 12.450
Number of comorbidities	0.24	1.19	0.00	1.27	0.721	4.124 to 7.668

Discussion

The delivery of patient-centered care has become an understanding of people's experiences and difficulties related to medication use.[23] This study assessed the variables related to drug burden in older adults with multiple comorbidities living with type 2 diabetes mellitus. Positively, this study also includes the majority with higher HbA1c values, which tended to show favorable results. Our study shed light on how different personal and psychological aspects influence the burden from a multifaceted point of view. According to the findings, older adults with multiple comorbidities who also experienced depression, polypharmacy, low self-efficacy of medications, high disease burden, and poor satisfaction with medication treatment were more likely to perceive the burden of medication treatments as high. Similar studies by Mostafavi et al. in Iran and Gonzalez et al. in America demonstrated that polypharmacy, disease burden, and depression have a greater perceived burden of medications among elderly patients with type 2 diabetes.[24,25]

The study highlighted the association of polypharmacy with the burden of medications, which increases the risk of drug interaction, adverse effects and non-adherence and negatively affects psychosocial well-being by causing cognitive decline and anxiety due to side effects. According to studies by Bourgeois et al. and Kirkman et al., multiple medication management has been associated with worse glycemic control in older adults. Scott et al. suggested that the de-prescribing and streamlining of drug schedules may lead to better outcomes and a higher standard of living.[26]

In general, the burden in our study was minimal. The first four items had an average score somewhat lower than what had been found in the study by Bekalu et al. in Ethiopia.[27] The multivariate analysis revealed that the variables entered into the model could account for about half of the variation in the burden of medications. Compared to the study by Akshatha et al., which solely considered the demographic and clinical characteristics of the participants, the percentage of explained variance was higher (range: 12% to 47.9%). This disparity could be explained by the addition of psychological variables to the account of the medicine load.[28]

Treatment satisfaction, with the highest regression coefficient in our model, significantly impacts the burden by influencing how patients view and use pharmaceuticals, ultimately affecting their decisions and outcomes. Previous research by Yang in China and Sendekie in Northwest Ethiopia has documented a bivariate relationship between medication burden and satisfaction with medication treatment.[29]

Self-efficacy, a key factor in change and disease management, is strongly linked to lower medication burden. Our research shows that higher self-efficacy in older adults with type 2 diabetes helps them manage challenges such as side effects, daily integration, and medication use, leading to better adherence to treatment and a reduced sense of medication burden. Similarly, Borson et al. have demonstrated that the burden of treatment burden was predicted by their level of self-efficacy in the treatment of chronic illnesses.[30] Our investigation further illustrated their strong correlations about drug treatments. To reduce the detrimental effects of the drug burden on patients' lives and health, healthcare providers should assist patients in developing medication self-efficacy. Consequently, influencing medication self-efficacy, social support, beliefs, and information about medications can have an impact on medication burden. Future studies may be necessary to confirm the possible indirect impacts of these variables on the burden on medicine, as they have not been examined in previous research.

Type 2 diabetic geriatric patients who have a high disease burden are more likely to find it difficult to manage the demands of multiple healthcare services. In addition to impairing mental and physical functioning, multimorbidity can further reduce an older person's ability to control his health. It is not unexpected that the

multivariate analysis showed a substantial positive correlation between the burden and disease burden. A similar study by Horne et al., Rayana et al. and Okere et al. shows that more chronic diseases, longer duration, higher severity, and low self-rated health have all been linked to higher perceived treatment burdens.[30-32]

Furthermore, our research revealed that not all individual physical conditions were substantially correlated with drug burden, except depression. A similar study by Lalwani RK in India indicated that the burden of treatment of a patient was correlated significantly with depression, dementia or severe mental health issues, but not with any specific medical condition.[33] This result would suggest that mental health issues play a significant part in exacerbating the need for medicines. However, in Morocco, research found that a significant increase in drug or overall treatment burden was predicted by having diabetes, atrial fibrillation, or hypertension.[34,35] Therefore, more research is needed to determine whether a person's physical state may be a contributing factor to their high drug burden.

The demographic characteristics of the patients and their drug burden were not found to be related in our study. The findings of earlier research are contradictory. Numerous research studies have shown a correlation between medication burden of medications and specific demographic characteristics, such as age, gender, marital and employment status. However, according to a recent study in East Uganda, the of patients of medication burden was the same regardless of their demographic characteristics. Additionally, the authors suggested that demographic characteristics could moderate the impact of patients' ability to manage their care on treatment burden.[36]

Previous findings by Fisher et al. and van Bastelaar et al. demonstrated that HbA1c (>7%) levels and medication burden had a significant positive correlation similar to our study. According to previous international reports by Aikens et al., the influence of DM medication regimens on the level of medication burden was statistically significant at ($p<0.05$).[37] Previous quantitative research suggested that insulin-treated patients have experienced a significantly higher burden of diabetes medications, which may directly affect the psychosocial variables.[38] Therefore, more research is required to determine the exact role of these components.

Study limitations

First, this is a cross-sectional study, making it difficult to determine the causal relationships. A longitudinal data set would be valuable to better understanding the causal relationships between psychosocial factors and medication burden. Future research should investigate the possible relationships between study characteristics and medication burden because it is a dynamic process that changes when new medicines are developed or chronic diseases develop. Second, the study's conclusions may be limited because the individuals were chosen from a single tertiary care facility. Third, elderly people with type 2 diabetes who have a high disease or medication burden may not be included in our study because they are unlikely to have the energy or time to participate in the volunteer study. Fourth, we excluded institutional / infrastructure factors, including continuity of care and quality of healthcare service, as well as interpersonal factors such as family concerns and consultation methods. For a more complete understanding of drug burden, these elements must be included in future research. Additionally, the study relied on self-reported data, which may have introduced biases in assessing adherence to medication and psychosocial variables. Objective measures, such as pill counts, electronic medication monitoring, or pharmacy refill data, could be used in future studies to obtain more accurate data.

Conclusion

The study shows that in elderly patients with type 2 diabetes who had multiple comorbidities, greater self-efficacy for medications and satisfaction with treatment were independently related to a lower drug burden, while depression, polypharmacy, and a higher disease burden were associated with a higher drug burden. It may be possible to identify geriatric patients who feel overwhelmed by their drug treatments by identifying the psychological aspects linked to the burden of medications. Type 2 diabetic geriatric patients with multiple comorbidities should receive support in medication self-management and personalized psychosocial therapies to reduce drug burden, improve medication adherence, and alleviate depression, potentially reducing the need for medication.

Declarations

Funding

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Author contributions

Conceptualization, H.W. and S.R.; Methodology, H.W.; Software, H.W.; Validation, H.W.; Formal Analysis, H.W.; Investigation, H.W.; Resources, H.W.; Data Curation, H.W.; Writing – Original Draft Preparation, H.W.; Writing – Review & Editing, H.W.; Visualization, H.W.; Supervision, H.W. and S.R.; Project Administration, H.W.

Conflicts of interest

The authors reveal no conflicts of interest.

Data availability

The datasets can be obtained on request from the corresponding author.

Ethical approval

This work was approved by the Institutional Review Board of Bhaarath Medical College and Hospital. The study was carried out from April to September 2024 (approval number 1056/2024).

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ORIGINAL PAPER

Determinants of time to first birth among women of childbearing age in Somalia – a survival analysis model

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ABSTRACT

Introduction and aim. The study evaluated factors and the significant indicators of time to first birth for reproductive aged women (aged 15–49 years) in Somalia.

Material and methods. The study considered SDHS, 2020. The log-rank test was used to assess the statistical significance of the observed difference between waiting time for first birth and various socioeconomic and demographic factors. The Cox proportional hazard model was applied to identify the influential factors for the waiting time to the first birth.

Results. The study established that about 71.19% of the respondents got married before the age of 20, while 28.81% did marry after the 20 years of age. Survival analysis revealed that the place of residence, region, age at first marriage, respondent's education, employment status, and Wealth quintile were statistically significant determinants of waiting time to first birth. The majority of the respondents (64.56%) of the total had their first birth between the ages of 15 and 24. Among these age groups, the highest number of first births occurs at the age of 19 years, with 11.98% of respondents falling into this category. The study also found that the different age groups revealed significantly lower hazards for first births as the age increased. Regarding regional differences, the study found significant variations in the probability of survival of the first birth. In particular, the Togdheer region exhibited a higher hazard ratio (HR=1.132979, p=0.030), indicating an increased risk of experiencing the first birth compared to the reference category. Other regions, such as Galgaduud (HR=1.140067, p=0.029) and Bay (HR=1.199272, p=0.018), also showed higher hazards. On the contrary, Woqooyi Galbeed did not show significant difference in survival probabilities (HR=0.972095, p=0.604) compared to the reference category.

Conclusion. Raising the minimum age for the first childbirth and promoting women's education are crucial for reducing child marriage rates in Somalia.

Keywords. age at first birth, reproductive age women, Somalia, survival analysis

Introduction

The decision to begin childbearing and the timing of the first birth represent two important transitions in female life.[1] The time to first birth varies from one woman to another, due to different individual, sociocultural, economic and environmental factors. We know that the first child is one of the most important events in a woman's life, often influenced by these factors. It indicates the beginning of intensive responsibilities of maternity and childcare. Around 1 in 10 childbirths worldwide is contributed by young mothers, with developing countries accounting for the majority of these cases, 95% of the share. Girls under 15 years of age account for 2 million (27%) of the 7.3 million births that occur in 18-year-

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old adolescent girls in developing countries.[2] The age at which a woman begins to have children has a direct impact on the total number of children she will have during her reproductive years, assuming no active use of fertility control methods, in regions such as sub-Saharan Africa, where contraceptive use is relatively low compared to developed nations, a younger age at the first birth tends to increase the likelihood of a higher number of children for a woman, as highlighted.[3,4]

The United Nations Population Fund (UNFPA) report states that early pregnancy can have severe consequences for adolescent girls.[5] In fact, globally, about 14 percent of adolescent girls and young women gave birth before the age of 18 years in 2021. It prevents them from continuing their education and livelihoods, in addition to affecting their health adversities that, in turn, delay their overall healthy development into adulthood. Especially, adolescent girls in the early stages of adolescence are at greater risk of health complications during pregnancy and delivery. About one in six young women globally gives birth before the age of 18 years.[5]

The report states that in South Asia, approximately 10% of adolescent girls and young women gave birth before age 18 between 2015 and 2021, while on the African side, this has more than doubled in sub-Saharan Africa. More than a quarter of adolescent girls and young women in western and Central Africa and Eastern and southern Africa have given birth before the age of 18 from the same period. That would be approximately 11 million young women in both regions.

Somalia has one of the highest rates of child marriage worldwide, with more than 1 million child brides.[6] This translates into the fact that the majority of girls in Somalia are married before they reach the age of maturity. This report further shows that four out of every ten young women in Somalia were married when they were children. Child marriage is more common in rural areas than in urban areas of Somalia, which means that it is more practiced in nonurban environments.

Except for Burkina Faso, the fertility rates fell in nearly all eight sub-Saharan African countries under observation during the period between the two surveys. Data indicate that this proportion decreased in all countries except Burkina Faso. In particular, Mali had the highest rate of teenage childbearing at 45%. On the contrary, Ghana, Kenya, and Zimbabwe had a considerably lower percentage with less than 25% of girls giving birth at an early age. Côte d'Ivoire recorded the biggest decline in the proportion of early first births between the surveys, falling from 41% to 28%. In Mali, the difference was only minor.[4]

The time to first birth among women of childbearing age varies between different regions and countries with median ages ranging from 15 to 20 years according to studies.[2,7–10] In Ethiopia, the median age at first birth was found to be 20 years, which is lower than the optimal age to give birth. Factors associated with early childbirth in Ethiopia include early age at first marriage, early sexual intercourse, high spousal age difference, and being Muslim.[7] The percentage of women who begin child delivery before reaching age 18 is approximately 36.80% in Nigeria. Some of the factors associated with the younger maternal age at the first birth in Nigeria include the place of residence in the North East region, Islamic religion, and the low literacy level in the community.[8] In Ghana, the median age at which women gave birth for the first time increased from 17 years in 1998 to 19 years in 2014.[8] Most women in most of the selected sub-Saharan African countries had optimally spaced, although a high proportion of births in Chad and the Democratic Republic of Congo had short birth intervals. In eastern and Southern African countries, long birth spacing was more common, with the highest rate expressed in Zimbabwe.[11] he residence in rural areas, occupation, early sexual intercourse, early age at first marriage, high spousal age gap, and unmet need for family planning factors are some of the factors associated with early child marriages, consequently leading to early births in Africa.[12,13]

Socioeconomic factors are considered contributing factors to the timing of first birth among women in sub-Saharan Africa. Some studies have established that variables such as educational status, age, residence, economic status, and knowledge about maternal health care determine the use of maternity care among adolescent mothers in the region.[14] The promoting female education can help delay the age of first birth, highlighting the significant role of education in shaping fertility behavior.[15] Further, in the matter of family formation, which differs throughout sub-Saharan Africa, differences in socioeconomic status have been shown to be related to the age at first marriage and first birth between different socio-economic status groups.[16] Furthermore, early childbirth, especially before full physical maturity, was associated with a higher risk of maternal death, evidence that the effect of early age at first birth impacts maternal health outcomes.[15] Budu et al. found that young women who gave birth for the first time between ages 20 and 24 years had a higher proportion of delivery with skilled birth attendance, emphasizing that the timing of first birth plays an important role in accessing obstetric care.[17]

On the other hand, Neonatal mortality remains a major challenge in sub-Saharan Africa, with 99% of the world's neonatal deaths occurring in poorer regions, particularly in sub-Saharan Africa and South Asia.[18] In 2018, sub-Saharan Africa had a neonatal mortality rate of 28 deaths per 1,000 live births.[18] Despite efforts to reduce neonatal mortality, disparities exist across the region. in Kenya, the rate of neonatal mortality in 2018 was 19.6 deaths per 1,000 live births, a decrease from 35.4 in 1975, while South Africa saw a more substantial

reduction from 27.9 to 10.7 deaths per 1,000 live births during the same period.[18] The main causes of neonatal deaths in both countries are preterm birth complications and intrapartum-related events.[18] Birth asphyxia is also a significant contributor, with the combined prevalence in sub-Saharan Africa estimated at 17.28%.[19] Factors such as low birth weight, primigravida status, and meconium-stained amniotic fluid were significantly associated with birth asphyxia.[19] To address these challenges, community-based breastfeeding promotion programs play a crucial role in improving neonatal survival.[18] Studies show varying prevalence rates for early initiation and exclusivity of breastfeeding across the region, with Southern Africa showing the highest rates.[18–20] However, regions like West and Central Africa show lower rates of early initiation and exclusive breastfeeding, both of which are crucial for improving neonatal health outcomes. Interventions should focus on increasing breastfeeding practices, particularly in regions with low rates, to reduce neonatal deaths and improve survival outcomes.

While global standards call for the legal age of marriage to be 18 years or older and for pregnancies to occur at an age that minimizes health risks for both mother and child, the opposite seems true in Somalia. By the age of 15 years, according to the Somali Health and Demographic Survey (SHDS) report 2020.[21] the percentages of married women aged 20-49 years are 16%. Another 34% in this age group were married by age 18 years, while 44% were married by the age of 20 years. The median age at first marriage among women aged 25 49 years is 20 years. These figures indicate that the early marriage rate in Somalia is quite high. This study tries to fill the knowledge gap by analyzing determinants of the timing of the first birth among young married women in Somalia. It will provide an understanding of what factors cause variation in this behavior; such knowledge is necessary to develop interventions that may eventually bring the ideal and actual situations closer to each other, thus improving reproductive health outcomes for women in Somalia.

Aim

The aim of this paper is to investigate the determinants that influence the time to first birth among young married women in Somalia, with a specific focus on identifying key sociodemographic, economic and cultural factors. Using survival analysis techniques, the study aims to understand how variables such as education level, marital age, employment status, and access to healthcare affect the age at which women have their first child. In addition, the paper explores contextual influences, including societal norms and regional disparities, to provide a comprehensive analysis of the factors that shape reproductive behavior.

Significance and novelty of the study

This study is novel, as it is the first to utilize SHDS data to analyze the timing of first births among Somali women. By applying survival analysis, it reveals critical socioeconomic and regional disparities influencing first-birth risks. These findings fill a significant research gap, providing actionable insights to improve maternal and newborn health outcomes, contributing directly to SDG 3.

Material and methods

Study area

Somalia is located in the Horn of Africa and covers a total area of 637,657 square kilometers. It consists of diverse landscapes, including plains, plateaus, and mountains. Somalia has the longest coastline in Africa, stretching approximately 3,333 kilometers from the northern tip, known as the Gulf of Aden, to the east and south, bordering the Indian Ocean. It shares borders with Kenya in the southwest, Ethiopia to the west, and Djibouti to the north, with few seasons fluctuations and regular temperatures ranging from 30°C to 40°C. Therefore, the study encompassed Somalia as a whole (including Somaliland), including its urban, rural and nomadic areas, as well as all geographic regions.

Study design and data source

The study will use a data set acquired from the Somalia National Bureau of Statistics (SNBS), which was obtained from the published reports of the Somali Demographic and Health Survey 2020.[21] The survey data collection began in February 2018 and was completed in January 2019 for urban and rural areas. The focus of the survey was limited to women aged 15 to 49 years who were of reproductive age. This survey, which is the first of its kind in Somalia, was conducted as part of the global DHS project. The data collected in this survey will provide detailed information on the determinant of time to first birth among women of childbearing age in Somalia. The study utilizes a survival analysis model. The author used secondary data from the SHDS.

Variables of the study

Dependent variable

The outcome variable in this research was the age in years at which a woman had her first child. The occurrence of a woman giving birth for the first time was considered an event.

Independent variables

This study examined several independent variables, including age at first marriage, education level, employment status, socioeconomic status, and community level factors including place of residence, health access, geographical region, and others.

Operational definitions
Event: giving first birth. (2) Censored: not giving first birth. (3) Survival time: the time is taken in years (age) from birth to give her first birth.

Sampling methods and sample size
The SHDS 2020 interviewed 16,486 women-11,876 married women and 4,610 never-married women, after cleaning; the final sample size is 11,579 ever-married women used in this Study.

Statistical analysis
Survival analysis is a collection of statistical procedures for the analysis of data in which the outcome variable of interest is time until an event occurs. By event, we mean birth, death, disease incidence or any designated experience of interest that may happen to an individual.[22–25] In summarizing survival data, two key functions are of central interest: the survivor function and the hazard function.

Survivor function
The survivor function is the probability that individuals or groups of individuals will survive beyond a particular time point, t; it is usually denoted as S(t). It gives an estimate of the survival probability over time. The usual practice is to estimate the survivor function non-parametrically using the Kaplan-Meier estimator, which calculates the probability of survival at each observed time. The survivor function is decreasing as time passes, which reflects the fact that survival is less and less likely to happen.

Consider a random variable, denoted "T," which is associated with survival times. The actual survival times are represented by the variable "t," and the probability density function of these survival times is denoted as "f(t)." We can define the cumulative distribution function, denoted as "F(t)".[26] which represents the probability that a randomly chosen subject will have a survival time less than a specified value"t" which is given by:

$$F\left(t\right) = P\left(T < t\right) = \int_0^t f(u)du, \quad where \quad t \geq 0 \tag{1}$$

Where
F(t) = P(that a woman has her first Birth before the time t),
The survivor function, represented by "S (t)," refers to the probability that a subject, chosen randomly, will survive for a time duration greater than or equal to a specified value "t," then

$$S(t) = P(T \geq t) = 1 - F(t), \text{ where } t \geq 0, \tag{2}$$

The survivor function "S(t)" is defined as the complement of the cumulative probability "P" that a woman has her first child before time "t."

So, $S(t) = 1 - P(a\ women\ has\ her\ first\ Birth\ before\ the\ time\ t)$ (3)

The relationship between the probability density function "f(t)" and the survivor function "S(t)" can be derived from equations. (2) and (3).

$$f\left(t\right) = \frac{d}{dt}(F(t)) = \frac{d}{dt}(1 - S(t)) = -\frac{d}{dt}S(t), \quad where \quad t \geq 0 \tag{4}$$

The hazard function h(t)
It is usually applied as a measure that quantifies the risk or probability of an individual experiencing the event of interest-a particular event like death-at a given time point. It is derived from the probability that an individual will experience the event at time t, since the individual has survived up to that time. In different fields, it is referred to by various names, such as conditional failure rate in reliability, force of mortality in demography, intensity function in stochastic processes, age-specific failure rate in epidemiology, inverse of the Mill ratio in economics, or simply the hazard rate. The hazard function provides information about the instantaneous potential for the event to occur per unit of time, given that the individual has survived up to time.

The hazard function $h(t|z)$ or simply $h(t)$ is defined mathematically as:

$$h\left(t\right) = \lim_{\Delta t \to 0+} \frac{1}{\Delta t} P\left\{t \leq T \leq t + \Delta t | T \geq t, Z = z\right\} \tag{5}$$

Where,
Z = m-Dimensional vector of covariates;
$h(t)$ = Hazard Function;
P = Conditional probability of failure;
t = Survived up to time;
T = random variable representing the survival time, which is nothing but the time-to-event;
t = given a small time period and
(t + Δ) = Time interval
Thus, the hazard rate and survival function are closely related by:

$$h\left(t\right) = \frac{f(t)}{S(t)} = -\frac{d}{dt} log(S(t)), \tag{6}$$

Or, to put it the other way around

$$S\left(t\right) = exp\left(-\int_0^t h(t)dt\right. \tag{7}$$

The integral in the expression above is known as the cumulative hazard function.[27] The cumulative hazard function can be expressed mathematically in different ways depending on the specific survival model used. Here are a few common expressions:

Non-parametric estimation (Kaplan-Meier method)
In nonparametric estimation, the cumulative hazard function is estimated using the Kaplan-Meier method.

The expression for the cumulative hazard function at time t is given by the following:

$$H(t) = -\log[S(t)] \quad (8)$$

where S (t) is the estimated survival function, representing the probability of surviving beyond time t.

Cox proportional hazard ratio

Cox regression A semi-parametric technique that is commonly used for survival data analysis that allows one to simultaneously assess the association between multiple covariates and survival, also termed Cox proportional hazards model.[28] The researcher used STATA version14 software to extract and process the data in order to perform survival analysis. To find missing values, the extracted data was subjected to a series of recording and cleaning procedures, such as frequency analysis, listing, and sorting. The Kaplan-Meier method, which is a nonparametric method to estimate survival probabilities over time, can be employed to estimate the median time to first birth and visualize the survival experience across different subgroups.[28,29] Moreover, when comparisons of two or more groups were made for survival experiences, the difference in the Kaplan-Meier survival plot was tested by a logarithmic rank test for its statistical significance. The logarithmic ranking test tells whether the survival curves of different groups differ significantly differ from each other.

Results

A total of 11,579 married women were interviewed in the SHDS 2020 data. Most of respondents (95.41%) had their first birth, while a small percentage (4.59%) had not yet given birth yet. The majority of the respondents (64.56%) of the total had their first birth between the ages of 15 and 24. Among these age groups, the highest number of first births occurs at the age of 19 years, with 11.98% of respondents falling into this category (Table 1).

Regarding the level of education, significant majority of respondents "uneducated" – meaning that they have no formal education, are the first to give their first child (84.37%), primary school is the next (11.89%), while women who have reached higher education are the last to give birth to their first child with only (0.79%). In terms of residence, Nomadics are the first to give birth for the first time, while rural residents are the next, and urban residents are the last to get their first birth (Fig. 1, Fig. 2).

The different age groups revealed significantly lower hazards for first births as the age increased. The risk levels for women aged 20–24, 25–29, 30–34, 35–39, 40–44, and 45–49 were 0.5025168, 0.3491268, 0.2619968, 0.1797551, 0.1313247, and 0.0845131, respectively, ($p<0.001$). These findings highlight the decreasing risk of first birth as women age (Table 3). The estimates for the age group 25 29 years are far lower in both the adjusted and unadjust-

Table 1. Univariate analysis of Socio-demographic distribution of the first birth among women of childbearing age in Somalia, SDHS, 2020 (n=11,579)

Variables	Categories	Weighted frequency	Weighted percent (%)
Age of first birth	0 (have first birth)	11,047	95.41
	1 (no birth)	532	4.59
	Total	11,579	100
Age group	15–19	399	3.45
	20–24	2,158	18.64
	25–29	3,537	30.55
	30–34	2,533	21.88
	35–39	2,063	17.82
	40–44	665	5.74
	45–49	224	1.93
	Total	11,579	100
Region	Awdal	630	5.44
	Woqooyi Galbeed	896	7.74
	Togdheer	955	8.25
	Sool	1,040	8.98
	Sanaag	1,001	8.64
	Bari	614	5.30
	Nugaal	709	6.12
	Mudug	717	6.19
	Galgaduud	581	5.02
	Hiraan	530	4.58
	Middle Shabelle	559	4.83
	Banadir	1,121	9.68
	Bay	260	2.25
	Bakool	736	6.36
	Gedo	578	4.99
	Lower Juba	652	5.63
	Total	11,579	100
Type of place of residence	Rural	3,130	27.03
	Urban	4,742	40.95
	Nomadic	3,707	32.01
	Total	11,579	100
Highest educational level	No education	9,769	84.37
	Primary	1,377	11.89
	Secondary	341	2.94
	Higher	92	0.80
	Total	11,579	100
Listening radio	At least once a week	772	6.67
	Less than once a week	283	2.44
	Not at all	10,524	90.89
	Total	11,579	100
Watching television	At least once a week	931	8.04
	Less than once a week	274	2.37
	Not at all	10,374	89.59
	Total	11,579	100
Wealth quintile	Lowest	2,796	24.15
	Second	2,440	21.07
	Middle	2,207	19.06
	Fourth	2,202	19.02
	Highest	1,934	16.70
	Total	11,579	100

ed models than the reference group of 15- 19 years. This is supported by the unadjusted risk ratio, which is 0.264 at a p<0.001 with a 95% CI of (0.235, 0.297); therefore, it is approximately 73.6% lower. In the fully adjusted model, the risk ratio increases slightly to 0.35, also with a p<0.001 and a 95% CI of (0.311, 0.393), which also represents a hazard ratio of -65% relative to the reference group. It shows that adjusting the HR from 0.264 to 0.35, after accounting for other factors, the risk is still significantly lower, but part of the reduction seen in the unadjusted model may be due to the effects of other covariates that have been introduced into the model.

The result shows that more than 70 % (> 8,243) of Somali women marry under the age of 20 while the rest marry over 20 year age (Table 2). Most of them reported that the husband had attended school at 82.25%, while 17.75% responded that their husband did not attend any school. On employment, 45.66% of the respondent husbands were employed in the last 12 months, while 54.34% were not. However, only a small proportion (0.92%) of the respondents themselves had worked during that period. Otherwise, (1.91%) of the respondents used contraceptive, while the majority (98.09%) were nonusers Table 2.

Regarding regional differences, the study found significant variations in the probability of survival of the first birth. In particular, the hazard ratio increases Togdheer, HR=1.123179, p=0.030; thus, this region is at a higher risk of having the first birth than the reference category. Correspondingly, Galgaduud with an HR of 1.140067 and a p-value of 0.029, and Bay with an HR of 1.199272 and a p-value of 0.018, also recorded higher hazards. On the contrary, Woqooyi Galbeed did not show significant difference in survival probabilities (HR=0.972095, p=0.604) compared to the reference category.

The influence of residence on first birth survival probabilities was also examined. The urban residence category did not demonstrate a significant difference (HR=0.9877183, p=0.629). However, the nomadic residence category showed a higher hazard ratio (HR=1.077211, p=0.004), suggesting an increased risk compared to the reference category.

Table 2. Distribution of reproductive factors of married female youths in Somalia, SDHS, 2020 (n=11,579)

Variables	Categories	Weighted frequency	Weighted percent (%)
Contraceptive use and intention	Users	221	1.91
	Non-users	11358	98.09
Age at first marriage	Less than 20 years	8243	71.19
	20 and more	3336	28.81
Husband worked in last 12 months	Yes	5287	45.66
	No	6292	54.34
Respondent worked in last 12 months	Yes	107	0.92
	No	11472	99.08

Table 3. Bivariate analysis of the sociodemographic distribution of first birth among women of childbearing age in Somalia, SDHS, 2020 (n=11,579)

Characteristics	Event observed	Event expected	Chi square	Df	p
Age in 5-year groups			3592.39	6	<0.001
15–19	329	85.660			
20–24	2041	997.420			
25–29	3344	2761.400			
30–34	2421	2553.120			
35–39	2031	2889.120			
40–44	657	1199.640			
45–49	224	560.640			
Total	11047	11047.000			
Region			150.17	15	<0.001
Awdal	587	692.560			
Woqooyi Galbeed	835	957.750			
Togdheer	921	865.810			
Sool	979	1000.380			
Sanaag	961	889.760			
Bari	596	550.360			
Nugaal	682	629.890			
Mudug	709	757.400			
Galgaduud	561	470.880			
Hiraan	509	628.700			
Middle Shabelle	523	500.620			
Banadir	1081	1081.710			
Bay	258	202.420			
Bakool	696	633.110			
Gedo	548	538.590			
Lower Juba	601	647.050			
Total	11047	11047.000			
Type of place of residence			51.25	2	<0.001
Rural	3011	3173.810			
Urban	4555	4693.430			
Nomadic	3481	3179.760			
Total	11047	11047.000			
Educational level			82.85	3	<0.001
No education	9319	9530.570			
Primary	1312	1099.140			
Secondary	327	285.070			
Higher	89	132.220			
Total	11047	11047.000			
Listening radio			2.52	2	0.2833
At least once a week	700	665.500			
Less than once a week	262	268.580			
Not at all	10085	10112.910			
Total	11047	11047.000			
Watching television			7.70	2	0.0213
At least once a week	872	899.150			
Less than once a week	255	220.510			
Not at all	9920	9927.340			
Total	11047	11047.000			
Wealth quintile			21.86	4	0.0002
Lowest	2704	2813.100			
Second	2325	2316.960			
Middle	2059	1916.740			
Fourth	2120	2094.050			

Highest	1839	1906.140			
Total	11047	11047.000			
Contraceptive use and intention			3.34	1	0.0677
Users	209	186.780			
Non-users	10838	10860.220			
Total	11047	11047.000			
Smokes cigarettes			2.77	1	0.0959
Everyday	77	64.920			
Not at all	10970	10982.080			
Total	11047	11047.000			
Age at first marriage			4327.16	1	<0.001
Less than 20 years	7753	4931.650			
20 and more	3294	6115.350			
Total	11047	11047.000			
Husband ever attended school			16.70	1	<0.001
Yes	9086	9228.710			
No	1961	1818.290			
Total	11047	11047.000			
Husband worked in last 12 months			24.51	1	<0.001
Yes	5030	4799.370			
No	6017	6247.630			
Total	11047	11047.000			
The respondent worked in last 12 months			7.32	1	0.0068
Yes	105	82.880			
No	10942	10964.120			
Total	11047	11047.000			

Discussion

The study identified key factors influencing the timing of first births among Somali women. Most of respondents (95.41%) had given birth, with the majority of first births occurring between 15 and 24, and the highest percentage (11.98%) at 19 years old. Educational attainment, type of residence, and age significantly impacted the timing of the first birth. Uneducated women had the first births (84.37%), followed by primary school graduates (11.89%), while higher educated women had the latest (0.79%). Nomadic women had the highest risk of early first births, followed by rural and urban residents. The risk of first births decreased significantly as age increased, with the adjusted risk ratio for the 25–29 age group being 65% lower than the reference group. Furthermore, significant regional variations were observed, with the Togdheer, Galgaduud, and Bay regions showing higher risks of first birth, while urban residence did not show a significant effect. Most women (more than 70%) married before age 20, and contraceptive use was minimal (1.91%). These findings underscore the influence of sociodemographic, regional, and behavioral factors on the timing of first birth in Somalia.

The proportional hazards model revealed that the probability of first birth decreased significantly with increasing age, as demonstrated by lower hazard ratios in older age groups. Women in regions such as Togdheer, Galgaduud and Bay were identified as having a higher risk for early first births compared to others. Similarly, contraceptive use was almost negligible (1.91%), reflecting further cultural and systemic barriers to family planning in the population. The same result applies in this research to a similar context in Uganda, Ethiopia and Nigeria.[8,13,30]

The median age at first birth among Somali Women is 18 years, so this study has reinforced the following studies.[2,7–10,12] all these studies have been done in Ethiopia, Sub-Saharan Africa Countries, On the other hand, factors associated with delayed first birth included higher education level and being within the richest wealth index. This finding was in line with the findings of Ethiopia Nigeria, Bangladesh and Iran.[7,8,10,13,31] The results for the age group 25-29 years indicate a significant reduction in hazard both in unadjusted and adjusted models compared to the reference group of 15-19 years. Unadjusted HR of 0.264, with a $p<0.001$ and a 95% CI of 0.235, 0.297, suggests approximately 73.6% lower risk. After adjusting for other variables, this result is similar to the studies in Ethiopia[12] and Bangladesh.[30]

Women living in rural areas are more likely than urban individuals to have children at a younger age, and early motherhood can be a desirable option in rural areas as an alternative to education, and the authors are similar in the results to research carried out in Uganda, Bangladesh and Ethiopia Respectively.[2,31,32]

The authors found different from most studies conducted in Africa as most of them classified regions by religion (Muslim, Christian, etc.), or Ethnic.[8,10,31] But this research is based on a Muslim community (for all of the regions of Somalia), and this is the reason behind the lack of contraceptive, because around 2% use contraceptive and 98% nonusers in the Somali community compared to Ethiopia (35% contraceptive users), Iran (76% contraceptive users) and Bangladesh (62% are contraceptive users) that have different religious approaches to Islam.[10,13,32] It leads to a decrease in its use, the Somali community's understanding of contraceptives is very low, and the lack of community awareness in addition to that, the culture of Somali society is very strict. Contraceptive use, age at marriage and husband's fertility desires show significant effects, reflecting cultural and personal behavior impacts.

Regional disparities in maternal and neonatal health risks are pronounced in areas such as Togdheer, Galgaduud, and Bay due to different socioeconomic and environmental factors. Early marriages and pregnancies are more prevalent in these regions, with many girls starting to have children at ages 15–19, a group at increased risk for pregnancy complications due to physical immaturity and limited access to healthcare. Rural settings exacerbate these challenges with inadequate medical facilities and emergency care, leading to increased rates of birth complications and neonatal mortality.

Table 4. Proportional hazard model on the determinants of time to first birth for the significant socioeconomic and demographic covariates, SDHS-2020

Background characteristics	Number of women	n= 11,579		Number of women	n=11,579	
	Unadjusted HR	p	95% CI	Adjusted HR	p	95% CI
Age in 5-year group	(ref: 15–19)					
20–24	0.492	<0.001	(0.438, 0554)	0.507	<0.001	(0.451, 0.571)
25–29	0.264	<0.001	(0.235, 0297)	0.35	<0.001	(0.311, 0.393)
30–34	0.193	<0.001	(0.171, 0.217)	0.26	<0.001	(0.23, 0.293)
35–39	0.132	<0.001	(0.117, 0.149)	0.178	<0.001	(0.157, 0.201)
40–44	0.096	<0.001	(0.084, 0.111)	0.13	<0.001	(0.113, 0.15)
45–49	0.063	<0.001	(0.053, 0.075)	0.084	<0.001	(0.07, 0.1)
Region						
Woqooyi Galbeed	1	0.583	(0.927, 1.145)	1	0.452	(0.862, 1.068)
Togdheer	1.03	0	(1.135, 1.396)	0.96	0.0750	(0.99, 1.22)
Sool	1.259	0.005	(1.045, 1.282)	1.1	0.517	(0.87, 1.072)
Sanaag	1.157	<0.001	(1.153, 1.416)	0.966	0.487	(0.935, 1.152)
Bari	1.278	<0.001	(1.144, 1.438)	1.038	0.101	(0.981, 1.236)
Nugaal	1.283	<0.001	(1.148, 1.432)	1.101	0.3450	(0.944, 1.181)
Mudug	1.282	0.073	(0.991, 1.233)	1.056	0.8740	(0.903, 1.128)
Galgaduud	1.105	<0.001	(1.257, 1.585)	1.009	0.0490	(1, 1.265)
Hiraan	1.412	0.428	(0.846, 1.073)	1.125	<0.001	(0.681, 0.869)
Middle Shabelle	.95300	<0.001	(1.098, 1.391)	0.7690	0.682	(0.91, 1.155)
Banadir	1.236	0.001	(1.069, 1.307)	1.025	0.068	(0.993, 1.226)
Bay	1.182	<0.001	(1.305, 1.75)	1.103	0.027	(1.02, 1.381)
Bakool	1.512	<0.001	(1.166, 1.453)	1.187	0.073	(0.99, 1.237)
Gedo	1.302	0.002	(1.071, 1.352)	1.107	0.42	(0.932, 1.183)
Lower Juba	1.203	0.111	(0.979, 1.229)	1.05	0.681	(0.869, 1.096)
Type of place of residence	1.097			0.976		
Urban	1	0.333	(0.977, 1.071)	1	0.829	(0.946, 1.046)
Nomadic	1.023	<0.001	(1.099, 1.212)	0.994	0.002	(1.031, 1.142)
Frequency of listening radio	1.154			1.085		
Less than once a week	1	0.298	(0.805, 1.069)	1	0.2	(0.78, 1.054)
Not at all	0.927	0.173	(0.878, 1.024)	0.906	0.733	(0.908, 1.07)
Frequency of watching television	0.948			0.986		
Less than once a week	1	0.013	(1.037, 1.371)	1	0.264	(0.938, 1.261)
Not at all	1.193	0.397	(0.961, 1.104)	1.088	0.594	(0.94, 1.115)
Wealth quintile	1.030			1.024		
Second	1	0.128	(0.988, 1.104)	1	0.249	(0.912, 1.024)
Middle	1.044	0.000	(1.055, 1.184)	0.966	0.386	(0.914, 1.035)
Fourth	1.118	0.073	(0.995, 1.115)	0.973	0.012	(0.867, 0.982)
Highest	1.053	0.901	(0.946, 1.065)	0.923	0.064	(0.866, 1.004)
Contraceptive use and intention	1.004			0.932		
Non-users	1	0.101	(0.778, 1.023)	1	0.098	(0.771, 1.022)
Age at first marriage	0.892			0.888		
20 and More	1	0.000	(0.262, 0.287)	1	<0.001	(0.288, 0.316)
Husbands desire	0.274			0.302		
More children	1	0.119	(0.988, 1.106)	1	0.008	(1.021, 1.146)
Fewer children	1.046	0.795	(0.845, 1.246)	1.082	0.023	(0.651, 0.968)
Don't know	1.026	0.169	(0.987, 1.074)	0.794	<0.001	(1.056, 1.152)
Husband/partner work	1.030			1.103		
No	1	0.000	(0.884, 0.953)	1	0.152	(0.929, 1.011)
Respondent worked	1			1		
No	0.78765	0.015	(0.649, 0.954)	0.85	0.101	(0.699, 1.032)
Highest educational	1		.	1		
Primary	1.223	0.000	(1.154, 1.296)	0.995	0.872	(0.935, 1.059)
Secondary	1.174	0.004	(1.052, 1.311)	0.894	0.062	(0.794, 1.006)
Higher	0.687	0.000	(0.557, 0.846)	0.54	<0.001	(0.435, 0.67)
Mean dependent var	19.802		SD dependent var		3.722	
Pseudo r-squared	0.030		Number of obs		11579	
Chi-square	5582.964		Prob > chi2		0.000	
Akaike crit. (AIC)	180563.538		Bayesian crit. (BIC)		180865.173	

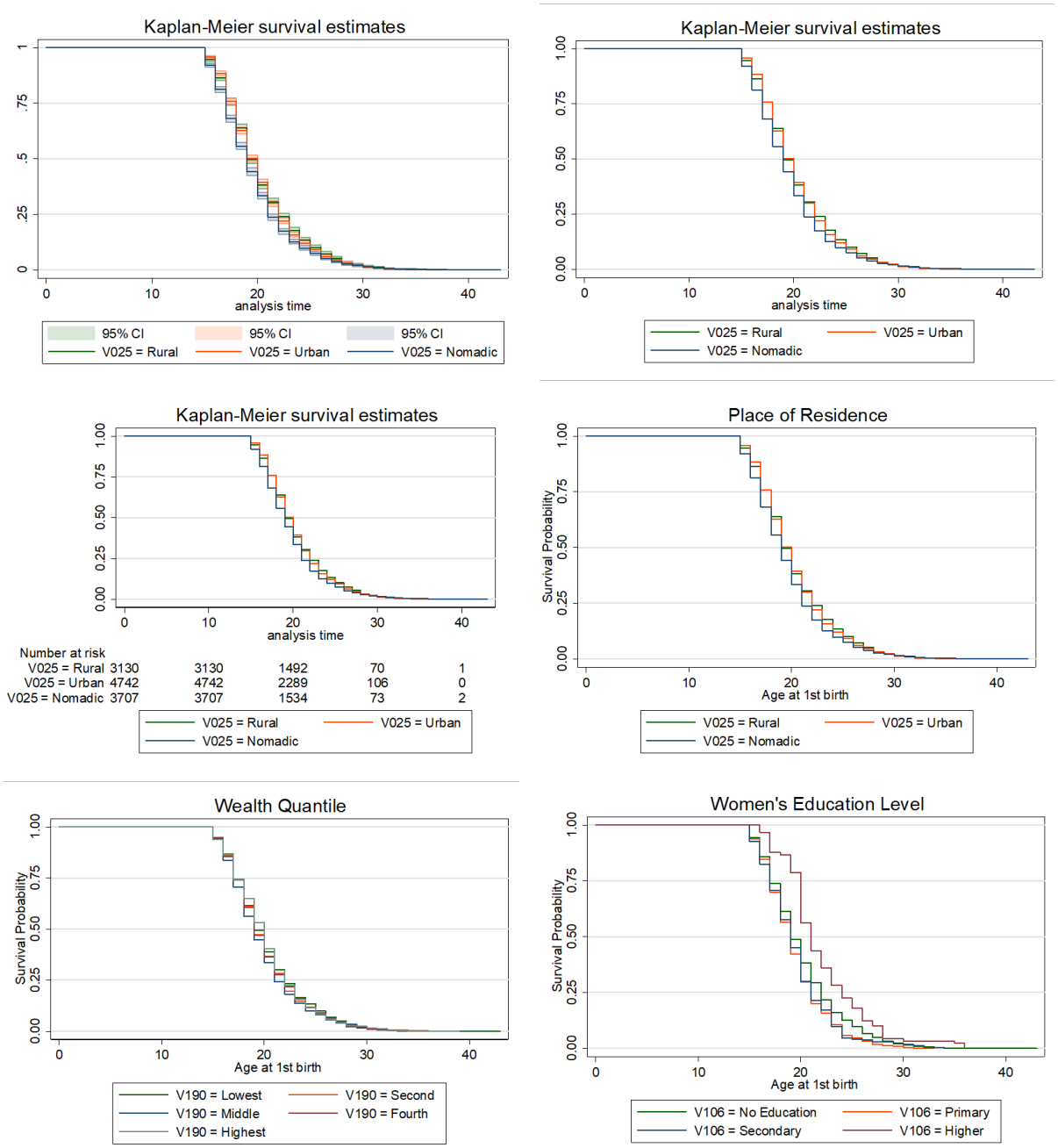

Fig. 1. Kaplan-Meier survival curves of timing at first birth by the characteristics of the selected respondents

This paper is the first from the SHDS to explore time to first birth among childbearing age women in Somalia, adding significant strengths to its contribution. However, due to security concerns, all strata in the Lower Shebelle and Middle Juba regions, along with the rural and nomadic segments in the Bay region, were omitted from the survey, which is the limitation of the paper.

When addressing the limitations of the Cox proportional hazards model in survival analysis, researchers have several alternative statistical methods and models at their disposal. Parametric Survival Models Accelerated Failure Time (AFT) Model, Bayesian Survival Analysis. These methodologies expand the analytical capabilities of researchers, allowing for more tailored and insightful survival analyses.

Conclusion

The paper explores 71.19% of the respondents who got married before the age of 20, while 28.81% did married after the age of 20 years. Survival analysis revealed that the place of residence, region, and age at the first marriage, respondent's education; employment status and the wealth quartile were statistically significant determinants of the waiting time to first birth.

This significant reduction in risk for women aged 25-29 years is statistically robust, suggesting a majority

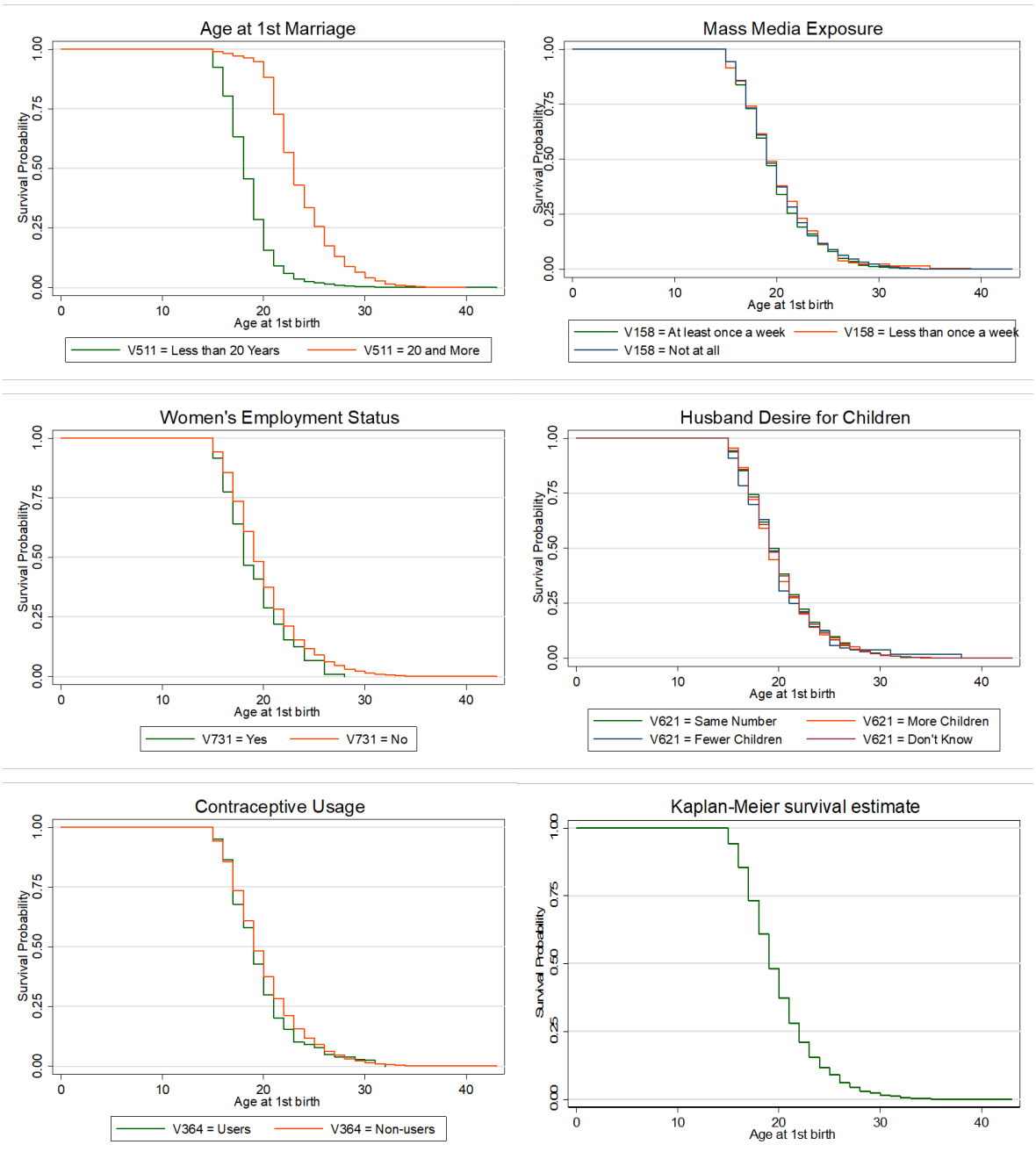

Fig. 2. Kaplan-Meier survival curves of timing at first birth by the characteristics of the selected respondents

protective effect that could be attributed to factors such as increased maturity, lifestyle changes, or other demographic characteristics prevalent in this age group. These findings are very important and could inform targeted interventions or further investigations of protective factors among this age group. I recommend setting this age as the lowest age of marriage in Somali society, so that you can find women who are highly educated, fully aware of the responsibilities of motherhood, and experienced in household and childbirth responsibilities.

For the Bay and Bakool, the survival analysis consistently reveals consistently higher risks, even after adjustments for other variables. Bay has a significantly higher unadjusted HR of 1.182 with statistically significant (p=0), and the risk remains significant with an adjusted HR of 1.103 and a (p=0.027). Similarly, Bakool shows the highest unadjusted HR of 1.512, also significant with a (p<0.001), which decreases slightly to an adjusted HR of 1.187 with a (p=0.073), yet remains elevated. These previous findings suggest that both regions still face higher risks than the reference region, highlighting the need for targeted health interventions and further research into the factors that drive these disparities.

According to the United Nations report on trends in Maternal Mortality (2000–2020), Somalia faces critical challenges, with one of the highest maternal mor-

tality ratios globally – 621 maternal deaths per 100,000 live births – accompanied by poor newborn outcomes, including 36 newborn deaths per 1,000 live births and 28 stillbirths per 1,000 births.[33] These alarming statistics underscore the urgency of addressing maternal and newborn health. In particular, the median age at first birth among Somali women is 18 years. Our study highlights that older maternal age correlates with significantly lower risks associated with first births. Combined with enhanced health interventions, this finding underscores the potential to improve maternal and newborn health outcomes in Somalia, directly contributing to the achievement of the Sustainable Development Goal 3.

This study underscores the prevalence of early childbirth among Somali women, with education and residential status emerging as significant determinants. Women without formal education and those living in nomadic settings are at increased risk of early first births. The findings also highlight considerable regional disparities, with specific regions exhibiting increased risks. Policymakers should prioritize interventions aimed at education and access to reproductive health services to delay first births, particularly in high-risk regions and among marginalized populations. Future research should adopt longitudinal designs and incorporate broader sociocultural factors to better understand and address the determinants of early childbearing in Somalia.

The authors advise that promoting education, especially completion of education up to the university level, not only delays the age at first birth, but also equips women with knowledge and resources to make informed decisions, ultimately improving maternal and child health outcomes. This study contributes to factors that influence the probability the understanding of survival of the first birth survival probabilities. The findings emphasize the importance of considering age, region, and residence when addressing maternal and child health outcomes. Policy makers and healthcare professionals should tailor interventions to target vulnerable age groups and regions with higher risks.

Declarations

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Author contributions

Conceptualization, A.O.A; Methodology, A.O.A and A.H.M.; Software, A.O.A and A.H.M.; Validation, A.O.A and A.H.M.; Formal Analysis, A.O.A and A.H.M; Investigation, A.O.A; Resources, A.O.A; Data Curation, A.O.A and A.H.M.; Writing – Original Draft Preparation A.O.A; Writing – Review & Editing, A.O.A; Visualization, A.O.A.; Supervision, A.O.A and A.H.M.; Project Administration, A.O.A.

Conflicts of interest

The authors declare that they have no competing interests related to this study.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval

Ethical approval was not required for this study as it utilized publicly available, de-identified data obtained with permission from the Demographic and Health Surveys (DHS) Program. The data can be accessed at: https://microdata.nbs.gov.so/index.php/catalog/50

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ORIGINAL PAPER

Therapeutic effect of naringenin on ethanol-induced liver fibrosis in rats

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ABSTRACT

Introduction and aim. Liver fibrosis, a progressive disorder marked by the surplus buildup of extracellular matrix proteins, frequently results from long-term ethanol intake. Our aim of study is to investigate how naringenin's antifibrotic properties impact ethanol induced liver fibrosis in rats.

Material and methods. Rats were divided into four groups: groups 1 and 2 received carboxymethylcellulose (CMC) containing 0.5% glucose, while groups 3 and 4 received 20% ethanol (6 g/kg of body weight) over a 60-day period. In the last 30 days, naringenin (50 mg/kg) was administered each day to groups 2 and 4.

Results. Rats treated with ethanol exhibited liver damage and fibrosis, leading to elevated serum concentrations of aspartate and alanine transaminases. Expression levels of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), alpha-smooth muscle actin (α-SMA) and related proteins were compared with the control group.

Conclusion. Ethanol-fed rats showed an increase in serum matrix metalloproteinases, TIMPs, α-SMA, transaminases, and other proteins compared to the control group. The administration of ethanol led to liver damage and fibrosis. During the final 30 days of the trial, the inclusion of naringenin in the diets of rats notably reduced the levels of α-SMA, MMP2, MMP9, TIMP1, along with serum levels of aspartate and alanine transaminase levels and significant differences were observed compared to control group.

Keywords. ethanol, fibrogenic factors, histopathology, liver damage, naringenin

Introduction

The WHO estimates two billion alcohol consumers worldwide, with 76.3 million having alcohol use disorders, leading to significant disease burden and mortality.[1,2] A percentage of heavy drinkers develop liver cirrhosis, making excessive use of ethanol an avoidable cause of illness. Alcoholics and heavy drinkers are more likely to develop cirrhosis.[3] Worldwide, excessive alcohol use causes cirrhosis, liver fibrosis, liver injury, fat accumulation, cell death, and decreased liver cell renewal, among other sociomedical and public health problems. The importance of prevention and treatment in public health is growing.[4-6] In this cycle, cells change into myofibroblasts, the matrix expands, fat, inflammation, fibrosis to permanent harm, and collagen types I and III in ECM(extracellular matrix), build up due to changes in the assembly or breakdown, resulting in 4,000 deaths annually in the UK, with two-thirds occurring before

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the age of 65.[7-10] Matrix metalloproteinases (MMPs) are proteases influencing various processes. in tissue remodeling and the breakdown of excess collagen. 17 of the more than 60 MMPs that have been sequenced are human MMPs. Growth factors, hormones, interactions between cells and the cell matrix, and inflammatory cytokines all alter their activity through transcription.[11,12]

Endopeptidases known as matrixins degrade extracellular matrix (ECM) proteins such as collagen and fibronectin, which are crucial for tissue remodeling, embryonic growth, and repair.[13] Their expression is regulated by growth factors, hormones, inflammatory cytokines, and interactions among cells within a matrix. In vertebrates, four TIMPs (TIMP 1–4) have been discovered; their functions in liver diseases are still not well understood and they are overproduced in liver fibrosis. TGF-β1 is a key factor in the progression and development.[14-21] Naringenin, a flavanone in citrus fruits, is used in traditional Chinese medicine and shows various health benefits, including anti-inflammatory and anticancer effects, with adults consuming approximately 68 g daily.[22]

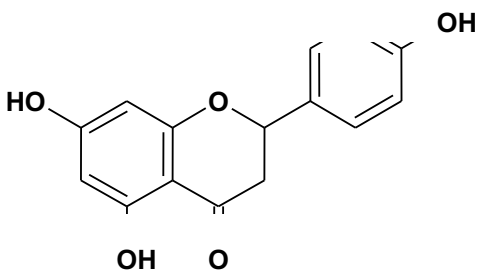

Fig. 1. Naringenin structure

Aim

The study investigates the effects of naringenin's impact on liver fibrosis from ethanol in rats.

Material and methods

Chemicals and reagents

Naringenin was sourced from Sigma, ethanol from E.I.D Parry, and antibodies for MMP and TIMPs from Santa Cruz.

Animals

Adult male albino Wistar rats (150–170 g) were obtained from Annamalai University and kept in regulated conditions featuring a 12-hour light/dark cycle, 50% humidity, and a temperature of 28°C, with the experimental protocols sanctioned by the Institutional Animal Ethics Committee (Reg. No. 160/1999/CPCSEA/557).

Study design

The study categorized the animals into four groups, each with eight rats on a standard pellet diet. Groups 1 and 2 received isocaloric glucose, while groups 3 and 4 received 20% ethanol for 60 days. Subsequently, group II received naringenin with CMC, and group IV received ethanol alongside naringenin (50 mg/kg/day), based on a previously published study, ensuring similar dietary conditions for groups 1 and 3. Since ethanol-induced liver fibrosis is prevalent in India, ethanol induction is used instead of CCl4 and bile duct ligation. The entire experiment lasted for sixty days. Fig. 2 shows the study design. Retroorbital punctures were used to obtain blood samples.

Animals received 30 mg / kg of ketamine intramuscularly, starved overnight, and blood samples were centrifuged in heparinized tubes to separate plasma.

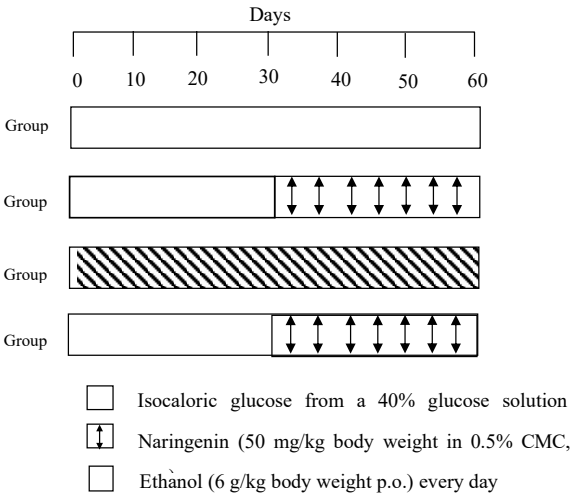

Fig. 2. Study design

Quantitative real-time PCR

Total RNA was obtained from tissues using the QIAGEN Total RNA Kit, and subsequently, DNase treatment was performed to eliminate genomic DNA. Reverse transcription utilized 200 units of Superscript reverse transcriptase, 2 μg RNA, and 0.25 μg random hexamer, incubated at 25°C and 50°C. Real-time PCR employed 3 μL cDNA with SYBR Green Master Mix and specific primase.

PCR primer design

S.No.	Symbol	Forward primer	Reverse primer
1	Alpha-SMA	CAGTTCTTTGGCCACACTCA	CCAGGATTAAGGCCGATGTA

Western blot analysis

Liver proteins were isolated using SDS-PAGE, followed by coating a PVDF membrane with the proteins. The membranes were immersed in 5% non-fat dry milk in TBS for one hour at room temperature prior to the addition of primary antibodies: MMP-9, TIMP-1, and MMP-2. Incubation overnight with Santa Cruz Biotechnology antibodies (1:1000 dilution) was carried out at 4°C, and secondary antibodies (1:5000 dilution) were added after three to five washes.

Liver histopathology and sirus red staining

After sacrifice, the liver is extracted and preserved in 10% formal saline for 48 hours for histological analysis. Subsequently, it was dehydrated with ethanol and water, cleaned utilizing xylene, and encased in paraffin. Sections, cut to a thickness of 5–6 µm, are stained with hematoxylin and eosin, mounted in neutral DPX, and analyzed microscopically.

Masson trichrome staining method for collagen

Liver slices were stained for collagen using Masson's trichrome, a method from 1929. Acid fuchsin, phosphomolybdic acid, glacial acetic acid, potassium dichromate, concentrated HCl, and quick green were among the reagents. The materials were dehydrated with ethanol after being absorbed in trays with distilled water, ethanol, and xylene. The slides were mounted in a resinous substance after cleaning with xylene.

Statistical analysis

Results presented as mean±SD for eight rats; significance $p<0.05$ (SPSS software, IBM, Armonk, NY, USA).

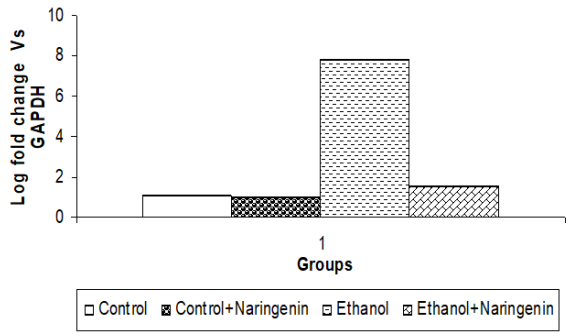

Fig. 3. Effect of narigenin and ethanol on alpha-SMA in rat livers

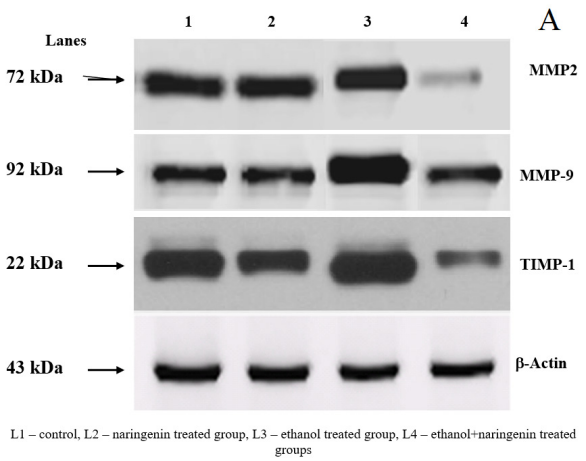

Fig. 4A. Effect of narigenin and ethanol on MMP-2, MMP-9, and TIMP-1 in the liver of control and experimental rats

Results

Hepatic fibrosis, an early pathological condition of cirrhosis, is caused by significant liver damage in several chronic liver illnesses. Cirrhosis is recognized to contribute to several types of liver cancer. Numerous experimental animal groups participated in immunoblot investigations of MMP-2 and MMP-9 (Figs. 3, 4A and 4B).

The results showed that rats given ethanol (group 3) had higher blood levels than rats in the control group (group 1). The combined administration of ethanol and naringenin resulted in a considerable reduction in the expression of MMP-2 and MMP-9. Western blot analysis was used to look at TIMP-1 in a number of experimental animal groups. TIMP-1 levels improved in rats compared to control rats.

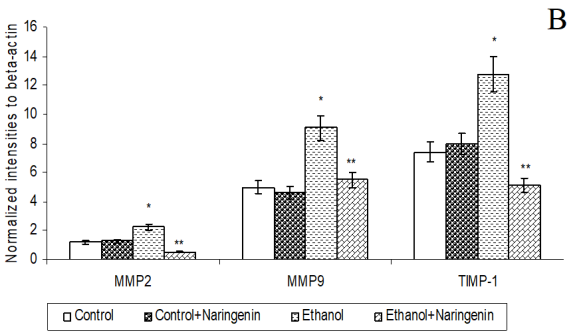

Fig. 4B. Naringenin and ethanol influence liver enzymes MMP-2, MMP-9

When ethanol and naringenin were combined, TIMP-1 expression somewhat decreased. The accumulation of normal chemicals in the extracellular matrix causes fibrosis. In order to provide a framework and maintain the regular operation of different liver cells, the liver extracellular matrix (ECM) provides structural support. Numerous biological functions depend on the regulation of collagen and ECM stability, which is strictly regulated by endogenous inhibitors (TIMPs). Several disorders arise as a result of dysregulation of MMP activity.[26] Additionally, it interacts with other molecules to carry out unrelated MMP processes, which are well documented in the invasion, migration, and development of cancer. Recent data clearly indicate that CD147 is important in PF, induces MMP, and transforms fibroblasts into myofibroblasts. In a number of fibrotic diseases, TIMPs and MMPs are essential.[27,28] The primary constituents of ECM29 can be broken down by gelatinase, which is made up of gelatinase A (MMP-2) and B (MMP-9). TIMP-1 is a naturally occurring tissue inhibitor of gelatinases that can prevent them from performing their gelatinolytic work.[30] We discovered that the livers of rats given ethanol expressed more MMP.

Alcohol consumption increases MMP activity by two mechanisms: ethanol oxidation through CYP2E1

produces reactive oxygen species that activate MMPs, and liver injury leads to cytokine release, further enhancing MMP activation and correlating with increased liver fibrosis.MMP-13 levels are elevated in various cancers, contributing to tumor progression and metastasis by cleaving collagens I, II, III and other ECM components. In addition to causing ECM remodeling, its action frequently results in the release of several angiogenetic and sequestered growth factors that promote the development, invasion, and angiogenesis of tumor cells.[32] Corresponding to activated stellate cells there after serving as a significant source of TGF-β, the primary mediator of MMPs during liver damage.[33] According to the above process, increased ROS generation of ROS and/or ECM in the group that received alcohol may be what caused the increased expression of MMPs. Naringenin may reduce MMPs and fibrosis by lowering the inflammatory response linked to alcohol-mediated liver injury due to its anti-inflammatory properties.

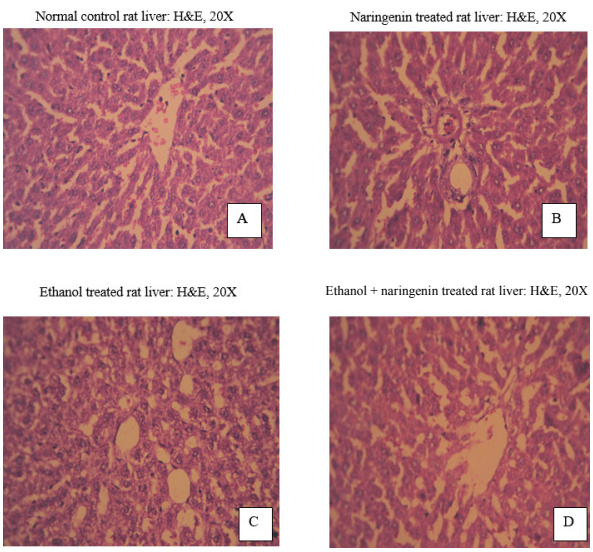

Fig. 5. Histopathological changes in the liver after ethanol administration

The data reported above suggest that naringenin inhibited the formation of hydroxyproline and the secretion of MMP by regulating HSC activity. This would help preserve the tissue's structural integrity by bringing MMP expression down to nearly normal. Figure 5 displays the liver's histological alterations. Hepatocytes in the liver of control rats were normal (Fig 5A).

Liver samples from rats given ethanol showed sinusoidal enlargement, ortal inflammation, and steatosis with micro- and macrovesicular alterations (Fig. 5C). The liver treated with ethanol and naringenin (Fig. 5D) exhibited normal histological characteristics with reduced and smaller fatty cysts. Rats treated with naringenin exhibited normal hepatocytes (Fig. 5B). These results aligned with our biochemical and cellular discovery that naringenin supplementation protects against ethanol-induced liver damage (Fig. 6). Serial sections were stained with Sirius red to visualize collagen. The liver of control rats did not show histological alteration (Fig. 6A). The livers of rats fed ethanol showed high collagen levels and clusters of collagen fibers surrounding the lobes. The central vein and the portal triad show a notable accumulation of collagen (Fig. 6C). Liver treated with ethanol and naringenin (shown in Fig. 6D) exhibited lower levels of collagen, reduced collagen deposition around the portal triad and central vein, a reduction in collagen fiber bundles, and decreased liver fibrosis scores. No alterations were observed in the histology of rats administered naringenin. These findings are consistent with the biochemical and cellular evidence that indicates that naringenin supplementation protects against ethanol-induced liver damage.

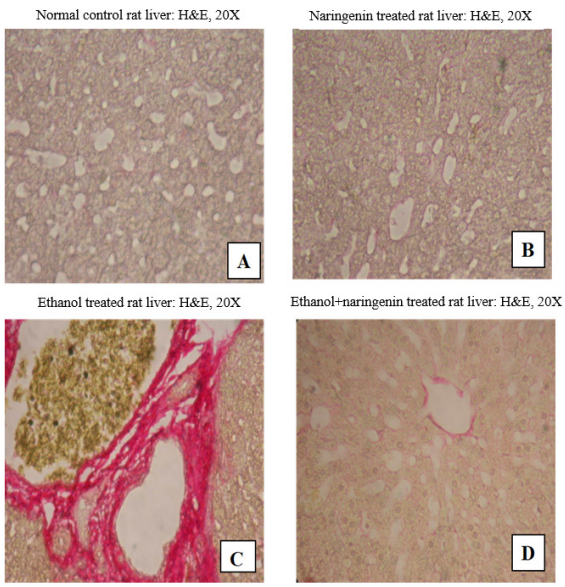

Fig. 6. Staining of collagen

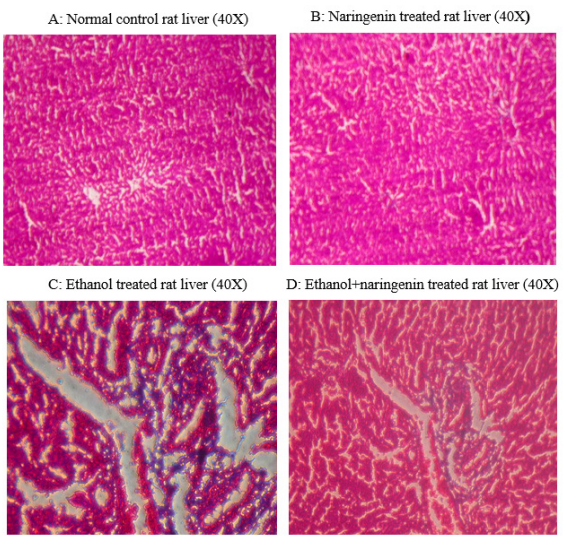

Fig. 7. Trichrome staining for collagen

Figure 7A shows the liver of the control rat, utilizing trichrome staining (red for collagen fibers), exhibits minimal collagen accumulation in the portal triad and central vein. Figure 7B presents rats exhibit collagen buildup and fatty changes in hepatocytes, while Figure 7C shows the livers show slight collagen accumulation around the veins, while hepatocytes remain within normal range after treatment.

Discussion

In our investigation, we observed elevated collagen levels and SMA expression in the livers of rats ingested ethanol. Liver disease injury caused by obstruction of the bile ducts has been associated with elevated levels of liver collagen.[35,36] In this context, it was shown that SMA-positive immunological cells are located primarily located in the portal ducts and fibrous septa align with collagen circulation.[37] Furthermore, SMA expression has been reported that SMA expression rises in the livers of rats fed ethanol, along with increased collagen levels in these livers. Elevated levels of collagen and SMA expression could be attributed to a higher count of SMA-positive cells in the liver. Furthermore, injured hepatocytes serve as significant sources of reactive oxygen intermediates, which are recognized for their ability to induce paracrine activation of stellate cells.[38-40]

In rats fed ethanol, naringenin supplementation markedly decreased the expression of collagen and SMA in liver. This could be due to reduced collagen levels, decreased oxidative stress, and suppressed HSC activation. Furthermore, rats treated with naringenin were found to exhibit reduced SMA expression levels in their livers.[41,42] The amount of naringenin used in this study could not reach the levels suggested for human treatment. Furthermore, the length of the study was inadequate to observe the full range of effects and long-term outcomes of naringenin treatment. Injection of ethanol can influence the level of liver fibrosis in the rat model. Variations in ethanol-induced fibrosis could influence the relevance of the findings. Not all aspects of a healthy liver might be reflected in the specific biomarkers and endpoints used to assess hepatic fibrosis and the efficacy of treatment. More studies will be conducted to determine the pharmacokinetic properties of naringenin.

Conclusion

The study suggests that naringenin positively impacts alcoholic liver fibrosis by regulating MMPs, TIMPs, and SMA, preventing ethanol-induced toxicity. Naringenin may improve liver histology and reduce fibrosis markers, potential therapeutic agent for liver fibrosis treatment. This study is limited by the inherent constraints of animal models, dosage parameters, and the need to further explore the safety profile and underlying mechanisms to establish naringenin's efficacy and safety of naringenin in human liver fibrosis. Additional research is needed, including clinical trials and studies addressing long-term effects and optimal therapeutic conditions.

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Declarations

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The authors did not receive funding.

Author contributions

Conceptualization, J.C.; Methodology, J.C.; Software, U.S.; Validation, J.C. and S.N.; Formal Analysis, J.C.; Investigation, J.C.; Resources, U.S.; Data Curation, U.S. and J.C.; Writing – Original Draft Preparation, U.S.; Writing – Review & Editing, K.M.; Visualization, J.C.; Supervision, S.N.

Conflicts of interest

No competing interests declared.

Data availability

Data sets from this study are available upon reasonable request to the author.

Ethics approval

Animal procedures ethically approved by Annamalai University (Reg.No: 160/1999/CPCSEA/557).

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ORIGINAL PAPER

Interleukin 6 as a key inflammatory predictor of gestational diabetes – clinical and biochemical evidence

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ABSTRACT

Introduction and aim. Gestational diabetes mellitus (GDM) is a prevalent metabolic disorder in pregnancy associated with significant maternal and fetal complications. Chronic low-grade inflammation is increasingly recognized as a key contributor to GDM pathophysiology, with interleukin 6 (IL-6) emerging as an important mediator of insulin resistance. The aim was to investigate the relationship between IL-6 levels and metabolic parameters in pregnant women with and without GDM, and to evaluate the diagnostic performance of IL-6 for distinguishing GDM.

Material and methods. A total of 45 pregnant women with GDM and 45 normoglycemic controls between 24 and 28 weeks of gestation were enrolled. Clinical data age, body mass index (BMI), gestational age, fasting insulin, oral glucose tolerance test (OGTT), and lipid profile were assessed. IL-6 was quantified by high-sensitivity enzyme-linked immunosorbent assay. Correlation analysis, multiple linear regression, and receiver operating characteristic (ROC) curve analysis were performed using SPSS.

Results. Women with GDM exhibited significantly higher IL-6 levels (25.35±11.76 ng/L) than controls (11.02±3.59 ng/L, $p<0.001$). IL-6 showed strong positive correlations with fasting insulin ($r=0.900$, $p<0.001$) and OGTT ($r=0.684$, $p<0.001$). Multiple regression indicated that gestational age, BMI, and total cholesterol were significant predictors of IL-6 in GDM ($p<0.05$). ROC analysis revealed an area under the curve of 0.923 ($p<0.001$) for IL-6, with a sensitivity of 88.9% and specificity of 78.3% at the cutoff of 13.243 ng/L.

Conclusion. Elevated IL-6 is strongly associated with insulin resistance and dyslipidemia in GDM, suggesting a potential role as an inflammatory biomarker for early risk stratification. Incorporating IL-6 measurement into GDM screening protocols may enhance diagnostic accuracy and facilitate timely interventions to improve pregnancy outcomes.

Keywords. gestational diabetes mellitus, insulin resistance, interleukin-6, maternal inflammation, metabolic dysregulation, pregnancy complications

Introduction

Gestational diabetes mellitus (GDM) is a common metabolic disorder characterized by glucose intolerance that first arises during pregnancy, with a global prevalence ranging from 5% to 20% depending on diagnostic criteria and population characteristics.[1] It has significant short- and long-term implications for both maternal and fetal health, including increased risk of hypertensive disorders, cesarean delivery, macrosomia, and future development of type 2 diabetes.[2]

A growing body of evidence indicates that inflammation plays a pivotal role in GDM, linking excess adiposity, insulin resistance, and placental hormonal changes to a pro-inflammatory state.[3] Among the

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various inflammatory mediators implicated in metabolic dysfunction, interleukin 6 (IL-6) has gained considerable attention for its multifaceted role in glucose homeostasis and insulin signaling.[4] IL-6 is primarily secreted by adipose tissue and the placenta in pregnancy, though it can also be produced by immune cells and skeletal muscle in response to stressors such as infection or hypoxia.[5] Elevated IL-6 concentrations have been observed consistently in pregnant women with GDM compared to those with normal glucose tolerance, supporting the hypothesis that chronic low-grade inflammation contributes to the development and progression of gestational dysglycemia.[5]

Mechanistically, IL-6 modulates insulin signaling through several pathways. Experimental models suggest that IL-6 can induce serine phosphorylation of insulin receptor substrate-1 (IRS-1), thereby impairing downstream insulin signaling and promoting insulin resistance.[6] In addition, IL-6 appears to regulate hepatic gluconeogenesis by influencing key enzymes, including phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, leading to increased endogenous glucose production.[7] During pregnancy, the placenta itself can be an abundant source of IL-6, and circulating levels tend to rise as gestation advances, further challenging maternal glucose homeostasis.[8]

Several clinical investigations have demonstrated a strong correlation between IL-6 and impaired glucose tolerance in pregnant populations. Nonetheless, most of these studies have focused solely on establishing this association, with limited exploration into the diagnostic utility of IL-6. In our study, we not only confirm the link between elevated IL-6 and GDM but also propose a specific cutoff value of ≥13.243 ng/L based on receiver operating characteristic (ROC) curve analysis. This threshold, which exhibits a sensitivity of 88.9% and a specificity of 78.3%, offers a valuable clinical tool for distinguishing GDM from normoglycemic pregnancies. By defining this diagnostic parameter, our work advances current understanding and holds promise for enhancing early screening strategies and guiding timely interventions in gestational diabetes management. Despite mounting evidence of IL-6 pathogenic significance, there is still a need to elucidate its precise relationship with classical metabolic and lipid parameters in pregnancy. Clarifying the interplay between IL-6, insulin resistance, and dyslipidemia may illuminate novel diagnostic or therapeutic avenues. Early identification of elevated IL-6 could help pinpoint those at the highest risk for GDM and enable timely interventions such as lifestyle modifications or pharmacotherapy.[8] Additionally, further exploration of IL-6 potential as a predictive biomarker may guide individualized management, ultimately improving maternal and neonatal outcomes.

Aim

Against this backdrop, the present study was designed to examine IL-6 levels in pregnant women with and without GDM, with particular emphasis on its correlation with insulin resistance indices and lipid abnormalities. Our investigation integrates both cross-sectional measurements and regression analyses to delineate factors influencing IL-6 in GDM, alongside a ROC curve assessment to determine the diagnostic accuracy of IL-6. By focusing on the multifaceted role of IL-6, we aim to contribute novel insights into the inflammatory underpinnings of GDM and to explore the potential clinical utility of this cytokine in enhancing early screening and risk stratification.

Material and methods

Study design and ethical approval

This study was conducted between September 10, 2024, and February 10, 2025, at Al-Najaf Al-Ashraf Teaching Hospital and Al-Hakim General Hospital in Najaf City, Iraq. The study protocol was reviewed and approved by the Institutional Review Board of the Najaf Health Directorate, Training and Human Development Center (Approval No. 33652), ensuring compliance with the ethical principles outlined in the Declaration of Helsinki.[9] Informed consent was obtained from all participants prior to enrollment in the study.

Study population and diagnostic criteria

Pregnant women between 13 and 27 weeks of gestation were screened. Those meeting the standard diagnostic criteria for GDM, based on the International Association of the Diabetes and Pregnancy Study Groups (IADPSG) 75 g oral glucose tolerance test (OGTT) thresholds (fasting plasma glucose ≥92 mg/dL, 1 h ≥180 mg/dL, or 2 h ≥153 mg/dL)[10], were enrolled as cases (GDM group). Pregnant women within the same gestational age range but without GDM served as controls. Exclusion criteria included known diabetes prior to pregnancy, hypertension, preeclampsia, multiple gestation, chronic inflammatory conditions, and use of corticosteroids or immunosuppressive therapy. A total of 45 pregnant women with GDM and 45 normoglycemic pregnant women were included in the final analysis. The sample size was determined using the formula for comparing two means. Based on an anticipated difference in IL-6 levels of 14.33 ng/L, a standard deviation of 8.7 ng/L, an alpha level of 0.05, and a power of 80%, the calculated minimum sample size per group was 6. To account for potential dropouts and to ensure adequate power, a total of 90 participants were recruited.[11]

Anthropometric and clinical assessment

Comprehensive anthropometric and clinical assessments were performed for all participants at the time of enrollment, which was between 13 and 27 weeks of

gestation. Maternal age, gestational age, and body mass index (BMI) were recorded and participants were categorized based on the World Health Organization (WHO) classification.[12] BMI was calculated using the formula: BMI=weight (kg)/height (m^2), with body weight and height measured using a calibrated stadiometer and digital weighing scale. It is important to note that these BMI values reflect maternal adiposity during the second trimester of pregnancy, rather than pregestational BMI.

Blood sample collection

Venous blood samples (5 mL) were collected from each participant at a single time point between 09:00 and 10:00 a.m. following a standardized 12-hour fasting period. Participants were enrolled at various stages of pregnancy, and the gestational age at the time of blood collection was recorded. For the purpose of analysis, participants were grouped according to their gestational age into the following categories: 13–16 weeks, 17–20 weeks, and ≥21 weeks. This approach enabled a cross-sectional analysis of IL-6 levels at different stages of gestation. Serum was separated by centrifugation at 3,000 rpm for 5 minutes at 4°C, aliquoted, and stored at -20°C until further processing.

Biochemical and biomarker analysis

OGTT

The 75 g OGTT was administered following IADPSG guidelines. Fasting, 1-hour, and 2-hour plasma glucose concentrations were measured using the Mindray Chemistry Analyzer (Shenzhen Mindray Bio-medical Electronics, China), providing precise and reproducible glucose measurements. Fasting plasma glucose (FPG) was measured as part of the 75 g OGTT protocol. To assess insulin resistance, the homeostatic model assessment of insulin resistance (HOMA-IR) was calculated for each participant using the formula:

HOMA-IR fasting insulin (mIU/L)/FPG (mmol/L)/22.5

For calculations, FPG values measured in mg/dL were converted to mmol/L by dividing by 18.

Glycated hemoglobin (HbA1c) measurement

HbA1c levels were quantified using a high-performance liquid chromatography (HPLC) method (Tosoh G8 Automated HPLC Analyzer, Tosoh Bioscience, USA), with results expressed as percentages (%), reflecting the average blood glucose levels over the preceding 2–3 months.

Lipid profile assessment

Serum lipid profile, including total cholesterol, triglycerides, HDL-C, and LDL-C, was evaluated using enzymatic colorimetric methods (Clinichem, Hungary) with the Biolis30i analyzer. Assays were calibrated daily, ensuring adherence to quality control protocols.

Fasting serum insulin

Insulin concentrations were measured through a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA) (BT Lab, China; Cat. No. E0010Hu). This method employed specific monoclonal antibodies for precise detection of serum insulin levels, with optical density measured at 450 nm using a Paramedical microplate reader (Italy).

IL-6

Serum IL-6 levels were determined using a Human IL-6 ELISA Kit (BT Lab, China; Cat. No. E0090Hu). The assay utilized a sandwich ELISA technique, incorporating wells pre-coated with anti-IL-6 antibodies. The colorimetric detection involved biotinylated antibodies and Streptavidin-HRP, with optical density measured at 450 nm. The assay featured a sensitivity of 1.03 ng/L and a detection range of 2–600 ng/L, providing robust data on the inflammatory status of participants.

Statistical analysis

All statistical analyses were conducted using SPSS software version 28 (IBM, Armonk, NY, USA). Prior to performing regression analyses, we assessed the assumptions of linear regression, including linearity, normality of residuals, homoscedasticity, and independence of observations. Scatter plots and normal probability plots were utilized to evaluate these assumptions, while the Durbin-Watson statistic was employed to assess the independence of residuals. When necessary, data transformations were applied to meet these assumptions.

Furthermore, potential confounders such as maternal age, BMI, and gestational age were included in the regression models as covariates to isolate the independent effect of IL-6 on metabolic parameters. Multicollinearity among predictors was examined by calculating the variance inflation factor (VIF), ensuring that all VIF values remained below 5, which supports the reliability of our estimates. These steps helped ensure that our statistical models were robust and that our findings are both valid and reliable.[13]

Results

In Table 1, no statistically significant differences were observed in maternal age (p=0.485) or gestational age (p=0.510) between the GDM and control groups. However, participants with GDM exhibited significantly higher BMI (p=0.0001), triglyceride (p=0.0001), and total cholesterol levels (p=0.030). Differences in HDL (p=0.064) and LDL (p=0.297) did not reach statistical significance. Although the HbA1c values did not differ significantly (p=0.153), the GDM group displayed markedly elevated fasting insulin (p=0.0001) and OGTT (p=0.0001) values, indicating pronounced glucose intolerance. Furthermore, GDM was associated

with a heightened inflammatory profile, as evidenced by significantly elevated IL-6 (p=0.0001).

Table 1. Comparison of clinical and biochemical parameters between GDM and non-GDM pregnant women

Variables	Pregnancy women Mean±SD		p
	GDM (HG) n=45	Controls (NG) n=45	
Age (years)	26.18±4.99	25.52±3.88	0.485
BMI (kg/m^2)	29.36±3.3	26.2±3.11	0.0001
Gestational age (weeks)	19.02±4.14	18.46±4.03	0.510
TG (mg/dL)	176.8±46.57	130.55±18.44	0.0001
Chol (mg/dL)	224.69±42.78	204.99±42.29	0.030
HDL (mg/dL)	51.2±11.68	55.84±11.91	0.064
LDL (mg/dL)	124.77±31.23	117.8±32.11	0.297
HbA1c (%)	5.41±0.36	5.27±0.5	0.153
Fasting insulin (mIU/L)	7.89±1.85	5.46±1.35	0.0001
OGTT (mg/dL)	159.43±15.89	81.45±5.69	0.0001
IL-6 (ng/L)	25.35±11.76	11.02±3.59	0.0001
HOMA1-IR	3.17±1.03	1.09±0.25	0.0001

In Figure 1, the mean IL-6 concentration was substantially higher among women with GDM (25.35 ng/L) than in those without GDM (11.27 ng/L), a difference that reached statistical significance (p<0.001). This notable increase in IL-6 suggests an intensified inflammatory state in GDM, potentially reflecting a pivotal pathophysiological mechanism.

In Table 2, fasting insulin, OGTT, and IL-6 levels in the GDM group increased significantly with advancing gestational age (p=0.0001 for each), indicating progressive insulin resistance and an augmented inflammatory response. By contrast, these parameters remained stable over the same interval in the control group (p>0.05), suggesting a consistent metabolic and inflammatory profile.

In Figure 2, IL-6 levels exhibited a strong positive correlation with both OGTT (r=0.684, p<0.001) and fasting insulin (r=0.900, p<0.001). These findings imply that elevated IL-6 is linked to deteriorating glucose tolerance and increased insulin levels, underscoring the crucial role of inflammation in exacerbating insulin resistance during pregnancy.

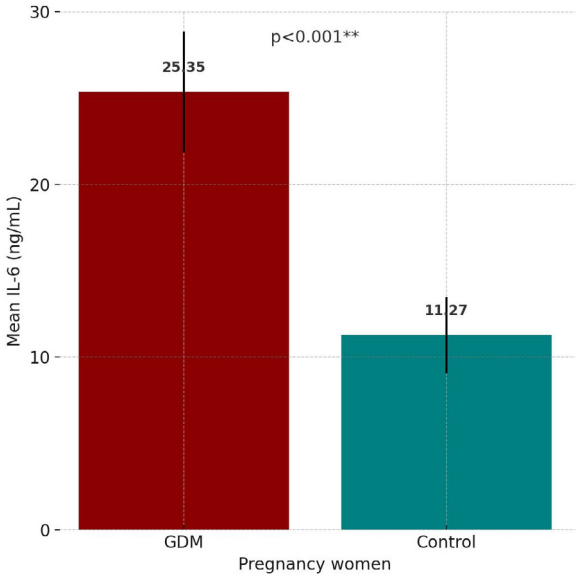

Fig. 1. Mean serum IL-6 levels in GDM versus control pregnant women

Table 2. Changes in fasting insulin, OGTT, and IL-6 levels by gestational age in GDM versus control pregnant women*

Pregnancy women		Mean±SD			
		13–16 weeks	17–20 weeks	≥21 weeks	p
GDM	Fasting insulin (mIU/L)	6.56±0.86	7.66±1.58	9.16±1.77	0.0001
	OGTT (mg/dL)	146.66±9.86	165.82±16.17	168.69±12.47	0.0001
	IL-6 (ng/L)	16.6±4.27	22.03±6.37	34.39±11.34	0.0001
Control	Fasting insulin (mIU/L)	5.58±1.42	5.89±1.38	4.91±1.11	0.159
	OGTT (mg/dL)	80.85±5.73	79.53±5.62	83.97±5.16	0.113
	IL-6 (ng/L)	11.35±4.15	10.41±2.82	11.08±3.48	0.780

* values represent mean±SD for each parameter across gestational age groups based on the single time point at which each participant's blood sample was collected (i.e., 13–16, 17–20, and ≥21 weeks) These groupings allow for a cross-sectional comparison of metabolic and inflammatory markers at different stages of pregnancy

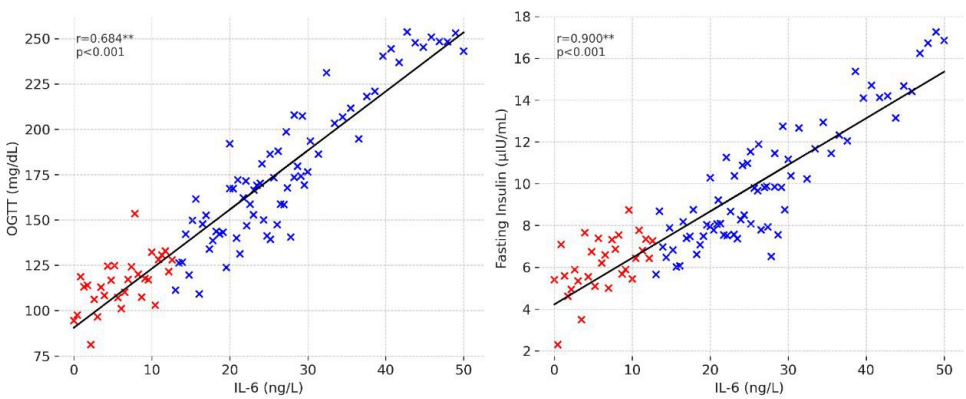

Fig. 2. Correlations between serum IL-6 levels and A: OGTT and B: fasting insulin in pregnant women, in these scatter plots, red dots represent women diagnosed with GDM and blue dots represent normoglycemic control women

Correlation analysis in Figure 3 demonstrated a significant positive association between IL-6 and HOMA-IR ($r=0.879$, $p<0.001$), further confirming the link between elevated IL-6 levels and insulin resistance in GDM. These findings are consistent with the observed relationships between IL-6, fasting insulin, and OGTT values.

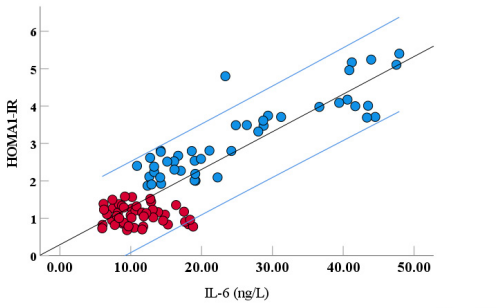

Fig. 3. Correlations between serum IL-6 Levels and HOMA-IR, in these scatter plots, red dots represent women diagnosed with gestational diabetes mellitus (GDM) and blue dots represent normoglycemic control women

Table 3 shows that among pregnant women with GDM, BMI ($\beta=0.451$, $p=0.010$) and gestational age ($\beta=0.399$, $p=0.017$) emerged as significant predictors of IL-6, whereas age did not exhibit a significant association ($\beta=-0.110$, $p=0.307$). These results suggest that both increased adiposity and advanced gestational age independently contribute to an elevated inflammatory status, as indicated by higher IL-6 levels.

Table 3. Linear regression analysis of demographic predictors of IL-6 in GDM

Pregnancy women	Predictors	Dependent variable: IL-6 (ng/L)		
		Standardized coefficients Beta	t	Sig.
GDM	Age	-0.110	-1.034	0.307
	BMI (kg/m²)	0.451	2.71	0.010
	Gestational age (weeks)	0.399	2.481	0.017

* This table presents the standardized beta coefficients, t-values, and significance levels (p) for demographic variables (age, BMI, gestational age) predicting serum IL-6 levels among women with GDM

In Table 4, of the lipid parameters analyzed, only total cholesterol significantly predicted IL-6 ($\beta=0.528$, $p=0.001$). Triglycerides ($p=0.362$), HDL ($p=0.535$), and LDL ($p=0.558$) were not significant predictors. These findings indicate that heightened cholesterol levels may critically influence the inflammatory processes in GDM, further highlighting the multifactorial pathophysiology of this condition.

Table 4. Linear regression analysis of lipid profile predictors of IL-6 in GDM

Pregnancy women	Predicators	Dependent variable: IL-6 (ng/L)		
		Standardized coefficients Beta	t	Sig.
GDM	TG (mg/dL)	0.139	0.923	0.362
	Chol (mg/dL)	0.528	3.450	0.001
	HDL (mg/dL)	-0.076	-0.625	0.535
	LDL (mg/dL)	0.089	0.591	0.558

Table 5, IL-6 demonstrated a strong diagnostic performance for distinguishing GDM, with an area under the curve (AUC) of 0.923 ($p=0.0001$; 95% CI: 0.873–0.973). A cutoff value of ≥13.2425 ng/L yielded a sensitivity of 88.9% and a specificity of 78.3%. These findings highlight IL-6 as a promising biomarker for identifying GDM risk, supported by the high accuracy depicted in the ROC curve.

Table 5. ROC analysis of IL-6 in differentiating GDM from control pregnant women

Predictors	Area under the curve					
	Area	Sig.	95% CI	Cutoff	Sensitivity	Specificity
IL-6 (ng/L)	?	0.0001	0.873-0.973	≥13.2425	0.889	0.783

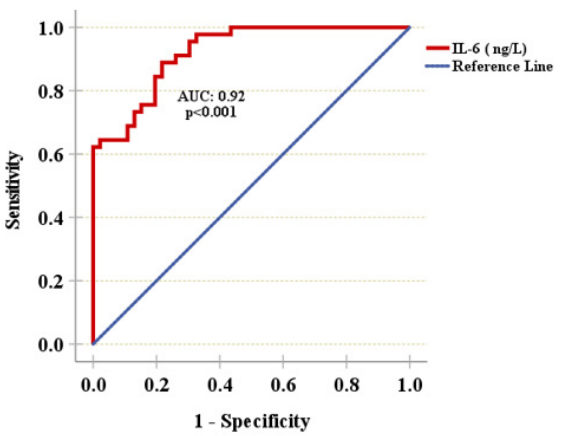

Fig. 4. ROC curve for IL-6 in GDM vs. non-GDM pregnant women

Discussion

The present study aimed to investigate the role of IL-6 in the pathophysiology of GDM by comparing clinical, biochemical, and inflammatory markers between pregnant women with and without GDM. Our data demonstrate significantly elevated IL-6 levels in the GDM group, alongside higher BMI, total cholesterol, fasting insulin, and impaired oral glucose tolerance. The strong positive correlations of IL-6 with fasting insulin and OGTT, coupled with our regression analyses, underscore IL-6 as a key inflammatory predictor of GDM.

Our findings revealed elevated IL-6 levels in GDM patients, aligning closely with previous studies that have demonstrated a comparable increase in this pro-inflam-

matory cytokine among diabetic pregnancies. (14)reported a similar pattern, showing nearly triple the IL-6 concentration in GDM women compared to healthy controls. Likewise,[6] observed a pronounced elevation in IL-6 among GDM participants, significantly surpassing levels seen in normoglycemic pregnancies. Although methodological differences, such as assay techniques and sample processing, can influence IL-6 measurements, the consistency across these studies underscores the robust association between heightened IL-6 and GDM.

Mechanistically, IL-6 is known to be secreted by adipose tissue and the placenta in response to heightened metabolic demands and insulin resistance in pregnancy.[16] Elevated IL-6 can exacerbate insulin resistance by interfering with insulin signaling pathways in both hepatic and skeletal muscle tissues, leading to increased hepatic gluconeogenesis and reduced peripheral glucose uptake.[7] In the context of GDM, expanding adipose tissue and placental size across gestation may further stimulate IL-6 production, This pro-inflammatory milieu can, in turn, amplify insulin resistance, creating a vicious cycle that contributes to hyperglycemia.[16]

Our findings also highlight the significant association between IL-6 and lipid abnormalities—particularly total cholesterol – a phenomenon that has been implicated in vascular inflammation and endothelial dysfunction.[17] Elevated cholesterol may promote oxidative stress and inflammation, thus driving IL-6 production and contributing to the progression of GDM.[18] In addition, the high BMI observed in GDM participants likely reflects excess adipose tissue, which is a key source of pro-inflammatory cytokines like IL-6.[16] These interrelated metabolic and inflammatory derangements point to the multifactorial etiology of GDM.

Building on our findings, the clinical implications of IL-6 measurement in GDM screening merit further consideration. Incorporating IL-6 as an adjunct to conventional glucose-based screening methods could enhance early risk stratification, particularly by identifying patients who might be missed by standard tests alone. The diagnostic cutoff of ≥13.243 ng/L, as derived from our ROC analysis, offers a promising threshold for flagging pregnant women at heightened risk of GDM. In practice, IL-6 assays could be performed concurrently with routine OGTT or fasting glucose measurements, thereby providing an additional layer of diagnostic accuracy and enabling earlier lifestyle or pharmacological interventions.

Looking ahead, future research should aim to validate the diagnostic utility of IL-6 in larger, multi-center, and longitudinal studies. Such studies could assess the predictive power of IL-6 over the course of pregnancy, examine its relationship with long-term maternal and neonatal outcomes, and potentially refine the optimal cutoff value for clinical use. Additionally, research exploring the cost-effectiveness and feasibility of integrating IL-6 screening into existing prenatal care protocols will be essential to inform its translation into clinical practice.

A key strength of this study is the focused investigation of IL-6 as both an inflammatory and diagnostic biomarker in GDM, encompassing correlations with metabolic parameters and lipid profiles. By establishing a specific cutoff for IL-6, we propose a potentially valuable clinical screening tool. The integration of comprehensive biochemical assessments, including insulin and lipid measurements, further strengthens our conclusions regarding the interplay between inflammation and metabolic dysfunction in pregnancy.

Despite the robust associations observed between IL-6 levels and GDM, several limitations of our study warrant consideration. First, the single-center design may limit the generalizability of our findings to broader populations, as patient demographics and clinical practices might differ in other settings. Second, the relatively small sample size (n=90) may reduce the statistical power and limit the detection of more subtle associations. These factors, combined with potential variability in IL-6 assay measurements, suggest that caution should be exercised when extrapolating our results. Future studies involving larger, multi-center cohorts are needed to confirm these findings and to further delineate the role of IL-6 in the pathophysiology and early diagnosis of GDM.

Nonetheless, we recognize that expanding the scope of inflammatory profiling could further enhance our understanding of GDM. Future research should consider analyzing the interactions between IL-6 and other emerging biomarkers, such as C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF-α), within the same patient cohort. Such studies could help determine whether IL-6 adds significant diagnostic and prognostic value over existing markers. Additionally, tracking pregnancy outcomes – both short-term (e.g., preeclampsia, preterm birth) and long-term (e.g., development of type 2 diabetes post-pregnancy) – would further underscore the clinical relevance of these inflammatory markers.

It would also be valuable to assess how IL-6 levels fluctuate across different stages of pregnancy. Our current study was limited to measurements between 24 and 28 weeks of gestation; however, evaluating IL-6 across all trimesters could identify critical windows when inflammatory changes are most predictive of GDM. Finally, while we controlled for key confounders such as BMI, age, and gestational age, other factors – including smoking, dietary habits, physical activity, and comorbid conditions – may influence IL-6 levels and should be addressed in future, larger-scale studies to improve generalizability.

While our study demonstrates that IL-6 levels are significantly elevated in women with GDM and suggests

a potential diagnostic cutoff of ≥13.243 ng/L, it is important to note that IL-6 is an acute-phase cytokine that lacks specificity. Elevated IL-6 is observed in a variety of inflammatory and metabolic disorders – including type 2 diabetes and obesity – and may be affected by factors such as dietary habits, physical activity, and stress. To mitigate these confounding effects, we excluded patients with known inflammatory conditions and controlled for variables such as BMI, age, and gestational age. Nonetheless, unmeasured factors (e.g., red meat consumption, exercise levels, and stress) could have contributed to the observed IL-6 levels.

Given these limitations, the diagnostic utility of IL-6 as a stand-alone marker for GDM must be interpreted with caution. Our study was not designed as a diagnostic trial with blinded protocols and focused sensitivity and specificity evaluations. Therefore, while our data support the potential of IL-6 as an adjunct biomarker, future studies employing specially designed, blinded protocols are warranted. In addition, exploring the interactions of IL-6 with other inflammatory markers – such as CRP and TNF-α – could enhance the specificity and clinical relevance of the inflammatory profile in GDM.

Conclusion

In conclusion, our findings indicate that elevated IL-6 levels are associated with gestational diabetes mellitus and may serve as a useful adjunct to conventional screening methods. However, because IL-6 is a non-specific marker affected by various confounding factors, further research using rigorously designed, blinded protocols is necessary to confirm its diagnostic value. Future investigations should also explore the combined use of IL-6 with other inflammatory biomarkers to improve the specificity of GDM screening and better predict both short- and long-term clinical outcomes.

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Declarations

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Author contributions

Conceptualization, S.H. and R.D. ; Methodology, S.H.; Software, S.H.; Validation, S.H., R.D., and A.U.; Formal Analysis, S.H.; Investigation, S.H.; Resources, R.D.; Data Curation, S.H.; Writing – Original Draft Preparation, S.H.; Writing – Review & Editing, R.D.; Visualization, A.U.; Supervision, A.U.; Project Administration, R.D.; Funding Acquisition, A.U.

Conflicts of interest

We declare that there are no conflicts of interest associated with this manuscript.

Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study protocol was reviewed and approved by the Institutional Review Board of the Najaf Health Directorate, Training and Human Development Center (Approval No. 33652).

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ORIGINAL PAPER

Significance of C-reactive protein to albumin ratio and thrombus burden in acute coronary syndrome

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ABSTRACT

Introduction and aim. Thrombus burden (TB) is a critical factor in the pathogenesis of acute coronary syndrome (ACS) pathogenesis with biomarkers such as C-reactive protein (CRP) and serum albumin that reflect systemic inflammatory and nutritional states. The CRP and albumin ratio (CAR) has emerged as a new composite marker, offering enhanced prognostic value. The aim of this study was to evaluate the association between CAR and TB in patients with ACS and to assess the predictive utility of CAR compared to CRP and albumin individually.

Material and methods. A hospital cross-sectional analytical study was conducted among 93 patients ages 18–60 years with ACS who underwent coronary angiography (CAG). CAR was calculated and its association with TB was analyzed.

Results. Of the participants, 9.7% had high tuberculosis. CAR, CRP, and albumin were significantly associated with TB ($p<0.001$). CAR showed the highest correlation ($r=0.728$) and perfect diagnostic accuracy (AUC=1), outperforming CRP (AUC=0.987) and albumin (AUC=0.030). High TB was significantly associated with the presentation of grade 1 TIMI and ST-elevated myocardial infarction (STEMI) presentation ($p<0.05$).

Conclusion. CAR is a reliable, accessible and independent biomarker for predicting TB in ACS, and its incorporation into standard clinical protocols could improve early risk stratification, therapeutic decision-making, and patient outcomes. More multicentric study are warranted to validate its broader clinical applicability.

Keywords. acute coronary syndrome, albumin, C-reactive protein, thrombus burden

Introduction

Acute coronary syndrome (ACS) characterized by sudden, reduced blood flow to the heart, causing symptoms includes unstable angina (UA), non-ST segment elevation myocardial infarction (NSTEMI), and ST segment elevation myocardial infarction (STEMI).[1,2] Despite advances in therapeutic strategies, ACS remains a significant global health problem.[3] The pathophysiology of ACS involves atherosclerotic plaque rupture, endothelial dysfunction, and thrombus formation, leading to partial or complete coronary arteries.[1,2] Among these thrombus formation following plaque rupture plays a critical role in determining the severity of myocardial ischemia and infarction.[4,5]

Thrombus burden (TB) is the amount of thrombus present in the blood vessel,[6] and it is a crucial factor in determining the severity of myocardial ischemia and infarction in ACS and is essential for risk stratification and management.[7] As patients with high TB are more likely to experience extensive myocardial damage, increased coronary artery obstruction, and an increased risk of major adverse cardiac events (MACE).[8] Accurate

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assessment of TB is crucial to determine the severity of ACS and guiding therapeutic interventions. Techniques like angiographic evaluation, intravascular ultrasound (IVUS), and optical coherence tomography (OCT) are used to visualize TB and assess its composition.[9]

Additionally, systemic inflammation is crucial in the pathogenesis of ACS, contributing to plaque formation, instability, and thrombus generation.[5,9,10] C-reactive protein (CRP), an acute phase reactant produced by the liver, is a biomarker of inflammation, associated with worse outcomes in patients with ACS.[11-14] Elevated levels of CRP are associated with increased mortality and a higher incidence of MACE.[15,16] However, relying on a single biomarker may not fully capture the complexity of inflammatory and thrombotic processes in ACS.

Recently, the ratio of C-reactive protein to albumin (CAR) has emerged as a novel prognostic marker that reflects both inflammation and nutritional status.[11–14,17,18] CAR provides a comprehensive understanding of the condition of a patient, including hypoalbuminemia,[19] which has been associated with worse outcomes in cardiovascular disease (CVD).[20,21] Furthermore, since both inflammation and malnutrition have been shown to influence the development of thrombotic complications, it is plausible that CAR may correlate with TB in patients with ACS.[16]

In various studies, CAR has been associated with high coronary artery resistance and significant predictor significance of CAR.[22,23] In another study, in patients with acute myocardial infarction (AMI), high CAR has been associated with larger infarct sizes, more extensive coronary artery disease (CAD), and poorer long-term outcomes.[11]

Aim

Despite these findings, our study addresses the limited evidence on the link between CAR and TB in ACS patients which remains underexplored. Although CRP and albumin have been linked to outcomes in ACS, no previous study has directly compared their CAR with angiographically quantified TB. Therefore, by evaluating CAR along with the standardized TIMI thrombus grading, our research uniquely integrates systemic inflammation, nutritional status, and direct measures of intracoronary clot load. This novel approach aims to determine whether CAR can serve as a readily available biomarker for early risk stratification in patients with ACS. With this background, the present study aims to investigate the relationship between CAR and TB in patients with ACS.

Material and methods

This hospital-based analytical cross-sectional study was conducted in the Department of General Medicine at the Mahatma Gandhi Medical College and Research Institute, Puducherry, during the period of November 2022 to September 2024. Ethical approval was obtained from the institutional Human Ethics Committee (IHEC) (MGMCRI/Res/04/2022/121) and all participants gave their written informed consent.

Inclusion criteria for study participants include age group of 18 to 60 years with signs and symptoms of typical chest pain and were diagnosed as STEMI, NSTEMI and ACS who requires coronary angiogram. patient who was treated with primary PCI was included. While patients diagnosed with UA, a history of coronary artery bypass grafting (CABG), active malignancy, infection and connective tissue disorder, and pregnant and lactating women were excluded from the study.

Sample size determination and sampling procedure

Assuming that the prevalence of high TB was 59.6% in ACS patients from the study conducted by Duman et al.,[17] the minimum calculated sample size was 93 using the formula

$$n \geq \frac{Z^2_{\frac{1-\alpha}{2}} \times p\,(1-p)}{d^2} = \frac{(1.96)^2 \times 0.596\,(0.404)}{(0.10)^2} = 93$$

($Z_{\alpha/2}$ – 1.96; proportion (p) – 0.596; precision (d)) – 0.1) with a two-sided confidence interval of 95% and 10% precision. This sample provides >80% power to detect a moderate correlation (r~0.30) between CAR and TB at α 0.05. The consecutive sampling technique was used to include all patients with inclusion criteria until the desired sample size was achieved.

Data collection procedure

Data collection was carried out using a semistructured clinical proforma which included demographic details, risk factor assessment, anthropometric and clinical examination. Electrocardiogram (ECG) was taken at the time of admission. Routine investigations, including complete blood count, renal and liver function tests, serum electrolytes, cardiac enzymes, and measurements of serum CRP and albumin levels, at the time of admission. After the initial evaluation, patients who required coronary angiography (CAG) were performed together with the procedure according to the clinical protocol, without any intervention of the study. Only patients who underwent CAG and satisfied the inclusion criteria were included in the final analysis. The CAR was calculated and compared with the CAG results to assess its association with TB and the degree of arterial occlusion (AO). Patients who did not satisfied inclusion criteria were also treated according to the ACS treatment protocol (American Heart Association (AHA) guidelines) without violating ethical issues.

Blood samples (5 mL) were drawn on admission where hsCRP was measured by immunoturbidimetry (Roche Cobas c501; CV <5%) and serum albumin was

assessed by the bromocresol green method (CV <4%). Further, CAR was calculated as

$$CAR = \frac{CRP\ (\frac{mg}{L})}{albumin\ (\frac{g}{dL})}$$

TB was classified as high and low based on the validated Thrombolysis In Myocardial Infarction (TIMI)[24] classification to allow an objective assessment of the severity of thrombotic in ACS. Grades 0–2 defined as 'low TB' and grades 4–5 as "high TB". Two independent interventional cardiologists, blinded to biomarker levels, classified TB and resolved the discrepancies (where grade 3 adjudicated by consensus). This stratification was helped to analyze its association with biomarkers such as CAR, CRP, and albumin and to evaluate their predictive utility for adverse outcomes.

Table 1. Sociodemographic characteristics and comorbid status of study participants*

Variables	Results n (%) or mean±SD
Age (in years)	
21–30	2 (2.2)
31–40	18 (19.4)
41–50	44 (47.3)
51–60	29 (31.2)
Mean±SD	46.20±7.938
Gender	
Male	56 (60.2)
Female	37 (39.8)
Smoking	
Yes	31 (33.3)
No	62 (66.7)
Systemic hypertension	
Yes	87 (93.5)
No	6 (6.5)
Diabetes	
Yes	86 (92.5)
No	7 (7.5)

* SD – standard deviation, n (%) = frequency, and percentages are presented in brackets

Statistical analysis

The data were entered into MS Excel (Ver_2007) and the statistical data were analyzed using SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY, USA) software. The normality of the data was assessed using Kolmogorov-Smirnov and Shapiro-Wilks tests and the outliers were handled by exclusion. Descriptive statistics for categorical variables were expressed as frequencies and percentages and for continuous variables as the mean and standard deviation or median without interquartile range (IQR). As for inferential statistics, the chi-square test and one-way ANOVA was used to find the association between TB and CAR. Values of $p<0.05$ were statistically significant. Receiver operating characteristic (ROC) curve and area under curve (AUC) analysis were used to determine the best cutoff point for the diagnosis and resolution of symptoms while on treatment. For all tests, a two-sided p value ≤0.05 was considered statistically significant. Diagnostic values based on AUC were 0.9–1.0 excellent test; 0.8–0.9 good test; 0.7–0.8 fair test; 0.6–0.7 poor test and 0.5–0.6 fail.

Results

The sociodemographic characteristics and the comorbidity status of the study participants are presented in Table 1. The mean CAR among the study participants was 9.38±10.22 and the remaining clinical profiles are presented in Table 2. Among study participants, 32 patients (34.4%) had STEMI, and the remaining 61 patients (65.6%) had NSTEMI in the ECG findings. TB was found among the study participants where 84 patients (90.3%) had low TB, while the remaining nine patients (9.7%) had a high level of TB (Fig. 1).

Table 2. Clinical profile of the study participants with ACS*

Variables	Mean±SD
C-reactive protein (mg/L)	24.77±20.38
Albumin (g/dL)	3.21±0.61
Troponin I (ng/L)	8808.71±13290.78
Low density lipoprotein (mg/dL)	139.86±36.36
High density lipoprotein (mg/dL)	33.16±5.19

* ACS – acute coronary syndrome, SD – standard deviation

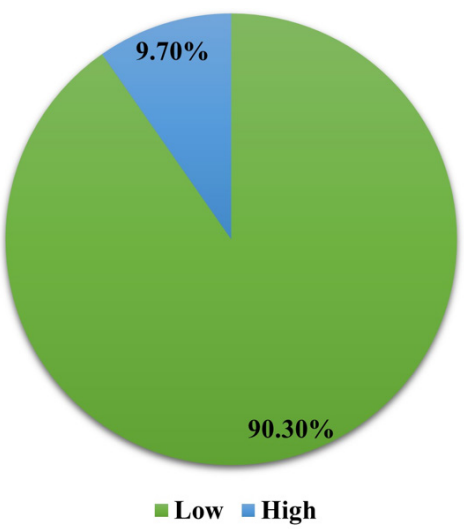

Fig. 1. Thrombus burden among the study participants

Table 3 shows the association of tuberculosis with other findings among study participants. Although the association between TB and ECG findings showed a significant relationship (p = 0.007) showing that the higher TB were more commonly associated with NSTEMI, which is typically associated with partial coronary occlusion, while STEMI often related to competitive occlusion, is mor frequently seen in patients with low TB. The

association between TB and CAR shows a statistically significant relationship, with a p-value of 0.001, indicating that higher TB is strongly associated with elevated CAR suggesting increased inflammation and poor nutritional status in patients with ACS patients (Table 4). Similarly, the association of CAR with other variables did not show a statistically significant result (Table 5).

Table 3. Association between thrombus burden and other variables among study participants*

Thrombus burden	Thrombus burden		p
	Low (n=84) n (%)	High (n=9) n (%)	
Gender			
Male	50 (59.5)	6 (66.7)	0.681
Female	34 (40.5)	3 (33.3)	
CAG TIMI grading			
Grade I	0 (0)	9 (100.0)	0.001
Grade II	25 (29.8)	0 (0)	
Grade III	59 (70.2)	0 (0)	
ECG findings			
STEMI	25 (29.7)	7 (77.8)	0.007
NSTEMI	59 (70.2)	2 (22.2)	

*chi-square test, CAG – coronary angiography, TIMI – thrombolysis in myocardial infarction, ECG – electrocardiography, STEMI – ST-elevated myocardial infarction, NSTEMI – non-ST-elevated myocardial infarction, n (%) – frequency and percentages were presented in brackets

Table 4. Association between thrombus burden and clinical profile among study participants

Variable	F value	p
CAR	102.369	0.001
Troponin I	0.345	0.55
CRP	76.824	0.001
Albumin	28.031	0.001

* ANOVA, CAR – C-reactive protein to albumin ratio, CRP – C-reactive protein

Table 5. Association between CAR and other variables among study participants*

Variables	F value	p
Troponin I	0.523	0.97
Gender	1.255	0.28
ECG	1.397	0.19
Age	1.227	0.30
Smoking	1.063	0.45
Diabetes	1.140	0.38

* ANOVA, ECG – electrocardiography

The ROC curve illustrates the diagnostic performance of CRP, albumin, and CAR in predicting TB, demonstrating the highest diagnostic accuracy (Fig. 2). Among the CAR markers, the curve is closest to the top-left corner, indicating superior sensitivity and specificity, making it the most reliable predictor. It emerges as the most effective parameter for distinguishing results, likely linked to its robust ability to reflect the balance of inflammation and nutritional status. Table 6 shows the AUC for TB with CRP, CAR, albumin and troponin I.

Table 6. AUC for CRP, albumin, CAR, troponin I with TB among study participants*

Test result variables	Area	Standard error	p	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
CRP	0.987	0.014	<0.001	0.960	1.000
Albumin	0.030	0.019	<0.001	0.000	0.067
CAR	1.000	0.000	<0.001	1.000	1.000
Troponin-I	0.425	0.087	0.459	0.253	0.596

*AUC – area under curve, CRP – C-reactive protein, CAR – C-reactive protein to albumin ratio

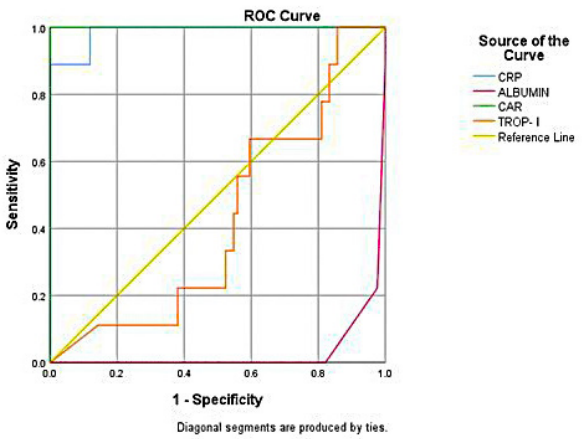

Fig. 2. Analysis of the ROC curve of TB with CAR, CRP, albumin, and troponin I

Discussion

The present study evaluated the significance of CAR as a predictor of TB in patients with ACS. The findings provide valuable information on the relationship between inflammatory markers, TB, and clinical outcomes in ACS. The study found significant associations between TB and CAR, CRP, albumin, TIMI grade, and ECG findings. CAR, with an AUC of 1.000, demonstrated excellent discriminatory power in predicting TB, outperforming individual biomarkers such as CRP and albumin. A strong positive correlation of tuberculosis was observed with CAR (r=0.728, p=0.001) and CRP (r=0.677, p=0.001) and a significant inverse correlation with albumin (r=-0.485; p=0.001). These findings suggest that CAR is not only a powerful prognostic biomarker in ACS but also reflects the systemic inflammatory state and the potential for thrombus formation.

TB plays an important role in the pathophysiology of ACS, contributing to vessel occlusion and myocardial damage.[5,10,25] The high rates of HTN and DM in

our study reflect their strong role as major risk factors in the pathogenesis of ACS. These comorbidities contribute to endothelial dysfunction and atherosclerosis, which are central to thrombus formation and coronary events. In addition, the hospital-based setting can capture more high-risk patients. The TB distribution shows that the majority of study participants (90.3%) had TB, while only 9.7% had a high TB. This suggests that most of the individuals in this cohort had less extensive coronary thrombus formation, which may correlate with less severe cases of ACS. The study by Souteyrand et al. also resulted that 11.8% of the patients had large TB, while remaining 88.2% had low TB, which was similar to our study.[26] Another study by Özkan et al., also resulted that 16.4% had massive TB.[27] These results align with prior studies indicating that inflammation plays a pivotal role in plaque instability, thrombus formation, and adverse outcomes in ACS.[28–30]

TIMI classification, a critical tool in assessing thrombus severity, aligns with the pathophysiological impact of high TB in worsening ischemia and altering coronary flow.[24] In our present study, TB assessed using the TIMI classification system during CAG, all patients with high TB were classified as TIMI grade I (100%), while those with low TB were predominantly in TIMI grade III (70.2%) and grade II (29.8%) (p=0.001) indicating a strong association between lower TIMI flow grades and higher TB. In our study, this significant association of TB with the CAG TIMI grade further reinforces its pathophysiological relevance in ACS events. This finding reflects the extent of luminal obstruction and impaired perfusion due to intraluminal thrombi, which has been associated with a worse prognosis in patients with ACS.[31–33] Further, a Pearson correlation analysis revealed a very strong association between TB and CAG TIMI flow grade (r=0.755, p=0.001) that highlights the diagnostic value of angiographic assessment in identifying patients with a high thrombotic load. The study by Kume et al. resulted that patients with high TIMI had higher TB compared to the patients with low TB.[34] These findings are consistent with previous literature that emphasized the clinical importance of quantifying TB for prognostication and to guide interventional strategies, including the use of thrombectomy or powerful antithrombotic therapy.[31–33] Thus, accurate evaluation of thrombuses is essential for optimizing ACS and improving patient outcomes.

The ECG itself does not directly quantify the thrombus load; it provides valuable indirect evidence of ischemic load and coronary obstruction. In our study, 65.6% presented with NSTEMI, while 34.4% of the patients had STEMI. This predominance of NSTEMI reflects the evolving trends in ACS presentations, where NSTEMI is increasingly recognized due to advances in diagnostic tools and heightened clinical vigilance.[27,35,36] The higher proportion of NSTEMI cases may suggest a more chronic atherosclerotic process with less abrupt plaque rupture.[36,37] A significant association was observed between TB and ECG findings in our study. Participants with low TB were predominantly diagnosed with NSTEMI, while those with high TB were more likely to have STEMI (p=0.007) in our patients. Furthermore, the correlation showed a modest but significant inverse relationship between the ECG findings and TB (r=-0.299, p=0.004) indicating that as TB increases, the probability of presenting with STEMI also increased. The study by Scarparo et al. showed that patients with high TB had STEMI with higher mortality compared to low TB in patients with NSTEMI.[25] Study by Coşkun et al., found that patients with high TB were higher in STEMI.[38] Scarparo et al. also showed that higher TB observed among the STEMI patients with increased 30-day mortality and 3-day MACE.[25] This finding highlights the interaction between thrombus composition, degree of coronary occlusion, and the severity of the infarction.[6,26] STEMI, characterized by complete occlusion of a major coronary artery, is typically associated with higher TB, reflecting its direct impact on myocardial perfusion, in contrast, NSTEMI, often involving partial occlusion, corresponds to a lower TB.[6,8,25,27] Thus, supporting that thrombus rich lesions are more common in total occlusions characteristics of STEMI events.

Both CRP and albumin, individually and in a combined ratio, are important for evaluating the inflammatory and nutritional status of patients with ACS.[15,17,21,23,29] The mean level of CRP and albumin among ACS patients in our study was 24.77±20.38 mg/L and 3.21±0.612 g/dL and CRP showed a strong positive correlation with TB (r=0.677, p=0.001),while albumin showed an inverse correlation (r=-0.485, p=0.001). Both were significantly associated with TB (p=0.001 for both). Furthermore, CRP exhibited a high predictive value for TB (AUC=0.987), while albumin alone had a very low discriminatory power (ACU=0.030), suggesting that albumin alone is not as reliable for thrombus risk. Elevated CRP levels in patients with ACS are associated with worse outcomes and have been shown to promote endothelial dysfunction, upregulated tissue factor and enhance platelet activation.[15,39,40] Conversely, albumin function as a negative acute phase protein with antioxidative and anti-inflammatory response are associated with increased cardiovascular mortality.[21,41–43] Together, CRP and albumin provide the complementary insights into the pathophysiological processes of ACS, where CRP reflects the inflammation and thrombotic activation, and albumin reflects systemic resilience and repair mechanisms. This interplay justifies the rationale behind using the CAR for enhanced prognostic accuracy in ACS management.

CAR has recently emerged as a powerful biomarker that reflects both inflammatory status and nutrition-

al reserve. In the findings of our study, the mean CAR was 9.38±10.222, highlights a significant inflammatory burden relative to nutritional status. CAR also showed a highly significant association with tuberculosis (F=102.369, p=0.001) and demonstrated the strongest correlation among the biomarkers evaluated (r=0.728, p=0.001). ROC analysis revealed an AUC of 1.000 for CAR, indicating perfect discriminatory power in the identification of patients with high RB, far surpassing individual predictive capacities. CAR makes it a superior marker for risk stratification in ACS, as elevated levels indicate a pro-inflammatory, prothrombotic state, closely linked with plaque rupture, endothelial injury, and intraluminal thrombus formation.[44,45] The study by Askin et al., showed that CRP or albumin levels had lower sensitivity and specificity when compared to CAR had higher specificity, but lower sensitivity.[46] Thus, CAR with its high diagnostic accuracy showed that it has potential to predict the TB in ACS patients, yet with intracoronary TB at high risk, had difficulty to predict by CAR.[46] Unlike single biomarkers, CAR integrates two opposing biological process, systemic inflammation and nutritional status, providing a more comprehensive view of disease burden. As an inflammatory marker of CRP and albumin alone has less diagnostic accuracy than compared to the combined marker.[46] Previous studies have validated CAR as a predictor of mortality and adverse cardiac events in patients with ACS.[39,41–43,45,47] The findings in our study strongly support its routine inclusion in clinical practice as a cost-effective, accessible marker to evaluate thrombotic risk and guiding therapeutic decisions in ACS.

The major strengths include that integrating CAR into existing risk scores which is TIMI that improved the calibration, and that even propose the prospective validation in the multicentre cohorts. Despite these encouraging findings, several limitations warrant careful consideration. Firstly, it was conducted in a single-centric study that poses constraints, which may limit the generalizability of the findings to broader populations. In addition, referral bias in a tertiary care setting could have inflated the prevalence of hypertension and diabetes and thus the observed biomarker associations. Second, the cross-sectional design precludes the establishment of causality between inflammatory markers, tuberculosis, and clinical outcomes. Additionally, the lack of long-term follow-up limits the ability to assess the predictive value of CAR for adverse cardiovascular events. Also, potential confounders including medications and time to angiography, were not captured. The grade 3 TB required adjudication, introducing potential classification bias despite blind dual review. Finally, we focused solely on CRP and albumin, hence the incorporation of additional inflammatory or nutritional biomarkers and the advancement of imaging techniques can further refine risk stratification.

To address these gaps, future multicentre prospective studies with larger, more diverse populations are essential. Longitudinal follow-ups must be incorporated to validate the prognostic significance of CAR for hard endpoints, such as reinfarction, heart failure, and mortality. Randomized trials could evaluate whether CAR-guided therapeutic intensification improves outcomes. Further studies could also explore the integration of CAR with other biomarkers and imaging modalities for comprehensive evaluation of thrombus risk in ACS patients.

Conclusion

Therefore, this study underscores the clinical relevance of integrating biochemical and angiographic markers, specifically CAR and stratification of TB in the evaluation and risk of patients with ACS. Our findings demonstrate a significant association between elevated CAR and increased TB, suggesting a direct relationship between systemic inflammation, impaired nutritional status, and the extent of coronary thrombosis. The findings advocate for the inclusion of CAR in routine clinical assessment due to its simplicity, cost-effectiveness, and diagnostic power.

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Declarations

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Author contributions

Conceptualization, V.R.; Methodology, V.R..; Software, P.V.R.I.; Validation, V.R.; Formal Analysis, P.V.R.I.; Investigation, P.V.R.I.; Resources, P.V.R.I.; Data Curation, P.V.R.I.; Writing – Original Draft Preparation, P.V.R.I.; Writing – Review & Editing, V.R.; Visualization, V.R.; Supervision, V.R.; Project Administration, V.R.

Conflicts of interest

The author(s) reported no potential conflicts of interest regarding the research, authorship, or publication of this article.

Data availability

All data generated or analyzed in this study are included in this published article.

Ethics approval

The Institutional Ethical Committee (MGMCRI/Res/04/2022/121) approved this study.

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ORIGINAL PAPER

Impact of melatonin on platelets during oxidative stress – an in vitro approach

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ABSTRACT

Introduction and aim. Platelets are susceptible to oxidative damage due to metabolic pathways and oxygen-rich environments. Antioxidants combat oxidative stress (OS) and are currently employed in therapeutics. Melatonin has potent antioxidant properties; however, it has not been explored in platelet OS models. This study investigates the effect of melatonin on platelets during 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced OS.

Material and methods. Platelets from Wistar rats (n=5) were grouped into controls (untreated), free radical-inducer (FRI: AAPH-treated), melatonin-treated (AO), and preincubated with melatonin and AAPH-treated (FRI+AO). OS and platelet markers were analyzed.

Results. Antioxidant defenses decreased in FRI, whereas increased in AO and FRI+AO. Lipid peroxidation (LPO) increased in FRI, whereas advanced oxidation protein products (AOPP) and metabolism increased in AO compared to controls. Superoxides, AOPP, and ATP secretion increased, whereas LPO decreased in FRI+AO compared to FRI. However, aggregation increased in FRI and AO compared to Controls, whereas decreased in FRI+AO compared to FRI.

Conclusion. OS models can give insights into the underlying redox status of the cells and modulations of antioxidants in platelets. The findings indicate that melatonin can modulate antioxidant defenses and alleviate OS in platelets. This study lays the foundation for further in vivo studies on platelet pathophysiology.

Keywords. antioxidants, melatonin, oxidative stress, platelets

Introduction

Oxidative stress (OS) is a primary causative factor that disrupts hemostasis and can potentially have pathological consequences. Reactive species can affect redox signaling and lead to cellular damage. These radicals damage the membrane by lipid peroxidation and also cause protein oxidation, eventually altering the functioning of proteins.[1] OS contributes to pathophysiological conditions like Alzheimer's, Parkinson's, cardiovascular diseases, and cancer.[2] Therefore, OS models simulate physiological conditions comparable to infections, diseases, stress, and drug toxicity. They can provide insights into the underlying redox mechanisms and their interaction with endogenous defenses.[3] 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) which can simulate the OS conditions in vitro.[4] It undergoes homolytic cleavage to form two carbon-centered radicals ($R^{\bullet}$) which produce peroxyl radicals in the presence of oxygen. These highly reactive radicals can initiate chain reactions, thereby rendering proteins and polyunsaturated fatty acids extremely prone to oxidative damage.[5]

Platelets are anucleate cells with a crucial role in hemostasis. Physiological processes in platelets contin-

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uously generate reactive oxygen species (ROS). ROS generation can overwhelm the endogenous antioxidant machinery and result in a redox imbalance. OS in platelets can lead to excessive activation, malfunction, or destruction.[6] This increases the risk of platelet-related disorders, such as thrombotic disorders and thrombocytopenia. OS can also shorten the lifespan of platelets by triggering apoptotic pathways.

However, antioxidants can enhance the antioxidant defenses in platelets. Vitamin C, vanillic acid, L-carnitine, coumaric acid, and other antioxidants have been studied for their beneficial effects on platelets.[7] Melatonin can penetrate the cell membrane to neutralize endogenous hydroxyl radicals and stimulate glutathione peroxidase.[8] In vitro studies have determined that 1 mmol/L melatonin can attenuate oxidative damage in different cell types.[9-12] Melatonin has proven beneficial in reducing lipid peroxidation and regulating intracellular calcium levels in platelets.[12-14] Although the effect of melatonin varies in physiological and pharmacological conditions, its role as an antioxidant has been widely studied.[15] However, its overall effect on oxidative stress, metabolism, and functions in platelets is unclear.

Melatonin can be a promising antioxidant due to its protective effects. It can prevent oxidative damage, stimulate platelet production, and enhance platelet function.[16-17] Melatonin supplementation can potentially modulate the OS situation and be beneficial in platelet disorders.

The rationale of this study is to investigate the effects of melatonin for potential therapeutic applications in platelet disorders. Studies indicate that melatonin promotes platelet production, attenuates oxidative damage, and prevents platelet apoptosis. Hence, melatonin could be a potential alternative therapeutic tool.

Aim

It is imperative to study OS in platelets to understand the underlying mechanisms using antioxidants for their targeted actions. This is the first study to elucidate the responses of platelets to melatonin in free radical-induced OS conditions, especially the interactions of peroxyl radicals with melatonin in platelets.

Material and methods

Reagents

2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), melatonin, Epinephrine, bathocuproine disulfonic acid disodium salt, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and bovine serum albumin were purchased from Sigma-Aldrich Chemicals (Bengaluru, India). Cytochrome C, β-Nicotinamide adenine dinucleotide disodium salt and collagen (ex. Marine Fish extrapure) was purchased from SRL Chem (Mumbai, India). Glucose reagent kit (GOD-POD method; Catalogue no. GLU-L-D-1153) was from Aspen Laboratories (Delhi, India).

Methods

Male Wistar rats (160–180 g) procured from Bioally Animal Facility and Research Pvt. Ltd. (Bengaluru, India) were used for this study. Experimental study was conducted according to the Institutional Ethical Committee regulations (1810/PO/RcBizbt/S/15/CCSEA).

The physiology of Wistar rats is similar to humans. These animals are bred and maintained in controlled laboratory conditions and exhibit minimal variations. Hence, they are valuable models for toxicological studies, and the outcomes can be directly related to experimental interventions.

Experimental design

The platelets (20 *Wistar* rats, n=5) were grouped as follows: (i) controls (untreated platelets); (ii) free radical initiator (FRI) (2 mmol/L AAPH for 30 min); (iii) antioxidant (AO) (1 mmol/L melatonin for 60 min); and (iv) FRI+AO (1 mmol/L melatonin for 60 min followed by 2 mmol/L AAPH for 30 min).

The samples were gently resuspended in Tyrode's buffer (pH 7.4) and stored at 22°C until analysis. These platelet samples were aliquoted in aseptic conditions and assessed for antioxidant defense, OS markers, and platelet functions.

Sample preparation

Blood collection

Male Wistar rats were mildly anesthetized, and blood was collected into polypropylene tubes containing CPDA-1 anticoagulant (citrate phosphate dextrose adenine).[18]

Isolation of platelets

Platelets were isolated from whole blood according to Carneiro et al.[19] Briefly, 8 mL of whole blood was spun at 1500 rpm at 22°C for 15 min. The supernatant containing platelet-rich plasma (PRP) was further centrifuged at 4000 rpm at 22°C for 15 min. The pellet containing platelets was resuspended in Tyrode's buffer.

Antioxidant defenses

Superoxide dismutase (SOD)

Platelet samples (100 µL) were mixed with 880 µL of 0.05 M carbonate buffer (pH 10.2 with 0.1 mmol/L ethylenediamine tetraaceticacid). 20 µL of 30 mmol/L epinephrine was added to it. The absorbance was read immediately at 480 nm for 4 min. The amount of enzyme that inhibits the oxidation of epinephrine by 50% is equal to 1 unit of SOD.[20]

Catalase
10 µL of absolute ethanol was added to 100 µL of the platelet sample. The mixture was placed in an ice bath for 30 min. 1200 µL of phosphate buffer (0.1 M) was added to the mixture. To this, 1250 µL of H_2O_2 (0.066 M) was added. The absorbance was read immediately at 240 nm up to 1 min. Catalase activity was determined using 43.6 M cm^{-1} as the molar extinction coefficient.[21]

Glutathione
Platelets (250 µL) were treated with 375 µL of 4% sulfosalicylic acid and vortexed. The mixture was spun at 2500×g for 15 min. The supernatant (40 µL) was treated with 280 µL of 10 mmol/L DTNB (Ellman's reagent), and the absorbance was measured at 412 nm.[22]

Total antioxidant capacity-cupric ion-reducing antioxidant capacity (TAC^{CUPRAC})
The platelet samples (5 µL) were treated with 195 µL of bathocuproine disulfonic acid disodium salt (BCS) (0.25 mmol/L), and the initial absorbance was measured at 490 nm. 50 µL of $CuSO_4$ solution (0.5 mmol/L) was added, and after 3 min incubation at room temperature (RT), 50 µL EDTA solution (0.01 M) was added. The final absorbance was measured at 490 nm. Total antioxidant capacity was calculated using uric acid as the standard.[23]

Oxidative stress markers
Superoxides
200 µL of cytochrome C (160 µmol/L) was added to 100 µL of platelet samples and the mixture was incubated for 10 min at 37°C. The absorbance was spectrophotometrically read at 550 nm to measure the reduction of cytochrome C.[24]

Nitrites
The platelet samples (100 µL) were mixed with 100 µL of Griess reagent. The mixture was incubated in the dark at RT. The absorbance was read at 548 nm. The amount of nitrites was determined using sodium nitrite as the standard.[25]

Thiobarbituric acid reactive substances (TBARS)
Platelets (100 µL) were placed in an ice bath for 10 min, and an equal volume of 20% (v/v) cold trichloroacetic acid (TCA) in 0.6 M HCl was added. The mixture was spun at 2000 rpm for 15 min and 40 µL of 0.12 M thiobarbituric acid (TBA) solution (in 0.26 M Tris at pH 7.0) was added to the supernatant. The mixture was boiled for 15 min and the samples were read at 532 nm.[26]

Protein sulfhydryls
Platelet samples (200 µL) were diluted with 1.5 mL of 0.08 M sodium phosphate buffer (0.5 mg/mL of Na_2-EDTA, and 2% sodium dodecyl sulfate). 0.1 mL of DTNB was added to the mixture and left for 15 min at RT. The absorbance was read at 412 nm.[27]

Advanced oxidation protein products (AOPP)
400 µL of samples were diluted with 1.2 mL of phosphate buffer and incubated with 200 µL potassium chloride (1.16 M). Absorbance was read at 340 nm immediately after adding 400 µL of acetic acid.[28]

Platelet functions and metabolism
Platelet aggregation
Platelet samples (100 µL) were incubated with collagen (100 µL, 2.0 µg/ml) for 10 min at 37°C in a V-bottom 96-well plate. The above procedure was repeated without collagen. The plates were subjected to shaking for 60 s, and the absorbance was recorded at 405 nm.[29]

ATP secretion
The platelet samples were incubated with 25 µL of collagen (2.0 µg/ml) for 10 min at 37°C. The mixture was treated with 1.2 M perchloric acid. The absorbance was measured at 260 nm. The amount of adenine nucleotides secreted was calculated using ATP as a standard.[30]

Glucose
Glucose was estimated using the Autospan Gold kit (Arkray, India), following the manufacturer's instructions based on the glucose oxidase-peroxidase (GOD-POD) method. The absorbance was read at 546 nm.[31]

pH
The pH of platelet samples was measured using the pH strips (Fisher Scientific).

Lactate dehydrogenase (LDH)
The platelet samples (10 µL) were treated with 1 ml of 4:1 mixture of reagent 1 (80 mmol/L Tris, 200 mmol/L NaOH, and 1.6 mM pyruvate) and reagent 2 (0.2 mmol/L nicotinamide adenine dinucleotide (NAD)) and incubated at 37°C for 3 min. The absorbance was measured at 340 nm for 3 min.[32]

Protein
500 µL of platelets were incubated with 2.5 mL alkaline $CuSO_4$ (50:1 mixture of 2% Na_2CO_3 and 0.5% $CuSO_4$) at RT for 15 min. 0.1 mL of Folin-phenol reagent (1:2 diluted in distilled water) was added to the above mixture. This was incubated at RT for 30 min. Absorbance was read at 660 nm. Bovine serum albumin (BSA) was used as the standard.[33]

Statistical analysis
Results are expressed as mean ±SE (n=5). One-way ANOVA was performed using GraphPad Prism 6 Soft-

ware (San Diego, CA, USA), followed by Bonferroni post-test. The values were considered significant at $p<0.05$.

Results

The variations occurring in FRI were compared against free radical-induced treated with melatonin antioxidant (FRI+AO) and controls. Variations occurring in melatonin antioxidant (AO) were compared against controls.

Antioxidant defenses

Superoxide dismutase activity decreased by 59% and 36% in FRI and FRI+AO, respectively, compared to the controls. SOD activity increased by 58% in FRI+AO against FRI (Table 1).

Catalase activity was 52% higher in AO compared to controls. It was 19% higher in FRI+AO compared to FRI (Table 1). Glutathione was 23% higher in AO than in controls (Table 1). TAC_{CUPRAC} was ~23% lower in FRI+AO than in controls and AO (Table 1).

Table 1. Antioxidant defenses in platelets exposed to AAPH-induced OS*

Groups	Superoxide dismutase (U/mg protein)	Catalase (U x 10^{-4}/mg protein)	Glutathione (mmol/mg protein)	Total antioxidant capacity (TAC_{CUPRAC}) (µmol uric acid equivalents/L)
Controls	5.11±0.5	10.00±2.3	3.64±0.5	840.2±123.7
FRI	2.06±0.2	7.65±2.0	3.37±0.3	766.93±64.6
AO	4.83±1.1	15.21 ±5.3	4.52±0.6	893.64±74.4
FRI + AO	3.26±0.8	9.14 ±2.3	3.56±0.2	658.00±56.5

* results are expressed as mean±SE (n=5), $p>0.05$

Oxidative stress

Superoxide levels were 41% and 53% higher than controls and AO, respectively. The levels were significantly higher by 75% ($p<0.05$) in FRI+AO compared to FRI (Fig. 1A).

Nitrites were 25% elevated in FRI+AO than in controls. However, it was maintained in all the other groups at similar levels (Fig. 1B).

TBARS increased in FRI by 12% and decreased in AO by 12% compared to Controls. It decreased by 24% in FRI+AO compared to FRI (Fig. 2A). AOPP was significantly higher (120%, $p<0.05$) in AO with respect to Controls. AOPP also increased by 66% in FRI+AO compared to FRI (Fig. 2B). P-SH levels were similar in all the experimental groups. However, it increased by 20% in AO compared to controls (Fig. 2C).

Platelet function and metabolism

Aggregation with collagen was significantly lower (~62%, $p<0.05$) in the FRI+AO compared to FRI and AO. Aggregation without collagen was similar in all other groups (Fig. 3B).

ATP secretion increased significantly in FRI+AO by 12% ($p<0.001$) compared to controls. It increased significantly by 17% ($p<0.0001$) and 8% ($p<0.01$) in FRI+AO compared to FRI and AO, respectively (Fig. 3B).

Glucose increased by ~20% in FRI and FRI+AO compared to controls (Fig. 4A). pH was similar in all the groups (Fig. 4B). LDH decreased by 17% in FRI compared to controls. There was an increment of 55% in FRI+AO compared to FRI (Fig. 4C).

Discussion

Free radicals play a crucial role in homeostasis and redox mechanisms in platelets.[4] AAPH generates peroxyl radicals that induce OS and is therefore employed to establish OS models. This study assessed the modulations in platelets by melatonin during AAPH-induced OS.

FRI vs. controls

Antioxidant enzymes act as the primary line of defense during OS and reflect the redox state of platelets. Peroxyl radicals can potentially inactivate the susceptible enzymatic active sites.[34] Therefore, superoxide dismutase (SOD) and catalase (CAT) lowered in FRI compared to controls. Notably, AAPH predominantly generates peroxyl radicals by thermal decomposition. These highly reactive radicals abstract hydrogen atoms from the lipids of the cell and its membrane to form lipid hydroperoxides (LOOH).[35] These lipid hydroperoxides are unstable and can decompose to form more reactive radicals, perpetuating the chain reaction of lipid peroxidation and leading to cell damage.[10] This corroborates with the higher TBARS levels, which also reflected in lowered TAC in FRI. Platelet aggregation increases during OS, activating a cascade of events leading to thrombus formation and posing a great risk in platelet disorders.[4] Aggregation in the presence of collagen increased in FRI, suggesting platelet stimulation due to redox changes. This can also be attributed to increased glucose metabolism and decreased LDH in FRI due to the aerobic state of platelets under in vitro conditions.

AO vs. controls

Melatonin, a powerful antioxidant, can penetrate the platelets due to its lipophilic nature. It stimulates endogenous antioxidant enzymes as seen in the increase in activity of SOD, CAT, and GSH in the AO. Melatonin directly scavenges hydroxyl radicals and indirectly detoxifies H_2O_2, superoxide, and peroxyl radicals through its metabolites.[12,36] This can be attributed to its powerful antioxidant capacity as it can bind to the Fe^{2+} ions and prevent the Fenton reaction. Therefore, melatonin lowers the susceptibility of membranes to oxidative damage and improves their stability.[37] Consequently, lipid peroxidation significantly lowered in melatonin-treated platelets compared to controls. Sulfhydryls are susceptible to oxidation and regulate the protein structure and function.[38] They are also

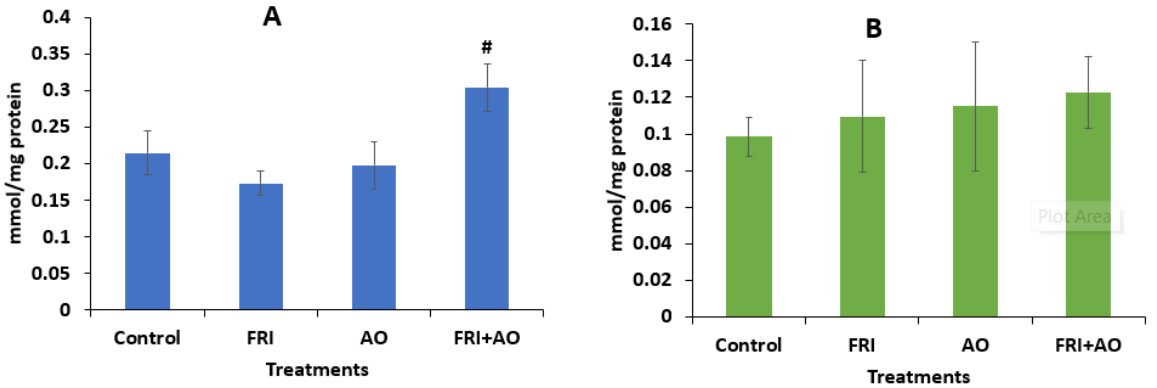

Fig. 1. A: Superoxides in platelets exposed to AAPH-induced OS expressed as mean±SE (n=5), B: Nitrites in platelets exposed to AAPH-induced OS represented as Mean±SE (n=5), # indicates significant difference with respect to FRI

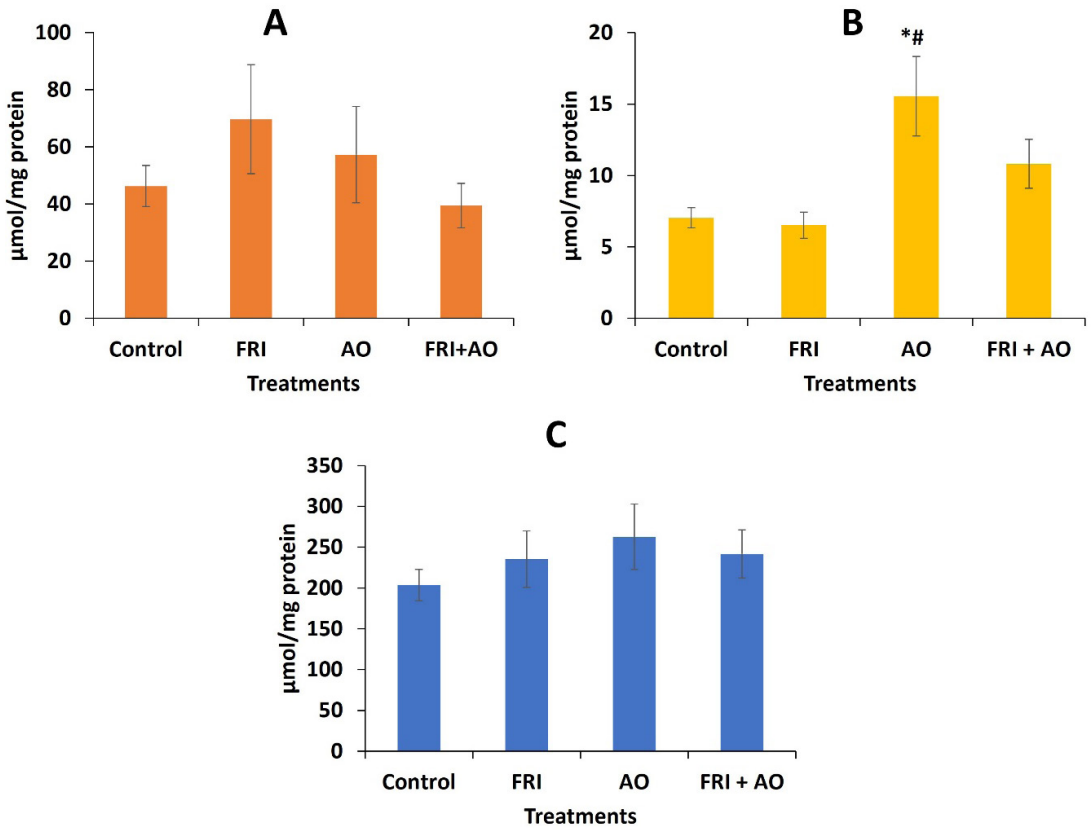

Fig. 2. A: TBARS in platelets exposed to AAPH-induced OS, B: AOPP in platelets exposed to AAPH-induced OS, C: Sulfhydryls in platelets exposed to AAPH-induced OS, results are expressed as mean±SE (n=5). * indicates significant difference with respect to controls, # indicates significant difference with respect to FRI

implicated in platelet activation and are required for adhesion via integrin αIIbβ3.[39] Protein sulfhydryls increased in AO, suggesting that melatonin was actively involved in protecting thiol groups by undergoing reversible oxidation. AOPP levels indicated that dityrosine linkages were formed, which can result in the cleavage of polypeptide chains and the formation of cross-linked protein aggregates.[40] AOPP levels increased in AO, suggesting that 1 mM melatonin could not prevent the formation of susceptible dityrosine linkages. Melatonin, as a hormone, can influence platelet functions in a concentration-dependent manner by altering its response to collagen and thrombin.[41] Melatonin increased the ATP secretion and aggregation (with collagen) in AO compared to the Controls, indicating platelet responsiveness to collagen and, clot formation. Melatonin regulates the pathways involved in platelet activation, thereby augmenting platelet responsiveness to agonists. Glucose and LDH levels were maintained in AO and controls, suggesting that melatonin did not intervene in platelet metabolism.

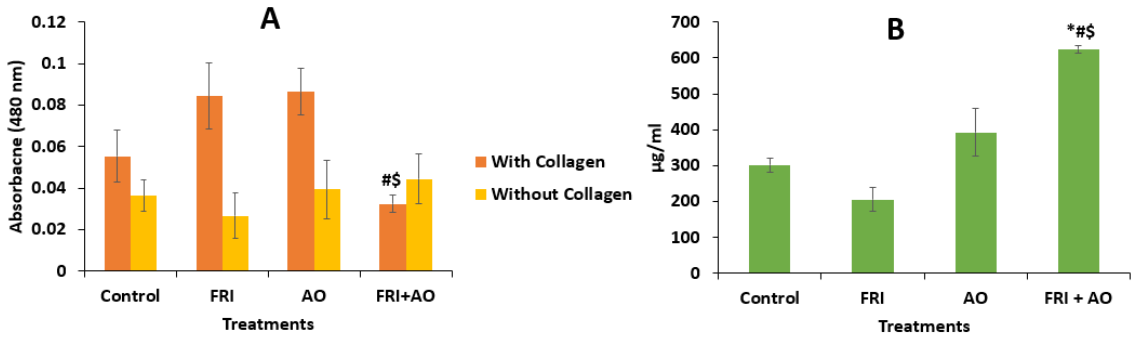

Fig. 3. A: Aggregation (with and without collagen) in platelets exposed to AAPH-induced OS, B: ATP secretion in platelets exposed to AAPH-induced OS, results are expressed as mean±SE (n=5), * indicates significant difference with respect to controls, # indicates significant difference with respect to FRI, $ indicates significant difference with respect to AO

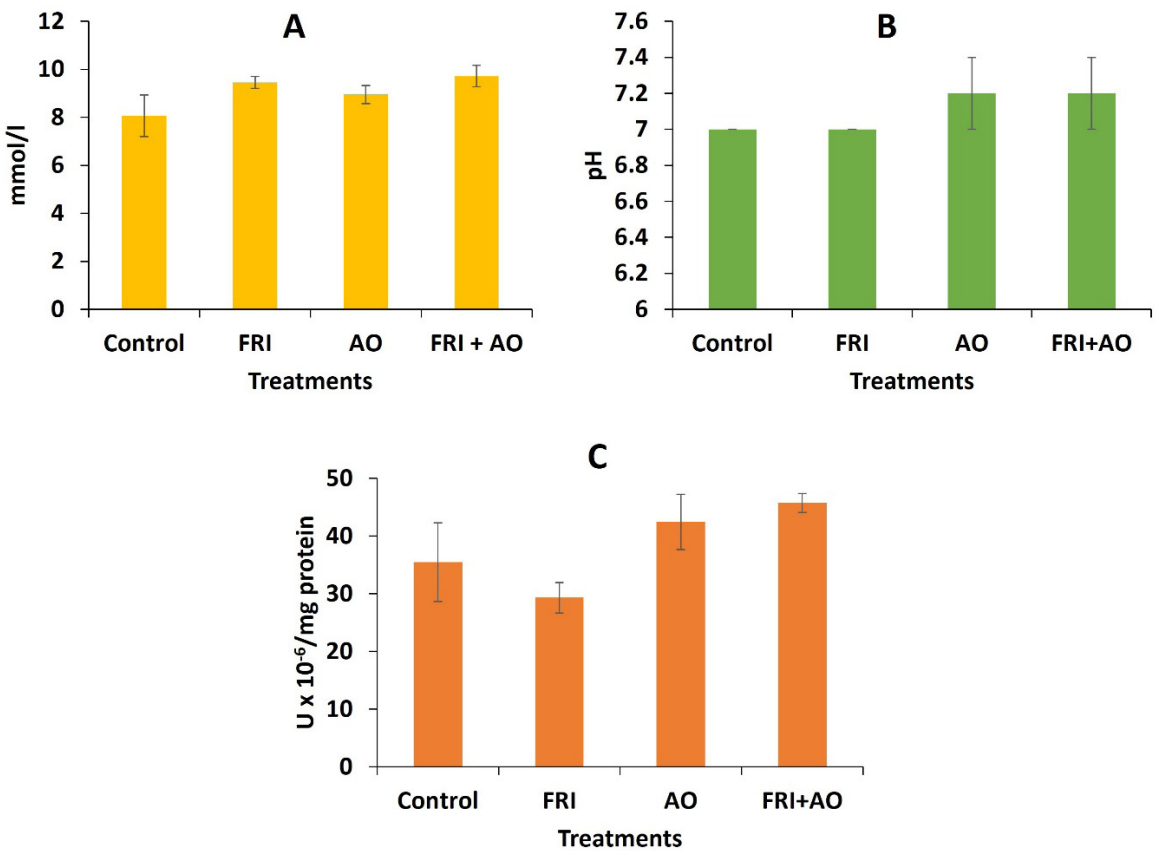

Fig. 4. A: Glucose in platelets exposed to AAPH-induced OS, B: pH in platelets exposed to AAPH-induced OS, C: LDH in platelets exposed to AAPH-induced OS, results are expressed as mean±SE (n=5), the changes between the groups were statistically insignificant

FRI+AO vs. FRI

The peroxyl radicals generated by AAPH are scavenged by melatonin using its two methoxy groups and an indole ring by a single-electron transfer (SET) mechanism.[34] Melatonin elevated the activity of SOD indirectly by increasing the production of superoxides in FRI+AO compared to FRI. CAT activity and GSH levels suggest that melatonin was involved in directly scavenging generated H_2O_2, as one molecule of melatonin is capable of scavenging two hydroxyl radicals. Melatonin has been protective against the catalase inactivation induced by AAPH, suggesting its involvement in reducing the oxidation of critical residues in these enzymes.[42] Nonetheless, elevated superoxide levels in FRI+AO signify the oxidative modulations due to AAPH in platelets, as superoxides are the primary and highly reactive species generated by physiological processes. Melatonin protects the lipids from oxidative damage by directly scavenging peroxyl radicals. It also actively protects the lipids due to their lipophilic nature, by altering the

membrane's physical properties at the peroxidation site.[12] TBARS decreased in FRI+AO, suggesting that lipid peroxidation was alleviated in platelets. This can be attributed to the SET mechanism, where peroxyl radicals are converted into stable molecules, thereby terminating the lipid peroxidation chain reaction.[34] Protein sulfhydryls were maintained in FRI+AO and FRI, suggesting that glutathione underwent reversible oxidation to protect SH groups to a certain extent.[4] However, ROS directly attacks the proteins by oxidizing certain amino acid residues, particularly those with sulfur-containing side chains, aromatic rings, or histidine residues.[1] AOPP increased in FRI+AO, suggesting that elevated superoxides might have rendered susceptible proteins to oxidation. Platelet aggregation in the presence of collagen significantly reduced in the FRI+AO owing to the increase in superoxide levels and LDH compared to FRI. ATP secretion significantly increased in the FRI+AO compared to FRI, indicating the increase in platelet sensitivity to collagen as a result of OS. This might be attributed to the biological defense mechanism of melatonin during OS by inhibiting prostaglandin synthesis, and can have implications in cardiovascular diseases.[43,44] Platelet metabolism was maintained as demonstrated by glucose levels. However, reactive species generated by AAPH affected platelet functions in FRI, and melatonin exposure benefited the platelets by modulating oxidative stress and platelet functions.

This study has potential clinical implications for platelet disorders. One of the major causes of platelet disorders is oxidative stress, as platelets actively generate ROS during activation. Antioxidants such as melatonin can modulate the physiological conditions and alleviate oxidative damage in platelets. The findings also indicate that melatonin can be explored in therapeutics for platelet disorders.

Study limitations

Melatonin is a hormone and is under constant regulation. A probable change in responses to physiological factors under in vivo conditions is possible.

Conclusion

Melatonin enhanced antioxidant defenses and attenuated lipid peroxidation in platelets at 1 mM concentration. It ameliorated the platelet function and maintained metabolism during AAPH-induced OS. These findings indicate that melatonin can modulate antioxidant capacity and alleviate oxidative stress in platelets. This suggests that melatonin plays a beneficial role in regulating redox mechanisms. Furthermore, in vitro platelet OS models provide insights into the implications of OS in platelet pathophysiology. Therefore, this study lays the foundation for further in vivo studies, which can contribute to therapeutic applications.

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Declarations

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Author contributions

Conceptualization, V.R.; Methodology, V.R.; Validation, V.R.; Formal Analysis, A.B.A. and M.C.R.; Investigation, A.B.A. and M.C.R.; Resources, V.R.; Data Curation, A.B.A.; Writing – Original Draft Preparation, A.B.A.; Writing – Review & Editing, V.R.; Visualization, V.R.; Supervision, V.R.; Project Administration, V.R.

Conflicts of interests

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethics approval

Animal care and maintenance were according to the guidelines of Institutional Ethical Committee (1810/PO/RcBizbt/S/15/CCSEA).

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ORIGINAL PAPER

Comparative diagnostic utility of leptin and resistin as inflammatory biomarkers in acute myocardial infarction

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ABSTRACT

Introduction and aim. Acute myocardial infarction (AMI) is a leading cause of mortality. Although traditional risk factors are known, adipokines, such as leptin (LEP) and resistin (RETN), are emerging as potential biomarkers involved in the inflammatory and metabolic processes underlying AMI. This study aimed to evaluate serum LEP and RETN levels in patients with AMI.

Material and methods. This case-control study included 60 patients diagnosed with AMI and 60 healthy controls recruited from the Nasiriyah Heart Hospital, Thi-Qar Province. Serum levels of LEP and RETN were measured using an enzyme-linked immunosorbent assay.

Results. AMI patients exhibited significantly elevated LEP (3.79±2.0 vs. 1.43±0.7 ng/mL, p<0.001) and RETN (606±325 vs. 289±160 ng/L, p<0.001) compared to controls. Both adipokines were positively correlated with high-sensitive troponin I (Hs-TnI), low-density lipoprotein (LDL-C), and triglycerides (p<0.05). ROC analysis demonstrated a high diagnostic accuracy for LEP (AUC=0.964; cutoff >2.23 ng/mL, derived from internal study data) and moderate accuracy for RETN (AUC=0.878; cutoff >305.9 ng/L). The sensitivity and specificity of the LEP were 93% and 92%, respectively.

Conclusion. LEP demonstrated high diagnostic accuracy in our cohort, and its clinical application requires validation in larger prospective studies. The association between RETN and AMI likely reflects inflammatory sequelae rather than predictive utility.

Keywords. acute myocardial infarction, leptin, resistin

Introduction

Acute myocardial infarction (AMI) remains a leading cause of global morbidity and mortality and is driven by complex interactions between metabolic dysregulation, inflammation, and cardiovascular (CV) risk factors.[1-3] While traditional biomarkers, such as troponin are central to the diagnosis of AMI, they lack specificity for the inflammatory and metabolic pathways integral to AMI pathophysiology.[4] This gap underscores the need for novel biomarkers that reflect these mechanisms to improve early detection and risk stratification. Recent projections indicate a substantial increase in CVD burden, with crude mortality expected to increase by 73.4% between 2025 and 2050, culminating in 35.6 million annual CV deaths by 2050.[5] In a published study of 19,781 patients with coronary artery disease (CAD), the prevalence of AMI was 23.3%.[5,6] Contrary to historical perceptions, emerging data highlight that the prevalence of AMI in low-resource settings is now rivaling that of developed nations, driven by urbanization and metabolic risk factors.[5]

Leptin (LEP), an adipocyte-derived hormone[7] was discovered in 1994 as a 16 kDa adipokine.[8] LEP, a 167 amino acids, is mainly synthesized by adipocytes from white adipose tissue and is transported via the circulatory system to the hypothalamus, where it modulates energy homeostasis and regulation of body weight reg-

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ulation.[9] In addition to its metabolic role, LEP exerts immunomodulatory effects by enhancing Th1 cell-mediated immune responses and attenuating Th2-associated inflammatory pathways. Furthermore, it stimulates the secretion of pro-inflammatory cytokines from mononuclear cells. The accumulation of evidence has underscored the involvement of LEP in the pathogenesis of autoimmune disorders.[10]

Resistin (RETN), a pro-inflammatory cytokine, was first identified in 2001 by a research group led by Dr. Mitchell Lazar who proposed its role in bridge the pathophysiological interplay between diabetes and obesity.[11] Originally recognized for its association with the pathological mechanisms of diabetes and its contribution to obesity-related metabolic dysregulation, emerging evidence has since expanded the functional relevance of RETN to include inflammation and immune modulation.[12] Recent studies have shown that RETN regulates the expression of pro-inflammatory mediators and directly stimulates the production of IL-6 and interleukin-8 (IL-8).[13]

Identification of effective biomarkers for AMI is crucial for improving patient outcomes and reducing mortality.[8] LEP and RETN, adipokines primarily secreted by adipose tissue, have been linked to CVDs through their roles in inflammation, insulin resistance, and lipid metabolism.[14] Elevated levels of both adipokines have been observed in coronary artery disease (CAD) and AMI, suggesting their involvement in atherosclerosis and plaque rupture.[15] While LEP and RETN have been linked to inflammatory and metabolic pathways, the exact mechanisms by which they contribute to myocardial injury and plaque rupture have not been fully elucidated.[16] However, prior studies have largely evaluated LEP and RETN in isolation, with limited comparative analyses of their diagnostic utility or adjustments for confounding factors such as body mass index (BMI).[17]

This study addresses these gaps by providing a head-to-head evaluation of LEP and RETN in patients with AMI, including ROC analysis to assess diagnostic accuracy, and adjusting for BMI to isolate its independent prognostic value. Furthermore, we explored their correlation with lipid profiles and high-sensitivity troponin I (Hs-TnI), offering novel insights into their synergistic contributions to the pathophysiology of AMI. By integrating these adipokines with established biomarkers, our findings aimed to refine risk stratification and highlight their potential as complementary diagnostic tools in the management of AMI.

Aim

This study aimed to evaluate and compare the diagnostic and predictive efficacy of LEP and RETN levels in patients with AMI and to assess their correlation with conventional biochemical markers, particularly Hs-TnI.

Material and methods

In this study a case-control study design was used. Samples were collected at Nasiriyah Heart Hospital in Thi-Qar province from individuals diagnosed with AMI, and samples were collected 8–12 h after symptoms appeared. The study population consisted of 60 patients divided into two categories: 29 male patients and 31 female patients. In addition, control group: healthy volunteers (n=60) were randomly recruited from the same community as the patients, without matching for age or sex. Sample collection was carried out between December 2024 and March 2025, and the study protocol was approved by the IRB (287/2024–December 11, 2024).

Inclusion and exclusion criteria

Patients were eligible for inclusion in the AMI group if they had a confirmed diagnosis of AMI, based on clinical presentation, electrocardiographic findings, and elevated levels of Hs-TnI equal to or exceeding 14 ng/L. Only people 18 years or older were included. Furthermore, blood samples were required to be collected between 8 to 12 hours after the onset of symptoms.

Participants were excluded if they had a history of chronic kidney disease, autoimmune disorders, or cancer. Further exclusion criteria included the use of corticosteroids or other medications known to affect adipokine levels. Pregnant or lactating women were also excluded from the study.

Sample collection

AMI patient sample collection consisted of performing a venous puncture to obtain 5 mL of peripheral blood, allowing coagulation at room temperature, and the collected sample was placed in a gel tube. The tubes were then centrifuged at 3000 rpm for 10 min to facilitate serum separation. The separated serum was then transferred to multiple Eppendorf tubes and stored at -20°C for further examination. Various factors, such as LEP, RETN, and Hs-TnI

Biomarker quantification methodologies

Human LEP (E-EL-H6017) and RETN (E-EL-H1213) concentrations were quantified using enzyme-linked immunosorbent assay kits (Biotek, Winooski, Vermont, USA) employing a sandwich methodology. Hs-TnI levels were analyzed using the AFIAS 6 fluorescence immunoassay system (South Korea), with a diagnostic threshold of 0.3 ng/L (negative: <0.3 ng/L; positive: 0.3 ng / L). The AFIAS 6 system facilitated ambient-temperature assembly and incorporated individual solid-phase receptacles, test strips, calibrators, and controls per sample. Lipid profiles were measured using a Mindray BS-230 clinical chemistry analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd, Shenzhen, China). The calibration protocols specified a two-point adjustment with a Min-

dray Human Multi-Calibrator and 9 g/L NaCl, traceable to the manufacturer's standards. Calibration was performed on the basis of reagent lot changes or as mandated by the internal quality control (QC) protocols. QC procedures require the analysis of two-tiered control materials (Mindray Human Assayed Control recommended) per sample batch, following calibration updates, reagent cartridge replacement, or maintenance interventions. The system automates all procedural steps to ensure standardized processing.

Statistical analysis

This study used an extensive statistical approach using Excel for data management and GraphPad Prism 9 (GraphPad Software, LLC, Boston, Massachusetts, USA). Descriptive statistics summarized categorical variables as frequencies/percentages and numerical variables as means with standard deviations (SD) and medians. Proportions and means were reported, and inferential analyses were performed (Chi-square or Fisher's exact tests for categorical associations and independent sample t-tests for numerical comparisons) between AMI patients and controls. The evaluated parameters included demographics, lipid profiles, hematological measures, cardiac biomarkers (Hs-TnI), and adipokines (LEP and RETN). The diagnostic performance of adipokines in distinguishing AMI from non-AMI cases was assessed using the ROC curve analysis.

Table 1. Comparative analysis of demographic characteristics between control subjects and patients with AMI*

Characteristic	Control (n=60)	AMI (n=60)	p
Age (years)			
Mean±SD	48.3±9.8	55.4±7.1	0.1 I
Range	28–62	39–77	
Gender			
Male, n (%)	29 (48%)	36 (60%)	0.2 C
Female, n (%)	31 (52%)	24 (40%)	
BMI (kg/m^2)			
Mean±SD	25.3±3.1	30±4.2	<0.001 I
Range	20–33	21.9–33	

* n – number of cases, SD – standard deviation, C– Fisher's exact test, I – independent samples t-test

Results

This study investigated the complex interplay between adipokines, lipid profiles, and cardiac biomarkers in patients with AMI. This study compared various parameters between AMI patients and healthy controls, including demographic characteristics, biochemical markers, hematological profiles, and adipokine levels. The findings revealed significant differences in these parameters between the two groups and in the metabolic and systemic disturbances associated with AMI. This study also examined the diagnostic efficacy of adipokine markers for AMI using ROC curve analysis. This comprehensive analysis provides valuable information on the pathophysiology of AMI and the potential role of adipokines as diagnostic markers and therapeutic targets in CVDs.

Table 2 shows that patients with AMI have significantly higher levels of total cholesterol, LDL-C, triglycerides (TG) and VLDL, with lower HDL-C compared to the control group. Patients with AMI also exhibited wider ranges in TG and LDL-C, indicating greater variability. These results highlight dyslipidemia in AMI patients, consistent with known CV risk factors.

Table 2. Comparison of mean values of lipid profile among control group and patients with acute myocardial infarction*

Characteristic		Control (n=60)	AMI (n=60)	P
Cholesterol (mg/dL)				
	Mean±SD	184.1±45.3	244±65.7	<0.001 I
	Range	112–298	129–429	
HDL (mg/dL)				
	Mean±SD	47.9±9.7	36.8±12.5	<0.001 I
	Range	33.5–71.6	14.2–69.7	
LDL (mg/dL)				
	Mean±SD	103.1±47.4	158.3±60.7	<0.001 I
	Range	42.5–137	76.53–195	
TG (mg/dL)				
	Mean±SD	154.6±60.6	191.6±86.4	0.02 I
	Range	57.3–280	72–448	
VLDL (mg/dL)				
	Mean±SD	30.7±11.7	37.4±17.8	0.02 I
	Range	11.4–56	14.4–89.6	

* I – independent samples t-test

Table 3. Comparative analysis of adipokine levels in control and AMI patients*

Characteristic		Control (n=60)	AMI (n=60)	P
Hs-TnI (ng/L)				
	Mean±SD	6.0±2.3	39.2±12.8	<0.001 I
	Range	1.9–11.8	17.5–68.9	
LEP (ng/mL)				
	Mean±SD	1.43±0.7	3.79±2.0	<0.001 I
	Range	0.5–3.9	2.0–12.1	
RETN (ng/L)				
	Mean±SD	289±160	606±325	<0.001 I
	Range	83–615	177–1197	

* I – independent sample t-test

Table 3 compares serum hs-TnI levels between control subjects and patients with AMI. AMI patients had significantly higher levels (39.2±12.8 ng/L) compared to the control group (6.0±2.3 ng/L), with a highly significant

difference (p<0.001), indicating a marked increase in Hs-TnI in AMI cases. Adipokine levels (LEP and RETN) in control subjects and patients with AMI AMI patients had significantly higher levels of LEP (3.79±2.0 ng/mL) and RETN (606±325 ng/L) levels compared to controls, with broader ranges in AMI. All comparisons are statistically significant (p <0.001), as shown in Figure 1.

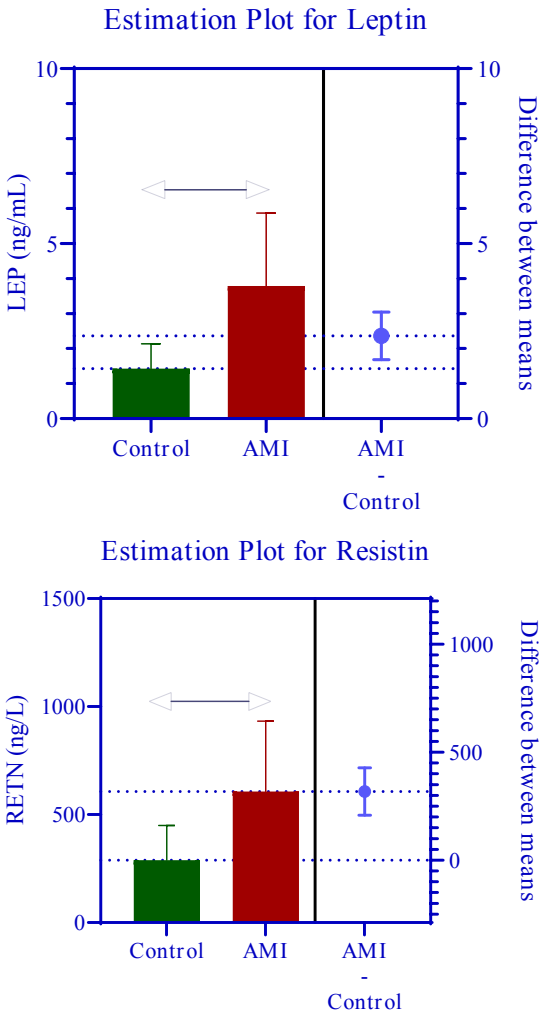

Fig. 1. Bar chart showing comparison of LEP and RETN between the control group and patients with AMI

Table 4. Diagnostic performance of LEP and RETN in AMI: a ROC curve analysis*

Variables	Cut-off value	Sens%	Spec%	PPV	NPV	Accuracy	AUC%	95% CI	p (AUC= 0.05)
LEP (ng/mL)	>2.23	93	92	93	92	85	96	0.89 to 0.99	0.001
RETN (ng/L)	> 305.9	93	70	79	89	70	87	0.78 to 0.94	0.001

* Sens – sensitivity, Spec – specificity, PPV – positive predictive value, NPV – negative predictive value, accuracy [(Sensitivity + Specificity) - 1], AUC – area under the curve, CI – confidence interval

Table 4 summarizes the receiver operating characteristic (ROC) curve analysis to evaluate the diagnostic efficacy of LEP and RETN in AMI (Fig. 2). LEP (cut-off >2.23 ng/mL) demonstrated the highest accuracy (85%) and area under the curve (AUC: 96%), with 93% sensitivity and 92% specificity, while RETN (cut-off >305.9 ng/L) had the lowest accuracy (70%) and AUC (87%). All biomarkers exhibited statistically significant discriminative power (p=0.001). In particular, LEP and RETN had high sensitivity (93%).

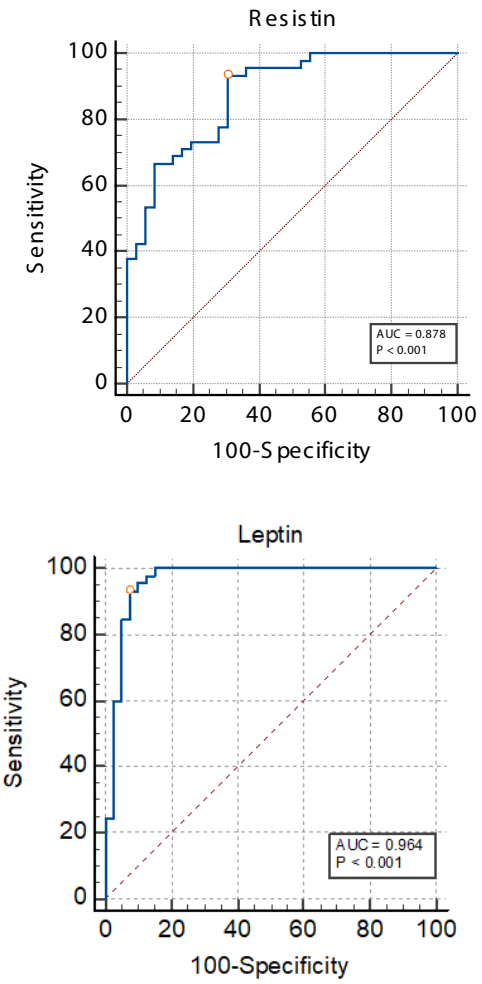

Fig. 2. ROC Curve chart of LEP, and RETN for diagnosing AMI in a sample of 60 patients and 60 controls

The Pearson correlation matrix that evaluates associations between lipid profiles and adipokines highlights patients with AMI-specific correlations of adipokines, demonstrating significant positive relationships between LEP and total cholesterol TC (r=0.428, p<0.001) and low-density lipoprotein (LDL: r=0.456, p<0.001). LEP was strongly and positively correlated with Hs-TnI (r=0.494, p<0.001). Although there was a significant positive correlation between RETN and LDL (r = 0.256, p = 0.032) and Hs-TnI (r = 0.527, p<0.001), RETN was positively correlated with LEP (r=0.525, p<0.001) and negatively correlated with HDL (r = 0.256, p = 0.026). The correlations between RETN and TC (r = 0.208,

p=0.062) and between HDL and LEP (r=-0.171, p=0.117) were not statistically significant.

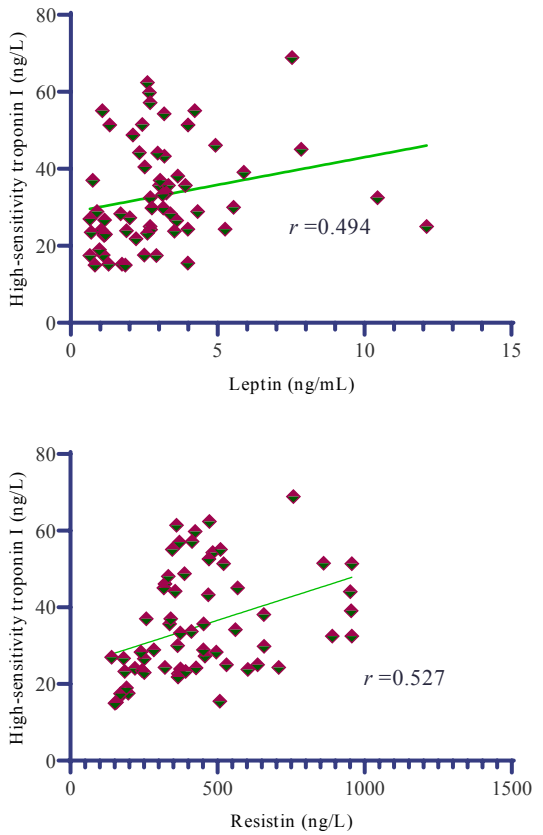

Fig. 3. Correlation chart of LEP and RETN with Hs-TnI in patients with AMI (n=60)

Table 5 presents the results of the logistic regression analysis evaluating the association of the adipokines LEP and RETN with AM. The model demonstrated (R^2=0.93) and discrimination, as evidenced by a near-perfect ROC AUC of 0.995 (95% CI: 0.942–1.000). LEP emerged as a statistically significant predictor, with a coefficient (B) of 9.676 (Wald=4.07, p<0.01) and an odds ratio of 43.7 (95% CI: 6.85–279.9), indicating a strong positive association with AMI risk. In contrast, RETN was not statistically significant in the model. ROC analysis demonstrated exceptional discriminative performance with an AUC of 0.995 (95% CI: 0.942-1,001). The classification accuracy was 94.5%, with 93.7% accuracy for negative cases and 95.1% for positive cases.

Table 5. Logistic regression analysis of the association of LEP and RETN with AMI (R^2=0.93)

Variables	B (coef)	Wald	Odds ratio	95% CI for odds ratio	p
LEP (ng/mL)	9.676	4.07	43.7	6.85 to 279.9	<0.01
RETN (ng/L)	Not significant in the model				
ROC AUC	0.995 (95% CI: 0.942 to 1.000)				
Classification accuracy	Negative cases: 93.7%, positive cases: 95.1%, overall: 94.5%				

Discussion

The study revealed significantly higher mean BMI values in patients with AMI than in controls, strengthening the established link between obesity and CV risk. This finding underscores the well-established association between obesity and increased cardiovascular risk in patients with AMI. Several studies support these findings, strengthening the well-documented relationship between higher BMI and an increased risk of CV events. A study conducted in the United States found that obesity is significantly associated with an increased risk of mortality in AMI patients.[18] Similarly, in Western Norway, obese individuals had an increased risk of AMI and CV death, which is consistent with previous results.[19] Furthermore, a study in Romania revealed that higher baseline BMI values, along with increased epicardial adipose tissue in specific coronary arteries, are strong predictors of sudden cardiac death and body fat distribution in CV health.[20] A study conducted in the United States identified what has been termed the "obesity paradox." Contrary to expectations, some studies have reported an obesity paradox in which a higher BMI correlates with a lower mortality from AMI. However, our findings align with the majority of evidence that underscores obesity as a risk enhancer.[21] Lipid abnormalities further differentiated AMI patients from controls, with significantly elevated levels of TC, LDL-C, TG, and VLDL and reduced HDL-C. These findings reinforce the well-established relationship between lipid abnormalities and the development of AMI. In Copenhagen, elevated LDL cholesterol levels were significantly associated with a markedly increased unconditional risk of MI and atherosclerotic CVD.[22] Furthermore, it was also confirmed in their study that elevated LDL levels were robustly associated with an increased risk of ASCVD, and lipid abnormalities, particularly elevated LDL-C and TG levels, contributed to a higher risk of CV events.[23] Another study by Ravnskov argued that high blood cholesterol levels are not the primary cause of CVD.[24]

The results of this study showed a significant increase in Hs-TnI levels in patients to AMI compared with controls. The pronounced elevation in Hs-TnI levels highlights its critical role in detecting subtle myocardial injuries, facilitating early diagnosis of AMI, especially in clinically ambiguous cases. The findings of this study highlight the enhanced sensitivity of modern Hs-TnI assays compared to traditional diagnostic methods.[25] This increase in the sensitivity of Hs-TnI assays has made them an invaluable tool in clinical practice, as it enhances diagnostic accuracy, even in cases where clinical symptoms may not be immediately apparent. Similarly, a study by Boeddinghaus (2020) supported the findings of this research, as it concluded that Hs-TnI offers high diagnostic accuracy in patients suspected of having MI, with clinical performance comparable to or

even exceeding that of traditional central laboratory assays.[26] Raber (2021) further substantiated these findings by reporting that higher concentrations of Hs-TnI were more strongly associated with AMI.[27]

The results of this study revealed significant differences in serum LEP adipokine levels between patients with AMI and control subjects. These findings suggest that both adipokines, which are involved in inflammation and metabolic regulation, may play an important role in the pathophysiology of AMI. The findings of this study were consistent with those of previous studies. For example, in Saint-Petersburg, Russia, elevated levels of LEP in obese patients were found to mediate the mechanisms of atherogenesis, metabolic damage, arrhythmogenesis, and myocardial ischemic damage in both experimental animal models and humans.[28] Similarly, in Pakistan, a positive correlation was found between serum LEP levels and AMI, particularly in smokers and obese patients; elevated LEP stems from the increased adipose tissue secretion as the amount of adipose tissue in the body increases, as does the level of LEP in the blood. LEP resistance also occurs in cases of obesity, and the brain may become resistant to the effects of LEP, leading to persistent feelings of hunger despite sufficient stored fat and metabolic disorders. High levels of LEP may be an indicator of disorders such as metabolic syndrome, in which LEP is produced in greater quantities by adipose tissue.[29] Another case-control study compared 40 patients with CAD and 40 healthy controls. Our study demonstrated a significant increase in LEP levels in CAD patients. Researchers noted a significant association between serum LEP level, BMI, and waist circumference, suggesting that it is an independent risk factor for cardiac disorders that are largely dependent on obesity.[30] A prospective study has provided evidence supporting the role of LEP as a risk marker, particularly in metabolic syndrome and inflammatory comorbidities. Although its diagnostic utility remains secondary to that of conventional biomarkers, its prognostic value for recurrent events and metabolic complications warrants further investigation.[31]

A study in Egypt found that serum RETN levels are elevated in patients with acute STEMI, further supporting the role of RETN in AMI.[32] Elevated LEP levels can help identify patients at higher risk of AMI and guide clinical decisions regarding treatment strategies.[33] A prospective case-control study demonstrated increased RETN levels in patients with AMI and established the prognostic significance of RETN for recurrent acute coronary syndrome (ACS). RETN levels are related to the extent of inflammation, highlighting their potential role as inflammatory markers in CV pathology.[34] A cross-sectional investigation involving 77 Saudi patients experiencing hypoxia classified participants into three cohorts: a control group, patients with normal BMI, and patients with AMI patients with heterogeneous BMI profiles. This study demonstrated significantly elevated serum RETN levels in patients to AMI compared with controls. Furthermore, under hypoxic conditions, RETN concentrations are markedly higher in obese patients with AMI than in non-obese AMI counterparts.[35] A meta-analysis of clinical investigations supports a link between elevated circulating RETN concentration and progressive severity of CAD. The study found a gradual increase in RETN levels in different CAD subtypes, with the lowest concentrations in unstable angina, intermediate levels in unstable angina pectoris, and the highest levels in acute myocardial infarction. Comparative analyses revealed a significant increase in standardized mean differences, indicating a proportional relationship between RETN elevation and CAD manifestation severity.[36] Increased serum RETN levels and a strong association with CAD severity showed a similar relationship with myocardial inflammation in RETN.[37]

In Abha, Saudi Arabia, serum LEP and RETN levels were independently associated with an increased risk of AMI. Both LEP and RETN levels are significantly elevated in patients with AMI. These results suggest that LEP and RETN could be important biomarkers to assess CV risk and may provide insights into the mechanisms underlying AMI.[38] There are several biological mechanisms by which LEP and RETN contribute to myocardial injury and inflammation. After MI, the innate immune system is activated, leading to the release of inflammatory cytokines, which improve LEP production from white adipose tissue, leading to elevated levels in circulation.[39] Infarction leads to ischemia/reperfusion, which increases the formation of reactive oxygen species (ROS) and induces further oxidative stress in cardiac cells, leading to a negative feedback loop that increases inflammation and cell damage. Furthermore, there is a direct effect on the myocardium, which binds to its receptors in cardiac cells (Ob-R), leading to hypertrophy of cardiac cells and increased proteolytic activity, contributing to impaired cardiac function.[40]

Elevated RETN levels in patients with AMI may indeed result from AMI-induced inflammation induced by AMI rather than serve as a precursor. RETN is secreted by macrophages and adipocytes in response to inflammatory signals (eg IL-6 and TNF-α) post-infarction. Furthermore, RETN impairs endothelial function (endothelial dysfunction) and increases vascular permeability, which promotes immune infiltration and increases the severity of local inflammation in the myocardium.[41] Regression models prioritize variables that explain unique variance, and LEP, with a stronger association with AMI, probably overshadowed the contribution of RETN. Additionally, the elevation of RETN may reflect systemic inflammation secondary to AMI rather than being an independent causal factor. This aligns

with studies suggesting that RETN acts as a reactive biomarker of inflammation (Tripathi et al., 2020; Zhang et al., 2017), while the mechanistic role of LEP in metabolic dysregulation may confer greater predictive utility. The ROC curve analysis conducted in this study suggests that LEP has considerable potential as a diagnostic biomarker for AMI, due to its high sensitivity and specificity. Furthermore, the combination of LEP and RETN can improve diagnostic accuracy, offering a more robust approach. Future investigations are required to assess the utility of adipokines in the stratification and management of risk of AMI patients.[42]

Study limitations and future directions

This study has limitations, including a modest sample size due to recruitment and resource limitations, which can limit its generalizability. Furthermore, the single-center design and recruitment from one hospital in this study can limit the generalizability of the findings to broader and diverse populations, while its cross-sectional nature precluded tracking temporal changes in adipokine levels after onset of AMI, obscuring insights into their dynamic roles in acute versus chronic phases. Future multicenter longitudinal studies should investigate temporal fluctuations in LEP and RETN levels after AMI, correlate these changes with long-term clinical outcomes (eg, heart failure and mortality), and incorporate comprehensive clinical covariates (e.g., BMI, age, sex, diabetes, and hypertension) to enable multivariate risk stratification.

Conclusion

This study revealed significantly elevated serum levels of LEP and RETN in AMI patients, which strongly correlated with dyslipidemia, systemic inflammation, and markers of myocardial injury such as Hs-TnI. In particular, LEP demonstrated superior diagnostic accuracy compared to RETN, closely aligned with Hs-TnI, and emerged as a promising complementary biomarker to enhance early detection of AMI and risk stratification. Although RETN elevation likely reflects inflammatory sequelae rather than predictive utility, both adipokines deserve further exploration in multicenter cohorts. Despite the diagnostic potential of LEP, its clinical translation requires validation through larger prospective studies to confirm its role alongside established cardiac biomarkers. These findings underscore the importance of integrating novel adipokine profiles into AMI diagnostics, while emphasizing the need for rigorous population-diverse research to refine their clinical applicability.

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Declarations

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Author contributions

Conceptualization: A.K.H. and B.R.A.; Methodology, A.K.H.; Software, A.K.H.; Validation, A.K.H., and B.R.A.; Formal Analysis, A.K.H.; Investigation, A.K.H.; Resources, A.K.H.; Data Curation, A.K.H.; Writing – Original Draft Preparation, A.K.H.; Writing – Review & Editing, A.K.H., and B.R.A; Visualization, A.K.H.; Supervision, B.R.A.; Project Administration, B.R.A., and A.K.H.; Funding Acquisition, A.K.H.

Conflicts of interest

The authors have nothing to disclose.

Data availability

All data supporting the study findings were obtained from the Nasiriyah Heart Hospital in the Thi-Qar province.

Ethics approval

The study protocol, subject information, and approval form were reviewed and approved by the main laboratory in Nasiriyah Heart Hospital, Iraq, in accordance with Document No. 287/2024 dated (11/12/2024) to obtain this approval.

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ORIGINAL PAPER

Association of interleukin-1β gene polymorphisms in the occurrence of gastric ulcer in Southern Odisha, India

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ABSTRACT

Introduction and aim. The occurrence of gastric ulcer (GU) is influenced by many factors including interleukin 1β (*IL-1β*) gene polymorphisms. In this study, the association of GU with *IL-1β* gene polymorphisms was investigated in patients with gastric disorders.

Material and methods. The patients were categorized into two groups, group 1: patients with normal impression in the upper-GI-endoscopy (n=135); group 2: patients with GU (n=135). Three single nucleotide variants (SNVs) of *IL-1β* gene [rs16944 (–511 C/T), rs1143627 (–31 C/T) and rs1143634 (+3954 C/T)] were investigated by PCR-RFLP method.

Results. The association of *IL-1β* gene polymorphisms and occurrence of GU along with other factors (socio-demographic and habits) showed patients with smoking, alcohol intake and NSAID use was high in group 2. For SNVs and occurrence of GU, rs1143627 (–31 'TT') genotype was significantly high in group 2 (21.48%) compared to group 1 (9.63%), while the other two genotypes were comparable. Further, there was no association of any genotypes with smoking, alcohol intake and NSAID use, except NSAID use with rs1143627 (–31 C/T) in group 1.

Conclusion. The findings revealed, the habits like smoking, alcohol intake and recurrent NSAID use may influence the occurrence of GU while, the *IL-1β* gene polymorphisms have minimal impact on the occurrence of GU.

Keywords. gastric ulcer, gene polymorphism, interleukin-1β, non-steroid anti-inflammatory drug

Introduction

Gastric ulcer (GU) is the most common gastrointestinal disease worldwide affecting about four million people annually with an estimated lifetime prevalence of 5–10% in general population.[1] Incidence of GU is associated with many confounding factors including food habits, alcohol consumption, smoking habit, use of non-steroid anti-inflammatory drug (NSAID), *Helicobacter pylori* infection, as well as various genetic polymorphisms etc.[2-5]

Several studies were carried out by taking the single nucleotide variants (SNVs) of interleukin 1β (*IL-1β*) gene against the development of ulcer in upper gastrointestinal (GI) tract especially for duodenal and GU. Three potentially functional SNVs: rs16944 (–511 C/T) and rs1143627 (–31 C/T) in the promoter region, and rs1143634 (+3954 C/T) in exon 5 of *IL-1β* gene have been widely studied and the association with GU are reported to be conflicting.[6-11] These SNVs have also been

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found to be strongly associated with the *H. pylori* infection with increased risk of gastric disorders.[6-12] Further the risk of GU were reported to be contradictory with respect to the genotypes of various SNVs in *IL-1β* gene in different populations worldwide.[6,8,13]

Aim

Keeping the paucity of information on the association of *IL-1β* gene polymorphisms with the occurrence of GU in the Indian population, this prospective study was designed with an aim to analyze the possible association of three SNVs of *IL-1β* gene [rs16944 (–511 C/T), rs1143627 (–31 C/T) and rs1143634 (+3954 C/T)] with the occurrence of GU in patients coming with gastric disorder to a tertiary health care facility of Eastern India.

Material and methods

This case-control (un-matched) study was conducted between March 2019 and February 2021 and included individuals presenting with symptoms of gastric disorders who were referred for upper GI endoscopy. All the consecutive cases were recruited except individuals with other chronic diseases and who refused to be a part of the study. The diagnosis of GU was established on the basis of endoscopic findings. Individuals with normal impression of upper GI endoscopy along with absence of an inflammatory process of any etiology were considered as control (group 1) and individuals diagnosed with GU were considered as case (group 2).

A total of 135 cases each in group 1 and group 2 were recruited considering the prevalence of *IL-1β* gene polymorphisms (variants) in GU patients of 40%, two sided confidence level of 95%, allowable error of 5% and odd ratio of 2. The sample size was calculated by using OpenEpi software, Version 3 (https://www.openepi.com/SampleSize/SSCC.htm). After taking informed consent for the study, 3 milliliters of venous blood was collected in EDTA vial from the individuals and were transported by coolants to the Multi-Disciplinary Research Unit (MRU) of M.K.C.G. Medical College for further *IL-1β* SNVs study. This study was approved by the Institutional Ethical Committee (IEC) of M.K.C.G. Medical College, Berhampur, Odisha (No. 819 /Chairman-IEC, M.K.C.G. Medical College, Berhampur-4).

The genomic DNA was isolated from peripheral blood sample by using phenol-chloroform isolation method. The isolated DNA was quantified and the quality was checked by spectrophotometer (DS-11+Spectrophotometer, DeNovix, Wilmington, DE19810 USA). On analysis, isolated DNA samples with a good purity ratio of 1.7 to 2.0 were allowed for polymerase chain reaction (PCR) and rest samples were re-processed with RNAse treatment where required.

Three SNVs of *IL-1β* gene [rs16944 (–511 C/T), rs1143627 (–31 C/T) and rs1143634 (+3954 C/T)] were investigated by PCR followed by Restriction Fragmented Length Polymorphisms (RFLP) as per the protocol described by Manchanda et al., and Ramis et al.[10,14] The list of primer used, PCR (time, temperatures and cycle) and respective restriction endonuclease (RE) along with resulted product and respective genotypes has been illustrated in Table 1. All the three RE (*Ava I, Alu I* and *Taq I*) used in this study were from New-England Biolabs. Both the PCR products and the RE digested product were run in 3% agarose gel electrophoresis followed by documentation in gel documented system using Image Lab software (GelDoc XR+, Bio-Rad, USA). The agarose gel picture of three SNVs of *IL-1β* gene [rs16944 (–511 C/T), rs1143627 (–31 C/T) and rs1143634 (+3954 C/T)] have been attached as supplementary files (Fig. 1, Fig. 2 and Fig. 3) respectively. For reproducibility in each run, we have used both the positive control and negative control investigated in our own samples. Again, 10 randomly selected samples were re-analyzed for all the three SNVs for further confirmation.

Table 1. Details of primer, PCR cycle, restriction enzymes and length of the products for the analysis of *IL-1β* gene polymorphisms

SNVs	Primers	Sequence	PCR cycle (35 cycles)	PCR product (in bp)	RE	DNA fragments and genotype
rs16944 (–511 C/T)	Forward	TGGCATTGATCTGGTTCATC	95°C for 30 sec 56°C for 30 sec 72°C for 30 sec	304	Ava I	CC 190, 114 CT 304, 190, 114 TT 304
	Reverse	GTTTTAGGAATCTTCCCACTT				
rs1143627 (–31 C/T)	Forward	AGAAGCTTCCACCAATACTC	95°C for 30 sec 56°C for 30 sec 72°C for 30 sec	239	Alu I	CC 239 CT 239,137,102 TT 137,102
	Reverse	AGCACCTAGTTGTAAGGAAG				
rs1143634 (+3954 C/T)	Forward	GTTGTCATCAGACTTTGACC	95°C for 30 sec 55°C for 30 sec 72°C for 30 sec	249	Taq I	CC 135,114 CT 249,135,114 TT 249
	Reverse	TTCAGTTCATATGGACCAGA				

Data collection and statistical analysis

A designed case format containing the set of information along with anthropometric and clinical parameters was filled-up for each study subject. All the data was presented by number followed by percentage. The comparison of variables (socio-demographic, habits, genotypes of respective SNVs) were made between the two groups of study participants. Further, the association of genotypes of three SNVs against various habits (smoking, alcohol intake and NSAID use) were analyzed. All the categorical data was analyzed by Chi square test of significance by using SPSS version 16 (IBM, Armonk, NY, USA). A p value of <0.05 was considered as statistically significant.

Results

During the study period, a total of 135 cases each in both the groups were considered for further analysis. There was no significance difference between the groups with respect to age, gender, occupation and place of liv-

ing. However, the number of cases with smoking, alcohol intake and NSAID use was significantly high in group 2 with GU compared to group 1 (Table 2).

Table 2. Comparison of variables (socio-demographic and habits) between the patients with and without gastric ulcer

Variables	Group 1 (without GU) (n=135)	Group 2 (with GU) (n=135)	χ^2 value, p
<40	38 (28.15)	28 (20.74)	3.367, 0.1857
40-60	43 (31.85)	48 (35.56)	
>60	54 (40.0)	59 (43.70)	
Gender			
Male	96 (71.11)	101(74.81)	0.469, 0.493
Female	39 (28.89)	34 (25.19)	
Occupation			
Service	30 (22.22)	57 (42.22)	8.398, 0.78
Business	14 (10.37)	9 (6.67)	
Farmer	40 (29.63)	32 (23.70)	
Others	35 (25.93)	26 (19.26)	
Housewife	16 (11.85)	11 (8.15)	
Place of living			
Urban	42 (31.11)	43 (31.85)	0.017, 0.896
Rural	93 (68.89)	92 (68.15)	
Smoking			
Yes	18 (13.33)	33 (24.44)	5.439, 0.02
No	117 (86.67)	102 (75.56)	
Alcohol intake			
Yes	32 (23.70)	47 (34.81)	4.026, 0.045
No	103 (76.30)	88 (65.19)	
NSAID use			
Yes	11 (8.15)	32 (23.70)	12.119, 0.001
No	124 (91.85)	103 (76.30)	

The genotypes and allelic frequency of three SNVs [rs16944 (–511 C/T), rs1143627 (–31 C/T) and rs1143634 (+3954 C/T)] of *IL-1β* gene were compared between group1 and group 2. Among the three, two SNVs, that is, rs1143634 (+3954 C/T) and rs16944 (–511 C/T), both the genotypes and alleles distribution were found to be comparable in group 1 and group 2 respectively. However, the 'TT genotype of rs1143627 (–31 C/T) was significantly high (χ^2 7.437; P value 0.024) in group 2 (21.48%) compared to group 1 (9.63%). Similarly, the 'T' allele was high in group 2 compared to group 1, however, it did not reach to a statistical significant level (χ^2 2.909; P value 0.088). The comparison of genotypes and alleles distribution of three SNVs of *IL-1β* gene between the two groups of patients has been depicted in Table 3.

Further, the habit like smoking, alcohol intake and NSAID use were compared among the genotypes of the three SNVs separately. On comparison, there is no specific significant association is observed among the variables and genotypes of all the three SNVs in both group 1 and group 2, except use of NSAID in group 1 for rs16944 (–511 C/T). Here 'CT' genotypes were reported to be low (zero) in those who had used NSAID (Table 4).

Table 3. Comparison of genotype and allele distribution of *IL-1β* gene polymorphisms between the patients with and without gastric ulcer

rs16944 (–511 C/T)	Group 1 (without GU) (n=135)	Group 2 (with GU) (n=135)	χ^2 value, p
CC	33 (24.44)	43 (31.85)	2.114, 0.347
CT	46 (34.07)	38 (28.15)	
TT	56 (41.48)	54 (40.00)	
C	0.41	0.46	0.508, 0.476
T	0.59	0.54	
rs1143627 (–31 C/T)	**Group 1 (without GU) (n=135)**	**Group 2 (with GU) (n=135)**	**χ^2 value,p**
CC	83 (61.48)	69 (51.11)	**7.437, 0.024**
CT	39 (28.89)	37 (27.41)	
TT	13 (9.63)	29 (21.48)	
C	0.76	0.65	2.909, 0.088
T	0.24	0.35	
rs1143634 (+3954 C/T)	**Group 1 (without GU) (n=135)**	**Group 2 (with GU) (n=135)**	**χ^2 value,p**
CC	91 (67.41)	84 (62.22)	1.073, 0.585
CT	34 (25.19)	37 (25.19)	
TT	10 (7.41)	14 (10.37)	
C	0.80	0.76	0.466, 0.495
T	0.20	0.24	

Table4. Association analysis of social parameters and the genotypes of *IL-1β* gene polymorphisms

	Group 1 (without GU) (n=135)				Group 2 (with GU) (n=135)			
rs16944 (–511 C/T)								
Variables	CC	CT	TT	χ^2 value, p	CC	CT	TT	χ^2 value, p
Smoking (Yes)	6 (33.33)	3 (16.67)	9 (50.00)	2.882,	6 (18.18)	11 (33.33)	16 (48.48)	3.766,
Smoking (No)	27 (23.08)	43 (36.75)	47 (40.17)	0.237	37 (36.27)	27 (26.47)	38 (37.25)	0.152
Alcohol intake (Yes)	8 (25.00)	14 (43.75)	10 (31.25)	2.216,	21 (44.68)	10 (21.28)	16 (34.04)	5.575,
Alcohol intake (No)	25 (24.27)	32 (31.07)	46 (44.66)	0.33	22 (25.00)	28 (31.82)	38 (43.18)	0.062
NSAID (Yes)	6 (54.55)	0	5 (45.45)	8.565,	9 (28.13)	8 (25.00)	15 (46.88)	0.826,
NSAID (No)	27 (21.77)	46 (37.10)	51 (41.13)	0.014	34 (33.01)	30 (29.13)	39 (37.86)	0.662
rs1143627 (–31 C/T)								
Variables	CC	CT	TT	χ^2 value, p	CC	CT	TT	χ^2 value, p
Smoking (Yes)	12 (66.67)	4 (22.22)	2 (11.11)	0.458,	17 (51.52)	12 (36.36)	4 (12.12)	3.061,
Smoking (No)	71 (60.68)	35 (29.91)	11 (9.40)	0.795	52 (50.98)	25 (24.51)	25 (24.51)	0.216
Alcohol intake (Yes)	23 (71.88)	8 (25.00)	1 (3.13)	2.799,	25 (53.19)	12 (25.53)	10 (21.28)	0.155,
Alcohol intake (No)	60 (58.25)	31 (30.10)	12 (11.65)	0.247	44 (50.00)	25 (28.41)	19 (21.59)	0.925
NSAID (Yes)	8 (72.73)	3 (27.27)	0	1.41,	12 (37.50)	13 (40.63)	7 (21.88)	4.197,
NSAID (No)	75 (60.48)	36 (29.03)	13 (10.48)	0.494	57 (55.34)	24 (23.30)	22 (21.36)	0.123
rs1143634 (+3954 C/T)								
Variables	CC	CT	TT	χ^2 value, p	CC	CT	TT	χ^2 value, p
Smoking (Yes)	14 (77.78)	4 (22.22)	0	1.942,	22 (66.67)	9 (27.27)	2 (6.06)	0.921,
Smoking (No)	77 (65.81)	30 (25.64)	10 (8.55)	0.379	62 (60.78)	28 (27.45)	12 (11.76)	0.631
Alcohol intake (Yes)	20 (62.50)	10 (31.25)	2 (6.25)	0.838,	28 (59.57)	14 (29.79)	5 (10.64)	0.235,
Alcohol intake (No)	71 (68.93)	24 (23.30)	8 (7.77)	0.658	56 (63.64)	23 (26.14)	9 (10.23)	0.889
NSAID (Yes)	6 (54.55)	4 (36.36)	1 (9.09)	0.934,	17 (53.13)	10 (31.25)	5 (15.63)	1.901,
NSAID (No)	85 (68.55)	30 (24.19)	9 (7.26)	0.627	67 (65.05)	27 (26.21)	9 (8.74)	0.387

Discussion

In this case-control study a total of 270 patients with symptoms of upper GI tract disorders advised for upper GI tract endoscopy were included. The anthropometric, socio-demographic, behavioral and three SNVs of *IL-1β* gene [rs16944 (–511 C/T), rs1143627 (–31 C/T) and rs1143634 (+3954 C/T)] were compared between the two groups (patients with and without GU) of patients.

Variables like age, gender, occupation, place of living and habit like smoking, alcohol intake, NSAID use were evaluated for the association of GU. There was no association between age, gender, occupation and place of living with GU. The occurrence of GU is significantly higher in patients with alcohol intake, smoking and NSAID use in many earlier studies. Smoking may lead to the development of ulcer in the upper GI tract and found to be reversible after stopping of smoking.[15-18] There was also inconsistent report on the association of smoking habit and occurrence of GU.[7,19] The frequent use of NSAID has been reported to be associated with ulcer disease due to its action on inhibiting the cyclooxygenase enzyme in the GI tract leads to a reduction of prostaglandin secretion and cytoprotective effects in gastric mucosa, increasing the susceptibility to mucosal injury.[20,21] The ulcer can be effectively treated by stopping the use of NSAID. However, Treatment may be recommended to speed up the healing process, else surgery may be needed.[22,23]

The association of three SNVsof *IL-1β* gene [rs16944 (–511 C/T), rs1143627 (–31 C/T) and rs1143634 (+3954 C/T)] with the occurrence of GU showed absent of any possible association with GU with two SNVs that is, rs16944 (–511 C/T) and rs1143634 (+3954 C/T). Similar interpretation was also reported in a study undertaken in Brazilian and Spanish Caucasian population, where the SNVrs1143634 (+3954 C/T) had no association with GU.[10,24] There is also contradictory result on the involvement of SNVrs1143634 (+3954 C/T), in which the 'TT' genotype found to be a genetic risk factor for *H. pylori* induced GU in Iranian population.[8] Further, rs1143634 (+3954 C/T) 'TT' genotype was also associated with peptic ulcer and 'T' allele (CT/TT) is correlated with gastritis.[8] The association of rs1143634 (+3954 'T') allele with gastric disorders have also been reported from the populations of African Americans and Caucasian, and Poland.[25,26]

For SNV rs16944 (–511 C/T) there was also inconsistent results. Briefly, In Chinese population the rs16944 (–511 'T') allele is more frequent and has association between peptic disease and *H. pylori*.[27] In Japanese population the rs16944 (–511 'C') allele is more dominant with advance atrophic gastritis.[28] There was no difference in the frequency of rs16944 (–511 'C' and 'T'allele) in Thai and Vietnamese populations in occurrence of chronic gastritis respectively.[28,29] In Korean population the rs16944 (–511 'C') allele leads to enhancement of production of *IL-1β* by gastric mucosa.[30] The presence of 'C' allele ('CC' and 'CT' genotypes) of rs16944 (–511 C/T) was associated with chronic gastritis and gastric cancer development especially individuals with *H. pylori*-infection in the Brazilian population.[12]

Unlike rs16944 (–511 C/T) and rs1143634 (+3954 C/T), the SNV rs1143627 (–31 C/T) was found to be associated with the GU in the present study, that is, the 'TT' genotype was significantly higher in group 2 with GU. The 'T' allele could act as a pro-inflammatory allele in rs1143627 (–31 C/T) and its presence may be an independent risk factor for gastric carcinoma. This was resulted by the study carried out in Korean population indicating the importance of rs1143627 (–31 'T') allele as well as *H. pylori* infection for the development of ulcer.[30] Few studies have reported with contradictory finding where presence of 'TT' genotype acts as a protector for *H. pylori* infection and gastritis.[31] Further the 'CC' genotype was more frequent in gastric carcinoma patients in Chinese population in Northern region.[27] The SNVs of *IL1 β* gene especially rs1143627 (–31 C/T) and rs16944 (–511 C/T) have been linked to the expression of IL-1β mRNA leads to the development of gastric inflammation as well as gastric carcinoma.[32,33] A detail comparative analysis on the association of *IL1 β* gene polymorphisms and gastric disorders has been illustrated as Table 5.

Table 5. Comparative analysis on the association of *IL-1 β* gene polymorphisms and gastric disorders

Studies	rs1143627 (–31 C/T)	rs16944 (–511 C/T)	rs1143634 (+3954 C/T)
Thai and Vietnamese populations[29]		No association with chronic gastritis	
Chinese population[27]	'CC' genotype with gastric carcinoma	'T' allele with peptic disease and H. pylori infection	No association
Korean population[30]	'T' allele in the development of gastric carcinoma	'C' allele enhanced the production of IL-1β by gastric mucosa	
African Americans and Caucasian[25]	No association	No association	'T' allele with multifocal atrophic gastritis
Turkish population[31]	'CC' and 'CT' genotype as an marker of Gastric Cancer		
Brazilian population[12]		'C' allele with chronic gastritis and gastric cancer	
Poles population (Poland)[26]			'T' allele with gastric disorders
Japanese population[28]		'C' allele with advanced atrophic gastritis	
Iranian population[8]			'TT' genotype with high risk of gastritis and peptic ulcer
Indian population (present study)	'TT' genotype with gastric ulcer	No association	No association

On comparison, rs16944 (–511 C/T) was found to be significantly associated with NSAID use in group 1 patients especially the 'CT' genotype. This may be due to the low sample size as there were only 11 cases who used NSAID in group 1 patients. It has been reported that, the use of aspirin may induce peptic ulcer as per a study carried out in Japanese Korean Ethnic group.[34] In a study carried out in Japanese population, rs1143627 (–31 C/T) and rs16944 (–511 C/T) were significantly associated with the development of peptic ulcer in cardiovascular diseases patients taking 100 mg of aspirin as well as those with *H. pylori* infection. However, the authors did not report any association in *H. pylori* negative patients suggesting *H. pylori* negative patients without past infection had no effect on development of aspirin related ulcer.[35] The information on which *IL-1β* gene polymorphisms are significant risk factors for the development of GU in individuals using NSAID are still lacking and need large-scale clinical studies. The current study has certain limitations like, lack of investigation of *H. pylori* in the study participants as *H. pylori* infection have a major impact on the GU, gastric cancer, peptic ulcer and gastritis.

Conclusion

The current study revealed the significant positive association of smoking, alcohol intake and NSAID use on the occurrences of GU. Secondly, the *IL-1β* gene polymorphisms found to have a minimal impact on the occurrence of GU in Indian patients that is, SNV rs1143627 (–31 'TT') genotype of *IL-1β* gene only was found to be associated with development of GU. Further, the possible effect of *IL-1β* gene polymorphisms on the occurrence GU was independent of other risk factors like smoking, alcohol intake, NSAID use etc. However, a large cohort study with *H. pylori* investigation, associated genetic polymorphisms and mRNA expression may be helpful for the better understanding of this genotype-phenotype relation in the study population.

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Declarations

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Author contributions

Conceptualization, S.A., P.P. and S.S.M.; Methodology, S.A., P.P. and S.S.M.; Validation, P.P. and S.S.M.; Formal Analysis, P.P.; Investigation, S.A. and P.P.; Resources, S.A., A.N. and P.P.; Data Curation, P.P.; Writing – Original Draft Preparation, S.A., A.N. and P.P.; Writing – Review & Editing, S.K.B., P.P. and S.S.M.; Supervision, P.P. and S.S.M.; Project Administration, S.S.M.; Funding Acquisition, S.A., P.P. and S.S.M.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

Data will be available on request.

Ethics approval

This study was approved by the Institutional Ethical Committee (IEC) of M.K.C.G. Medical College, Berhampur, Odisha (No. 819 /Chairman-IEC, M.K.C.G. Medical College, Berhampur-4).

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ORIGINAL PAPER

The knowledge of society regarding health-promoting behaviors in oncology

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ABSTRACT

Introduction and aim. Enhancing awareness, early detection, and fostering health-seeking behavior is imperative to address the growing problem of cancer, advocating for basic health education from an early age to reduce morbidity and mortality.

Material and methods. The survey was carried out in two forms: paper and online. The research tool was a questionnaire, consisting of 25 closed-ended questions.

Results. While 95.5% denied the presence of personal cancer, 63% reported family history. Despite awareness of the impact (83% women; 81% men), 44% consume fast food monthly. Self-examination rates are low: only 37% perform it regularly; 45% of men lack knowledge of testicular examination.

Conclusions. Health campaigns across all age groups are necessary to promote cancer prevention and early detection, with a focus on educating both men and women on self-examination due to inadequate knowledge levels.

Keywords. cancer prevention, health behavior, health promotion

Introduction

Cancer is the second most common cause of death in developed countries. Awareness of risk factors affecting the formation and development of cancer is an important element in the fight against this disease, as proper health-promoting behaviors can significantly reduce the risk of developing cancer. The best results would come from the promotion of health at an early age, which could influence the quality and lifestyle that accompany a person over the years, and thus the risk of the disease. Health awareness encompasses knowledge of cancer prevention, health behaviors, factors detrimental to health and the resulting risks, as well as ways to prevent and eliminate them.[1] Contrary to popular belief, currently only a small proportion of cancers are conditioned by the inheritance of certain genetic predispositions. The vast majority of these diseases result from the long-term accumulation of DNA damage. The accumulation of such abnormalities in genetic material causes cells to start dividing uncontrollably. Scientists emphasize that a healthy lifestyle can limit the accumulation of such damage.[2] Given the currently available methods of cancer prevention, it is necessary to impart sound knowledge on the significant risk factors that occur in the later years of life

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for women and men. Above all, it is important to make the public aware of how crucial leading a healthy lifestyle is in reducing the risk of cancer.[3] By analyzing data from the National Cancer Registry from 2010 to 2020, a clear upward trend in cancer incidence can be observed. The number of cases increased, as has the number of cases and deaths per 100,000 inhabitants. The same upward trend is also observed in the age-standardized morbidity (mortality) rate, which determines how many diseases (deaths) would occur in the studied population if the age structure of this population were consistent with the age structure of the population accepted as the standard (in this comparison: the standard world population and the standard European population). Increasing awareness and promoting proactive health behaviors can have a significant impact on cancer prevention and improving public health outcomes.

Aim

The aim of this work will be to examine the role of increasing awareness of risk factors, early detection, and promoting a healthy lifestyle in the context of the growing incidence of cancer. We want to investigate what educational strategies can be implemented to effectively inform society about key aspects of cancer prevention and early detection, aiming to reduce the number of cases and associated mortality.

Material and methods

We organized a questionnaire survey containing 25 questions, filled out by 315 adults, including 220 female participants and 95 male participants. Our study obtained positive opinion of the Bioethic Committee of Rzeszow University (Resolution number: 2022/099 date; 07 December 2022).

The study was conducted in paper and online form (according to the level of computerization of the participants), distributed at different locations: high schools, universities, seniors' clubs, and health department venues. The survey form included questions concerning age, sex, education, relationship status, place of residence, and occupation, as well as knowledge about neoplasm / cancer,, family history of neoplasm/cancer and awareness of various pro-health behaviors, the implementation of which allows reducing the chances of cancer. Both paper and online surveys were completed anonymously through sheets that do not require the submission of personal data and through anonymous Google Forms surveys.

The geographic area of the study comprises two Polish provinces: Podkarpackie and Małopolskie.

Participation in the study was voluntary.

The choice of the number of 315 respondents in this study is statistically justified. The sample size was selected to ensure representativeness and the ability to generalize the results to the population of residents of the Podkarpackie and Małopolskie provinces. In addition, the use of both paper and online versions of the questionnaire, as well as the selection of respondents from different age groups, genders, and education levels increases the representativeness and diversity of the sample, which positively affects the reliability of the results.

The survey was carried out over a three-month period and involved people who voluntarily expressed their willingness to participate by completing a questionnaire. Random sampling was used among the enrolled participants, allowing a representative survey group. The inclusion criteria were originating from the above-mentioned Voivodeships, high schools, universities, membership of senior clubs and attendance to health department sites. The only exclusion criterion was not agreeing to complete the survey.

The data analysis took into account the comparison of results in different groups of subjects according to variables, i.e., age, place of residence, and level of education. The data obtained were processed and analyzed using the Statistica package.

Results

The vast majority of respondents denied having or currently having cancer (95.5%; n=314).

Significantly, 63% (n=198 (Table 1) of the respondents indicated that there was a family history of cancer. Still, 7% (n=22) (Table 1) of the respondents state that alcohol abuse does not affect the development of cancer. A large group of respondents (94%; n=296) (Table 1) answered in affirmative to the question of whether smoking cigarettes and other tobacco products can contribute to the development of cancer. Of the respondents, 91% (n=286) (Table 1) believe that excessive sunbathing can contribute to the development of cancer. However, up to 70% of respondents report that they regularly expose their bodies to sunlight. Furthermore, 65% (n=204) (Table 1) of the survey participants agreed with the statement that lack of physical activity can lead to the development of cancer. Also in this group of respondents, when asked "Do you do physical activity?" 51 people, representing 16% (n=50) answered that "no".

A group of 208 people, representing 66% (n=207) of the respondents, associated good hygiene with a reduced incidence of certain cancers. In the group surveyed, 77% (n=242) (Table 2) believe that fast food can increase the risk of cancer. When asked how often they eat fast food, respondents reported: 44% (=138) 12 times a month; 16.5% (=51) 12 times a week. On average, 82% (n=258) agreed that a good, healthy diet reduces cancer risk. Of these, 83% (n=261) of women believe that an adequate diet can reduce the risk of cancer. Similarly, 81% (n=255) of the men surveyed also indicate a correlation between diet and reduction in cancer risk.

Approximately half (49.6%; n=156) of the respondents agreed with the statement that viral diseases may be responsible for the development of cancer.

Of the 314 people in the survey group, only 209 know how to perform a testicular/breast self-examination. When asked whether respondents conducted such self-examination, only 37% (=116) indicated a yes answer. It should be noted that 28% (n=88) of the women in the survey group do not know how to perform such an examination and 45% (n=141) of the men report that they do not know how to perform a testicular self-examination (Tables 3 and 4).

When asked which factors they thought had the greatest impact on their health, respondents indicated, in order, lifestyle (49.6%; n=156), genetic factors (35%; n=110), physical environment (3%; n=40), health care (3.5%; n=11) (Fig. 1).

The responses regarding lifestyle correspond in percentage to the health fields. However, we see disagreements about genetic factors, physical environment, and health care. In our study, they are 35%, 3% and 3.5%, respectively, and according to Lalonde's concept, they are 16%, 21% and 10%.

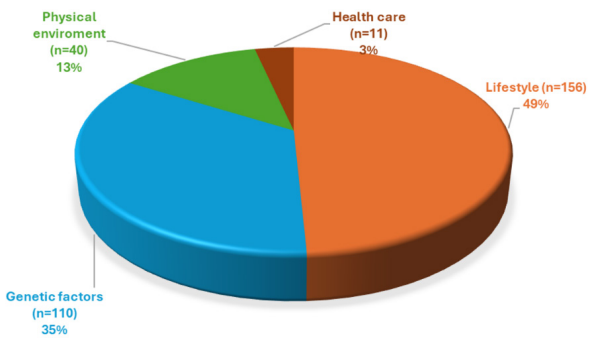

Fig. 1. Factors that have the greatest impact on cancer incidence according to respondents

Table 1. Demographic characteristics of the respondent group (n=314)

Demographic feature	Category	Number (n)	Percentage (%)
Age	14–17	118	37%
	18–24	72	23%
	25–34	2	1%
	35–50	20	6%
	50–64	37	12%
	65+	65	21%
Gender	Male	95	30.2%
	Female	219	69.8%
Marital status	Married	80	25%
	Single	195	62%
	Divorced	5	2%
	Widow	30	10%
	In separation	4	1%
Cancer history	Yes	14	5%
	No	300	95%

Table 2. Knowledge of cancer prevention

Question	Answer	Number (n)	Percentage (%)
Do you think viruses can cause cancer?	Yes	156	50%
	No	42	13%
	Don't know	116	37%
In your opinion, can excessive sunbathing contribute to the development of cancer?	Yes	287	91%
	No	7	2%
	Don't know	20	7%
Do you think eating fast food can increase your risk of cancer?	Yes	244	78%
	No	29	9%
	Don't know	41	13%
In your opinion, can an appropriate diet reduce the risk of cancer?	Yes	244	78%
	No	29	9%
	Don't know	41	13%
Do you know how to perform a testicular self-examination/breast self-examination?	Yes	209	66.5%
	No	105	33.5%

Table 3. Prognostic factors related to the knowledge of society about health-promoting behaviors in oncology

Factor	Category	Question	Answer	Number (n)	Percentage (%)	p
Age	<65	Do you think alcohol abuse can contribute to cancer development?	Yes	212	67.5%	0.02
			No	15	4.5%	
			Don't know	22	7.5%	
	≥65		Yes	42	13.5%	
			No	7	2.0%	
			Don't know	16	5.0%	
Education level	Higher	In your opinion, can performing certain professions increase the risk of developing cancer?	Yes	38	12.0%	<0.001
			No	5	1.5%	
			Don't know	10	3.0%	
	Lower		Yes	235	75.0%	
			No	3	1.0%	
			Don't know	23	7.5%	
Marital status	Married	Do you regularly perform testicular self-examination/ breast self-examination?	Yes	41	13.0%	0.008
			No	39	12.0%	
	Single		Yes	76	24.0%	
			No	155	51.0%	

Table 4. Comparison of selected responses by gender

Question	Women (n=219)	Men (n=95)	Remarks
Do you think that an appropriate diet reduces cancer risk?	83% (181)	81% (77)	Similar high awareness in both groups
Do you know how to perform breast/testicular self-examination?	72% (158)	54% (51)	Significant difference; women show higher awareness
Do you regularly perform self-examinations?	41% (90)	27% (26)	Women more often declare regular self-examinations
Can a lack of physical activity lead to cancer?	68% (149)	58% (55)	10 percentage points difference
Does fast food consumption increase the risk of cancer?	79% (173)	76% (72)	Similar results
Does alcohol abuse affect cancer risk?	92% (202)	85% (81)	Women more often indicate the relationship
Can excessive exposure to the sun lead to cancer?	93% (204)	88% (84)	High awareness in both, higher among women

Discussion

The survey results reveal a significant gap between awareness and practice of cancer prevention among the respondents. Although most understand that factors such as smoking, excessive tanning, alcohol abuse, and physical inactivity can contribute to the development of cancer, many do not take steps to minimize these risks. Despite a high awareness of the factors that contribute to cancer, there appears to be a gap between knowledge and practice, as evidenced by discrepancies in self-examination and lifestyle choices.

The phenomenon of an increase in cancer incidence is a consequence of changes in the demographics of society and the evolution of risk factors. At the same time, the cost of treating cancer patients is increasing, which poses a challenge to effective prevention. It is estimated that up to 40% of cancer cases in Europe could be prevented by adequate understanding of risk factors and preventive measures.

The survey included 315 respondents, of whom 219 (69.8%) were women and 95 (30.2%) were men. This gender imbalance may have influenced the results of the survey, especially in terms of health awareness and declared preventive behaviors. Numerous previous studies show that women are statistically more likely to participate in preventive activities, are more interested in health topics, and show a higher level of awareness of health risks, including cancer. This may lead to an overestimation of the overall level of knowledge and pro-health attitudes observed in the survey, which does not necessarily reflect the actual state of awareness in the general population. Additionally, in the context of questions on self-examination (e.g. breast or testicular self-examination), the unequal participation of men and women may have affected the overall result, for example, the higher number of responses from women, who are more likely to know and perform breast self-examination, may have hidden the low level of knowledge among men about testicular self-examination. Therefore, the results obtained should be interpreted with this imbalance in mind.

International studies show that the level of knowledge of breast and testicular self-examination can vary significantly depending on socioeconomic and cultural conditions. Examples from countries such as Pakistan and Nigeria indicate extremely low levels of awareness and practice of self-examination, especially among men. This kind of data underscores that effective health education requires not only imparting knowledge, but also taking into account broader contexts such as access to information, level of education, and meeting basic life needs. In countries with lower socioeconomic status, limited access to preventive care can exacerbate health inequalities, highlighting the need for integrated public health interventions that include education, systemic support, and improved living conditions.

Regular physical activity can significantly improve the survival rate of cancer patients. The authors point out that physically active individuals have a lower risk of disease recurrence and cancer death, as well as a better quality of life. These findings underscore the need to incorporate recommendations for physical activity into care plans for cancer patients, during and after treatment.[4]

Organizations operating around the world have developed guidelines that indicate the importance of diet and a healthy lifestyle in the prevention of cancer. Reducing alcohol consumption, as well as introducing fruits and vegetables and fiber, are documented factors that can change the risk of developing cancer. Unfortunately, there are barriers that make it difficult for society to adopt the guidelines. This is a way of conveying knowledge, among others, through the media. Knowledge about prevention is not always conveyed in a clear and understandable way. Therefore, despite the development of cancer prevention guidelines and the proven benefits that result from them, public involvement in preventive behaviors is low.[5]

A healthy lifestyle, which includes proper eating habits, physical activity, and avoidance of risk factors such as smoking, is reflected in specific metabolic profiles that can affect cancer risk. The authors showed that people characterized by the "metabolic signature" of a healthy lifestyle had a significantly lower risk of colorectal cancer. These findings underscore the importance of prevention efforts aimed at promoting healthy habits that can translate into metabolic changes that promote the reduction of cancer risk.[6-8]

Despite the relatively high level of awareness of the negative effects of fast food on health, there is a marked discrepancy between knowledge and actual eating habits. A similar phenomenon has also been noted in studies conducted in other countries, including the United States, where the percentage of people who declare regular consumption of fast food remains high despite the recognition of its harmfulness. This type of discrepancy indicates that knowledge alone is not enough to change health behavior. This may be due to convenience, the availability of such foods, as well as established cultural habits. Therefore, for effective prevention, not only educational efforts are needed, but also strategies that support real lifestyle change, for example, by promoting healthy alternatives, creating a favorable eating environment, or introducing regulations that limit the promotion of unhealthy foods.

A 2018 study highlights the important influence of diet on the anticancer immune response, highlighting the role of nutrients in modulating the gut microbiome and reducing inflammation. These findings suggest that appropriate diet interventions can promote the body's natural defenses and reduce the risk of developing can-

cer. In the context of the use of information technology and artificial intelligence in health management, these observations open up the possibility of using mobile apps, digital platforms, and AI algorithms to monitor dietary habits, personalize dietary recommendations, and identify risk factors early. Integrating such solutions into healthcare care can increase the effectiveness of preventive measures and support the conscious development of healthy lifestyles.[9]

Studies indicate that physical interventions, particularly those that combine physical activity with manual lymphatic drainage (MLD), show promising results in reducing the incidence of secondary lymphedema in patients with breast cancer. These programs, which ran from 4 months to 5 years after treatment, lasted from 3 weeks to 12 months and demonstrated significant improvements in physical function, reduced arm swelling, and better recovery. In particular, programs that combined MLD with exercise produced better results, with participants showing a lower incidence of SL and faster recovery compared to control groups.[10]

For comparison, in another study conducted among the urban community of Karachi (Pakistan), it was found that up to 43% of women and 96% of men were unable to perform breast or testicular self-examination.[11]

The results differ even more compared to regions with worse socioeconomic conditions, eg, among young Nigerian men, where the knowledge of how to perform a testicular self-examination is only 1.3%. Furthermore, none to the respondents of the cited study performs this test regularly (compared to 35% in our study). This emphasizes the need to improve quality of life and the ability to meet basic needs to improve knowledge about health and its threats.[12]

78% of the respondents stated that eating fast food can increase the risk of cancer. In addition, 44% of the respondents stated that they eat fast food once or twice a month and 16.5% once or twice a week. These data can be compared with a study conducted in the state of the Wisconsin, where 38.75% of respondents reported eating fast food 1 3 times a month, while 19,39% reported eating fast food once or twice a week. These results show that despite the awareness that fast food is harmful to health, many of the Poles and Americans surveyed use this form of eating out.[13]

These findings point to the need for more intensive education and support in translating knowledge into daily health habits, which can help reduce the risk of developing cancer.

Given the systematic increase in the number of cancer cases, it is becoming important to place a primary emphasis on prevention. Preventive measures not only benefit individuals but also form an important part of health policy strategies. Prevention not only improves the general health situation of the population, but also effectively reduces health care costs. Primary prevention, which targets healthy individuals, and secondary prevention, which aims to control risk factors in exposed individuals, represent an integrated approach to maintaining individual and societal health. In combination, these two forms of prevention play a key role in effective health management. An important prerequisite for the success of screening programs is not only broad coverage of high-risk populations but also awareness-raising. Education is an indispensable component of preventive measures, performing not only the function of raising awareness of health, but also significantly influencing health behavior, including regular screening at specific intervals. Therefore, investing in educational programs becomes crucial for effective cancer prevention, both at the individual and social levels.[6]

There is a growing consensus that environmental factors play a key role in the development of cancerous lesions, acting not only as external threats but also as factors that can directly affect the genetic material of human cells. Epidemiological studies highlight the interaction between environmental exposures and genetic susceptibility, revealing a complex relationship that contributes to cancer risk. Most importantly, this risk is not evenly distributed around the world; Geographic variation in environmental conditions means that some populations may be more exposed to specific carcinogens depending on where they live.

This geographic disparity underscores the need for location-specific cancer prevention strategies. Understanding how carcinogens in the environment are distributed and how they interact with local ecosystems and populations is fundamental to developing evidence-based, targeted health policies. Aligning prevention efforts with regional risk profiles can increase the effectiveness of public health interventions, reduce health inequities, and ultimately contribute to reducing the global burden of cancer. Therefore, integrating environmental monitoring with cancer prevention initiatives should be considered a key component of comprehensive health strategies.[14]

Survey analysis (National Cancer Institute's Health Information National Trends Survey) revealed that after controlling for differences based on gender, race, age, income and education, attention to health news was significantly associated with knowledge about cancer risks associated with food and smoking, but not for knowledge about exercise, sun or alcohol.[23]

It is now known that environmental factors are a major threat to the formation of cancerous lesions. Epidemiological studies indicate that the influence of these factors can also affect the genetic material of cells. This relationship is closely related to the spread of carcinogens in different geographic zones. This means that geo-

graphic areas with environmental differences may be exposed to specific carcinogens, which in turn affect the risk of cancerous changes in body cells. Understanding this link is the key to developing effective prevention strategies and protecting health from the effects of harmful environmental factors.[15]

Responsible use of social networking tools can help spread accurate information, generate important new scientific discoveries, share diagnostic, treatment, and follow-up protocols, and compare different approaches on a global scale. In a society that trusts scientists and doctors, the former act as providers of facts based on solid research. As such, it is our responsibility as health professionals to be leaders in the conversation on social media platforms and to provide accurate and helpful information to those looking for answers. By doing so, we can effectively shape public dialogue and support the public in making informed health decisions.[16]

The increasing prevalence of ultraprocessed food (UPF) consumption has become a pressing public health concern, particularly because of its association with an increased risk of cancer and other noncommunicable diseases. A growing body of evidence suggests a consistent and significant association between high UPF consumption and the incidence of various cancers, including colorectal, breast, and pancreatic cancers.[20] These findings point to the broader implications of dietary patterns shaped by industrialized food systems.

It is worth noting that a large UK cohort study further supports these observations, indicating that elevated UPF intake may not only contribute to a higher incidence of cancer, but also increase cancer-related mortality, particularly for ovarian cancer in women.[21] These associations underscore the need to reassess current dietary environments and food policies.

From a preventive health perspective, these insights underscore the importance of promoting access to fresh and minimally processed foods while implementing regulatory measures targeting UPF reformulation, taxation, and marketing. Such interventions could play a key role in mitigating modifiable cancer risk and promoting healthier eating habits at the population level. As such, UPF consumption is not only a nutritional issue but also a key component of cancer prevention strategies in modern societies.

An analysis of the survey (National Cancer Institute's Health Information National Trends Survey) found that after adjusting for demographic variables such as gender, race, age, income, and education, interest in health news was significantly associated with awareness of modifiable cancer risks associated with eating and smoking, but not with risks associated with physical activity, sun exposure, or alcohol consumption. These findings suggest that the media tends to emphasize certain behavioral risks more than others, which may lead to an uneven public understanding of cancer prevention strategies. As Stryker et al. pointed out, the type and frequency of cancer-related topics presented in newspapers significantly shape the public's knowledge. In particular, lifestyle factors such as diet and smoking are more frequently discussed and thus better recognized by the public, while other important risk factors, such as inadequate exercise or excessive sun exposure, remain underrepresented in media narratives.[22] This selective exposure challenges comprehensive cancer education and points to the need for more balanced communication strategies. Public health campaigns should aim to broaden the scope of information spread through mass media, ensuring that less-publicized but equally important risk factors are also addressed. Incorporating a wider range of modifiable behaviors into educational efforts can promote a more holistic awareness and promote preventive action in many areas of cancer risk.

In response to this challenge, taking policy measures aimed at reformulating products, introducing taxation and marketing restrictions associated with ultra-processed foods, while promoting fresh or minimally processed foods, is becoming a key component of primary cancer prevention. Many countries have already implemented these aspects in their official dietary recommendations, adopting the precautionary principle. Such initiatives are not only at controlling the nutritional composition of the products consumed, but also at forming healthy eating habits in the population. Limiting access to ultra-processed foods can help reduce the incidence of risk factors associated with the development of cancer and other chronic diseases. These measures are an important step toward building more health-focused societies, while also following a prevention-based approach to public health.[17]

Incidence rates of breast, prostate, and colorectal cancers increase with socioeconomic development. Due to their high frequency, these cancers are significant contributors to the overall increase in cancer incidence rates worldwide. The increasing prevalence of these cancers, along with other Group A cancers, can be attributed to risk factors that become more common as countries progress from lower to higher levels of socioeconomic development. These development-related risk factors include smoking, alcohol consumption, overweight and obesity, physical inactivity, delayed fertility, and older age at the beginning of the pregnancy.[18]

To reduce the incidence of cancer among workers, preventive strategies must be implemented in high-risk workplaces. Effective prevention of occupational cancer requires a thorough understanding of carcinogenic substances. Like many other areas of healthcare care, occupational health has benefited from information technology solutions that improve prevention, early detection, treatment, and overall efficiency and cost-effectiveness with-

in the healthcare system. Information technology plays a crucial role in the healthcare field, especially in relation to occupational cancer. High-quality data on occupational cancer and its causes can help identify workers' groups at higher risk. This information is vital for policymakers, managers, physicians, patients, and researchers. As a result, vulnerable workers can undergo screenings and receive targeted preventive interventions.[19,20]

It should be emphasized that due to the cross-sectional nature of the study, no conclusions can be drawn regarding cause-and-effect relationships. The data collected only represent relationships observed at a single point in time and do not allow us to determine the direction of influence between variables.

Conclusion

Based on the results and discussion, several key conclusions can be drawn. The research conducted revealed a varied level of knowledge on health promotion behaviors among respondents, evident in differences related to both age and marital status. A significant issue is the lack of awareness of the role of physical inactivity and hygiene in the context of cancer risk. Interestingly, married individuals and widowers/widows less frequently in any physical activity compared to single individuals, among whom a higher number participate in intense physical activity (over 5 hours per week).

A positive aspect is that approximately half of the respondents are aware of the crucial impact of lifestyle on health and cancer risk. However, a similar proportion of respondents demonstrated low awareness of the link between viral infections and cancer development. Significant differences in the level of knowledge about testicular/abdominal self-examination were observed depending on marital status, with individuals currently or previously in a marital relationship showing greater knowledge in this area.

The findings unequivocally indicate an urgent need to strengthen primary prevention efforts. A considerable proportion of the respondents are unaware of the fundamental role of sports and regular physical activity in the prevention of cancer and promoting a healthy lifestyle. Similarly, awareness of the connection between certain cancers and viral infections preventable through vaccination is not sufficient. Taking effective action requires, first and foremost, intensifying education and raising public awareness across all age groups, from schoolchildren to seniors. Educational campaigns reaching a wide audience through schools, elderly care facilities, and the distribution of informational materials in public spaces have the potential to bring about significant change.

It is important to emphasize that raising awareness of cancer risk should focus on promoting a healthy lifestyle, regular screening, and limiting exposure to carcinogens, which is crucial to improve both prevention and treatment outcomes. Regular screenings and genetic tests for individuals at higher genetic risk enable early detection and intervention.

Effective cancer control requires collaboration across different medical fields, as well as public participation and support from health policies. Investments in prevention, both primary (promoting healthy lifestyles, vaccinations, education) and secondary (early detection), constitute an integrated approach to improving public health and reducing healthcare costs. Research on the impact of diet on cancer risk suggests that balanced nutrition may play a protective role, and further studies on gene-environment interactions, including food compounds like isoflavones, may provide valuable information for prevention and treatment.

Furthermore, it is necessary to develop and implement educational strategies tailored to specific demographic groups and their levels of knowledge and marital status, as observed differences suggest the need for personalized interventions. There is also a need to investigate the reasons for the lower levels of physical activity among married individuals and widows / widows and to develop programs encouraging these groups to engage in regular activity. Increasing emphasis on education on the role of viral infections in cancer development and the promotion of vaccination as an effective preventive tool is crucial. Consideration should be given to incorporating education on testicular and breast self-examination into standard medical consultations and information campaigns, taking into account the specific informational needs of men. The results suggest that the media coverage of cancer prevention may not be sufficiently comprehensive, and efforts should be made to provide more balanced information on all relevant risk factors and preventive methods.

These conclusions have significant implications for the formulation of public health strategies, highlighting the need for targeted and evidence-based educational initiatives and interventions that promote healthy lifestyles and regular screening to reduce cancer risk and burden on the healthcare system.

Declarations

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Author contributions

Conceptualization, A.A. and B.S.K.; Methodology, Kl.Z and Ka.Z.; Software, A.S. and P.S.; Validation, A.L., N.Z. and S.S. ; Formal Analysis, A.S.; Investigation, M.J.; Resources, S.S.; Data Curation, C.L., M.J. and J.F.; Writing – Original Draft Preparation, A.A.; Writing – Review & Editing A.A.; Visualization, A.A.; Supervision, B.S.K.; Project Administration, S.S.; Funding Acquisition, P.S.

Conflicts of interest

The authors declare that they have no conflict of interest.

Data availability

The data sets generated and/or necessary during the investigation are available from the authors.

Ethics approval

Our study obtained a positive opinion from the Bioethics Committee of Rzeszow University (Resolution number: 2022/099 date; 07 December 2022).

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ORIGINAL PAPER

Ultrasonography of the salivary glands in the diagnosis of Sjögren's syndrome secondary to rheumatoid arthritis – a probabilistic approach

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ABSTRACT

Introduction and aim. To evaluate the role of salivary gland ultrasound (SGUS) in differentiating rheumatoid arthritis (RA) patients with or without Sjogren's syndrome (SS) using a probability method and to study the relation between secondary SS (sSS) and RA disease characteristics.

Material and methods. One hundred RA patients with disease duration ≥5 years underwent detailed history taking, examination, routine laboratory testing, Schirmer's test, unstimulated salivary flow rate and SGUS of the 4 major salivary glands using Salaffi and Outcome Measures In Rheumatoid Arthritis Clinical Trials (OMERACT) scores.

Results. Patients sum with probabilities for sSS ≤20% and ≥80% were (39/100) before and (90/100) after SGUS with a highly significant difference ($p<0.001$).There was significantly more frequent carpal tunnel syndrome (CTS), longer RA disease duration and higher anti-cyclic citrullinated peptide antibodies (anti-CCP) in RA patients with sSS compared to those without($p<0.05$). There was highly significant agreement between Salaffi and OMERACT scores in gland evaluation by kappa test. The highest ultrasound OMERACT score of SG showed significant positive correlation with both Disease Activity Score-28-Erythrocyte Sedimentation Rate (DAS-28 ESR score) and ESR in RA patients.

Conclusion. Secondary SS is frequent in RA patients especially in association with longer disease duration, higher anti-CCP antibody titer and CTS. SGUS is a useful tool that helps diagnosing and grading the severity of SS in RA. SS severity correlates with RA disease activity.

Keywords. rheumatoid arthritis, salivary gland ultrasound, secondary Sjogren's syndrome

Introduction

Determining the exact prevalence of Sjogren's syndrome (SS) is very difficult as the diagnostic criteria frequently change. Although SS was looked upon as a rare disease in the past, it is now regarded as the second most common autoimmune disease after rheumatoid arthritis (RA).[1]

RA is the most common connective tissue disease associated with SS. RA may present with several extra-articular manifestations which might include sicca symptoms which are important for the diagnosis of secondary SS (sSS). However, other symptoms of SS are usually mild and uncommon.[2,3] RA patients with sSS have a worse course with higher morbidity and mortality than RA patients without sSS.[4]

Salivary gland ultrasound (SGUS) is an effective, easy, safe, non-invasive, and inexpensive tool to assess

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parotid and submandibular salivary gland parenchymal abnormalities accompanying SS.[5] Introduction of novel treatments necessitates finding tools for the early diagnosis of the disease.[6]

The most specific finding in ultrasound is the parenchymal inhomogeneity. Several US scores were developed considering other items as glandular size, posterior gland border, contour regularity, cyst size, echogenic bands, and hypoechogenic zones.[7]

Aim

This study aimed to evaluate the role of salivary gland US in differentiating RA patients with or without SS by using probability method and to study the relation between sSS and RA disease characteristics.

Material and methods

This cross-sectional study included 100 RA adult patients diagnosed according to the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) Classification Criteria for RA.[8] Disease duration of RA was 5 years or more. They were recruited randomly from the rheumatology outpatient clinic and inpatient internal medicine department, Ain Shams University Hospitals between January 2021 and January 2023. Patients with other connective tissue diseases, history of head and neck radiation, hepatitis viral infections, acquired immunodeficiency disease (AIDS), pre-existing lymphoma, sarcoidosis, graft versus host disease, use of drugs including anticholinergics, neuroleptics, antidepressants, antihypertensive drugs or those with known other primary or secondary salivary glands diseases were excluded from the study.

Ethics approval

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Ain Shams Univeristy in 2019 (FWA00017585).

For all patients detailed medical history and clinical examination with assessment of RA disease activity by Disease Activity Score 28 (DAS 28- ESR) were done.[9] Laboratory workup included: Complete blood count (CBC) measured on a Siemens ADVIA 2120i hematology analyzer (Siemens Healthcare diagnostic, Erlangen, Germany), erythrocyte sedimentation rate (ESR) by placing sample into sedimentation measurement stand (BD Seditainer™ Manual ESR BD), C-reactive protein (CRP) was measured quantitatively (0004956842190c501V9.0) by Immunoturbidimetric assay using Roche/Hitachi Cobas c systems, reference value <5mg/L, rheumatoid factor (RF, 0020764574322c501V8.0) was measured quantitatively by an immunoturbidimetric assay using Roche/Hitachi Cobas c systems, reference value <14 IU/mL and anti-cyclic citrullinated peptide (anti-CCP, 0511567100V4) was measured by electrochemiluminescence immunoassay "ECLIA", Roche diagnostic, GmbH, Mannheim, Germany, reference value <20 IU/mL. Anti-Ro (SSA) antibodies performed using ELISA for all patients to exclude SS/RA overlap with cutoff for positive value ≥20 IU/mL.[10] Schirmer's test without topical anesthesia was performed by placing The end of a special filter paper strip inside the lower eyelid of each eye. The eyes were closed gently for 5 minutes without rubbing, Both eyes were tested at the same time. The paper was removed after 5 minutes, its moisture was measured. A score < 10 mm in 5 minutes is accepted as normal. A score of less than 5 mm in 5 minutes indicates a tear deficiency[11] and unstimulated salivary gland flow rate test (USSFR) were performed by asking the patient to spit all saliva possible over 15 min without gustatory provocation. A volume of <1.5 mL of saliva in 15 min is considered abnormal.[12]

Salivary glands ultrasound (both parotids (PG) and submandibular (SMG)) was done using both qualitative and quantitative assessment by both Salaffi and Outcome Measures In Rheumatoid Arthritis Clinical Trials (OMERACT) scores.[13,14] All ultrasound scans were performed by 2 expert rheumatologists (the first and third authors) who were blinded to the clinical data to minimize bias and used 3–13 linear array transducer and E-Saote Mylab Six machine. Length and width were measured in the longitudinal and transverse planes for the parotid and SMG, and the surface area was computed as (length × width)/2, Normal parotid gland surface area is around 3–4 cm^2, normal SMG surface area around 1–2 cm^2.[15]

According to the Salaffi score, grade 0=normal homogeneous glands; grade 1=small hypoechogenic areas without echogenic bands; grade 2=multiple hypoechogenic areas measuring 2 mm with echogenic bands; grade 3=multiple hypoechogenic areas measuring 2–6 mm with hyperechogenic bands; and grade 4=multiple hypoechogenic areas measuring >6 mm or multiple calcifications with echogenic bands.[13]

According to the OMERACT score; grade 0=normal appearing SG parenchyma, grade 1=minimal change: mild inhomogeneity without hypo/anechoic areas, grade 2=moderate change: moderate inhomogeneity with focal hypo/anechoic areas, grade 3=severe change: diffuse inhomogeneity with hypo/anechoic areas occupying the entire gland surface.[14]

The patient was considered abnormal if at least one parotid or one SMG showed an US score of at least 1. Then the highest score for each gland and patient (Salaffi & OMERACT scores) was recorded. Also, the sum score (simple addition) of the four and the three glands scores (Salaffi & OMERACT scores respectively) for each patient was calculated.

The diagnostic role of US for SS in RA was evaluated by probability method depending on an expert rheumatologist evaluation before and after parotid and SMG US using 5 points scale with 80 % or more meaning very high probability and 20% or less meaning very unlikely.[16] Thus parameters for SS diagnosis were: 1) symptoms of dry eye, 2) dry mouth, 3) positive Schirmer's test and 4) abnormal USSFR, in 5) RA duration ≥ 5 years (long standing RA). For example those with RA more than 5 years, positive symptoms of dry eye and dry mouth, and abnormal both Schirmer's and USSFR tests were considered as having ≥ 80% probability of secondary SS before US . On the other hand, those with RA more than 5 years, positive symptoms of dry eye and dry mouth, and normal or borderline both Schirmer's and USSFR tests were considered as having 40-60% probability of secondary SS before US. After US showed abnormality, according the reported number and severity /grade of salivary glands abnormality in each patient, we increased the probability of SS by 20-40%. To our knowledge, despite commonly used in clinical practice and search work no proved validation was available from previous studies .

Ultrasound was also used to detect dominant hand metacarpophalangeal joints (MCP) erosions by using the Power Doppler (PD) ultrasound device MyLab^{TM}-Six (E-Saote company) with 6–18 MH probe, PD pulse repetition frequency was 500–750 Hz. The first through the fifth MCP joints of the dominant hands were scanned. Each joint was scanned in both the longitudinal and transverse planes from radial to ulnar sides on both volar and dorsal aspects. A definite ultrasound erosion was defined as a cortical "break" or defect with an irregular floor seen in longitudinal and transverse planes. The sizes of definite erosions were measured using electronic calibers and a semiquantitative scale (small erosion≤2 mm, moderate erosion=2–4 mm, and large erosion≥4 mm).[17] The sum of number of erosions per patient was recorded as erosion index and used to reflect the joint damage.

Statistical analysis: Data was coded and entered using statistical package SPSS version 2021 (IBM, Armonk, NY, USA). Data was summarized using number and percent for qualitative variables, mean±SD for quantitative variables. Comparisons between groups were done using Chi-square tests or Fisher exact tests when appropriate for qualitative variables. Comparison between quantitative normally distributed variables was done using independent sample T-tests, analysis of variance (ANOVA) with multiple comparison post Hok test. Comparison between quantitative variables which were not normally distributed were done using Kruskal Wallis test and Mann-Whitney test. Correlations were done to test for linear relation between variables. Kappa agreement measures were used to test for agreement between variables. p value ≤0.05 was considered as statistically significant.

Results

The commonest extra-articular manifestations of RA among the enrolled patients were carpal tunnel syndrome (CTS) in 62% of patients, rheumatoid nodules in 9%, generalized lymphadenopathy in 8%, and interstitial pulmonary fibrosis (IPF) in 6% of patients. All RA patients were on disease modifying antirheumatic drugs (DMARDS) (conventional, biologic and targeted synthetic), and steroids. Table 1 shows RA patients characteristics.

Table 1. Characteristics of the enrolled 100 RA patients*

Parameter n (%) or mean±SD (range)		RA patients (n=100)
Gender (F/M)		99/1
Age (years)		43.64±9.08 (25–65)
Disease duration (years)		11.3±4.89 (5–27)
No. swollen joints		0.93±1.94 (0–12)
No. tender joints		9.4±8.17 (0–28)
VAS (0–100)		50±25.51 (10–90)
DAS-28 ESR score		4.78±1.45 (1.6–7.3)
Remission (<2.6)		8
Low disease activity (2.6-3.2)		11
Moderate disease activity (>3.2-5.1)		36
High disease activity (>5.1)		45
ESR (1st hour in mm)		37.48±19.47 (8–100)
CRP (mg/L)		7.23±3.92 (2–22)
Rheumatoid factor (RF) (IU/mL)	Positive	96
		60.2±50.26 (8–328)
anti-CCP antibodies (IU/mL)	Positive	99
		130.75±79.74 (10–363)
WBCs (10^3 cells/mL)		6.68±2.27 (3.8–13.4)
Hb (g/dL)		11.2±1.19 (8–13.9)
PLT (10^3 cells/mL)		261.74±77.74 (122–436)
AST (U/L)		21.97±5.84 (12–40)
ALT (U/L)		17.98±5.14 (10–35)
Serum creatinine (mg/dl)		0.75±0.16 (0.4–1.2)

* No. – number, VAS – visual analogue scale, ESR – erythrocyte sedimentation rate, CRP – C-reactive protein, anti-CCP – anti-cyclic citrullinated peptide, WBCs – white blood cells, Hb – hemoglobin, PLT – platelets, AST – aspartate aminotransferase, ALT – alanine transaminase

By musculoskeletal US, erosions were detected at 111 out of 200 examined sites and in 48 out of 100 patients, 88.2% of these erosions were metacarpal versus 11.7% at proximal phalanx. Erosion index mean±SD was 1.13±1.66 with range 0–7. Fifteen/111 erosions (13.5%) were detected in first MCP joints in 100 RA patients, mostly radial, In second MCP joints, 47 erosions (42.3%) mostly radial, in third MCP joints, 14 erosions (12.6%) mostly dorsal and radial, in fourth MCP joints, 3 erosions (2.7%) mostly dorsal and in fifth MCP joints, 32 erosions (28.8%) mostly ulnar.

Regarding sSS,72% of the enrolled patients had SS positive parameters; 58% had dry eyes, 45% had dry mouth, 39% had positive Schirmer's test, 35% had abnormal USSFR. SS symptoms duration ranged from 0

to 3 years with mean of 0.92±0.75. Two patients only had just positive anti-Ro antibodies.

Using these parameters to propose a probability of sSS in RA before and after SGUS, the results were as follows: Before SGUS the probability of sSS was ≤20% (highly unlikely) in 26% of patients, ≥80% (highly likely) in 13% of patients, 20-40% in 26% of patients, 40-60% in 21% of patients and 60-80% in 14% of patients. After SGUS, the probability of sSS was ≤20% in 51% of patients, ≥80% in 39% of patients, 20-40% in 5% of patients, 40-60% in 1% of patients and 60-80% in 4% of patients. Tables 2 and 3 show SGUS findings in 100 RA patients and Kappa agreement test between Salaffi and OMERACT scores of 4 salivary glands.

Table 2. Salivary gland ultrasound findings among the enrolled 100 RA patients

Parameter n (%) or mean±SD		Left parotid	Right parotid	Left submandibular	Right submandibular
Contour	Regular	99	99	97	96
	Irregular	1	1	3	4
Inhomogeneity	Positive	27	27	32	29
	Negative	73	73	68	71
Echogenic bands	Positive	27	23	10	9
	Negative	73	77	90	91
Posterior border	Visible	95	97	98	98
	Invisible	5	3	2	2
Surface area (mm^2)		10.28±2.99	9.79±2.88	9.79±2.88	4.8±1.09
Spot size (mm)		1.68±0.75	1.87±1.32	1.87±1.32	1.33±0.58
Salaffi score	Normal (0)	80	77	72	73
	Abnormal	20	23	28	27
	1	1	1	2	3
	2	10	11	18	17
	3	9	11	8	7
	Total score	0.48±1	0.56±1.07	0.62±1.04	0.58±1.01
OMERACT score	Normal (0)	73	73	68	71
	Abnormal	27	27	32	29
	1	9	6	7	5
	2	17	17	23	22
	3	1	4	2	2
	Total score	0.46±0.81	0.52±0.92	0.59±0.91	0.55±0.90

The kappa agreement test between the Salaffi and OMERACT scores showed a highly significant agreement between the 2 scores in detection of normal and abnormal glands (Table 3). Both scores detected normal glands in 57 patients and abnormal glands in 39 patients. However, there was a disagreement between the two scores in only 4 RA patients, in whom abnormality was detected by OMERACT score only. Regarding the left parotid gland, both scores detected 73 normal and 20 abnormal glands, with 7 glands recorded as abnormal by OMERACT score only. Also in the right parotid gland, both scores detected 73 normal and 23 abnormal glands, with 4 glands recorded as abnormal by OMERACT score only. As for the left submandibular gland, both scores detected 68 normal and 28 abnormal glands, with 4 glands recorded as abnormal by OMERACT score only. In right submandibular gland, both scores detected 71 normal and 27 abnormal glands, with 2 glands recorded as abnormal by OMERACT score only.

Table 3. Ultrasound findings of all four salivary glands and Kappa agreement test between Salaffi and OMERACT scores of 4 salivary glands

		n (%) or mean±SD (range)
Normal/abnormal patient by Salaffi score	Normal	61
	Abnormal	39
Normal/abnormal patient by OMERACT	Normal	57
	Abnormal	43
Highest Salaffi score	0	61
	1	3
	2	15
	3	21
	0.96±1.27 (0–3)	
Highest OMERACT score	0	57
	1	7
	2	29
	3	7
	0.86±1.06 (0–3)	
Sum scores of 4 glands per patient by Salaffi score		2.22±3.35 (0–12)
Sum scores of 4 glands per patient by OMERACT score		2.10±2.93 (0–10)
No. of glands affected per patient		1.13±1.45 (0–4)
	0	57
	1	3
	2	22
	3	6
	4	12

Kappa agreement test between Salaffi and OMERACT scores of 4 salivary glands

		RA patients by Salaffi score		p	Kappa
		Abnormal n (%)	Normal n (%)		
RA patients by OMERACT score	Abnormal	39 (100)	4 (6.6)	<0.001	0.9
	Normal	0	57 (93.4)		

Patients with probabilities for SS ≤20% and ≥80% were considered certain or near certain for secondary SS and their sum before SGUS was 39/100 and after SGUS was 90/100, with a highly significant difference ($p<0.001$). On the other hand, patients with probabilities 40–60% were considered uncertain or vague; and were recorded in 21/100 of patients before SGUS and in 1/100 of patients after SGUS with a highly significant difference ($p<0.001$), as shown in Figure 1.

There was a statistically significant more CTS, longer RA disease duration and higher anti-CCP antibody titer in RA patients with secondary SS compared to those without secondary SS ($p<0.05$). Also, there was statistically highly significant higher percentage of RA patients with secondary SS who had longer SS symptoms duration, dry eye, dry mouth compared to RA patients without secondary SS ($p<0.001$), as shown in table

4. None of RA patients without SS had positive Schirmer's test or abnormal USSFR. No significant difference between the 2 groups in surface area of the 4 salivary glands measured by US (p>0.05), data not shown.

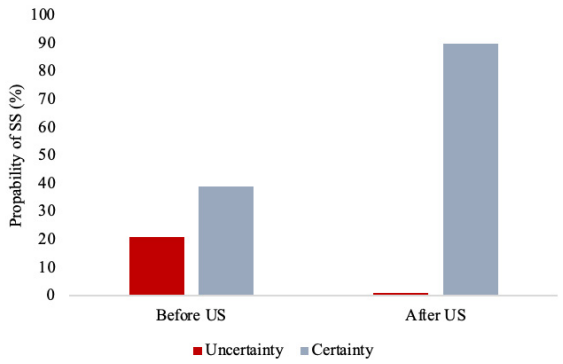


Fig. 1. Comparison between percent of RA patients in area of certainty (with probabilities for SS ≤20% and ≥80%) and uncertainty (with probabilities for SS 40–60%) before and after SGUS

Table 4. Comparison between RA patients with and without secondary SS after SGUS

RA parameters n (%) or mean±SD		RA patients without SS (probability ≤20%) (n=51)	RA patients with SS (probability ≥80%) (n=39)	P
RA disease duration (years)		10.35±4.82	12.36±4.61	0.02
anti-CCP antibody (IU/mL)		114.71±74.99	154.66±79.65	0.01
SS symptoms duration (years)		0.47±0.5	1.46±0.72	< 0.001
Dry eye		20 (39.2%)	29 (74.4%)	<0.001
Dry mouth		14 (27.5%)	29 (74.4%)	<0.001
Schirmer's test	Positive	0 (0%)	32 (82.1%)	< 0.001
	Negative	51 (100%)	7 (17.9%)	
USSFR	Positive	0 (0%)	32 (82.1%)	< 0.001
	Negative	51 (100%)	7 (17.9%)	
Carpal tunnel syndrome	Positive	23 (45.1%)	32 (82.1%)	< 0.001
	Negative	28 (54.9%)	7 (17.9%)	

Despite higher percentage of RA patients with secondary SS compared to those without secondary SS as regards active RA (94.9% versus 88.2%), positive RF (97.4% versus 94.1%), ESR (mean ,42.03 versus 34.24), CRP (mean, 7.8 versus 6.9 mg/L) and presence of erosions (46.2% versus 43.1%), differences did not reach statistical significance. Also, there was no statistically significant difference between RA patients with and without secondary SS as regards age, gender, disease activity, number of swollen joints, number of tender joints, VAS, DAS 28-ESR, RF titre, anti-CCP antibody positivity, drug intake including steroids and erosion presence or index (p>0.05) (data not shown).

There was a statistically significant difference between RA patients with different grades of disease activity measured by DAS28-ESR score as regards right PG OMERACT score being highest in patients with high disease activity, p=0.04.

Table 5. Correlation between mean of DAS28-ESR score, ESR, CRP and salivary glands US scores (Salaffi and OMERACT) in 100 RA patients*

	DAS28-ESR score		ESR		CRP	
	r	p	r	p	r	p
Left PG Salaffi score	0.07	0.43	0.1	0.2	0.1	0.08
Left PG OMERACT score	0.15	0.11	0.1	0.1	0.1	0.2
Right PG Salaffi score	0.2	0.04	0.1	0.09	0.2	0.04
Right PG OMERACT score	0.2	0.01	0.1	0.06	0.2	0.02
Left SMG Salaffi score	0.06	0.5	0.08	0.4	0.02	0.8
Left SMG OMERACT score	0.05	0.56	0.1	0.2	0.06	0.5
Right SMG Salaffi score	0.1	0.07	0.1	0.07	0.03	0.7
Right SMG OMERACT score	0.1	0.1	0.1	0.1	0.05	0.5
Highest Salaffi score	0.1	0.1	0.1	0.07	0.1	0.3
Highest OMERACT score	0.1	0.04	0.2	0.02	0.1	0.1
Sum scores of 4 glands per patient by Salaffi score	0.1	0.1	0.1	0.08	0.09	0.3
Sum scores of 4 glands per patient by OMERACT score	0.1	0.06	0.1	0.06	0.1	0.08
No. of glands affected per patient	0.1	0.07	0.1	0.09	0.1	0.2

* No. – number, ESR – erythrocyte sedimentation rate, DAS-28 – Disease Activity Score 28, CRP – C-reactive protein, PG – parotid gland, SMG – submandibular gland

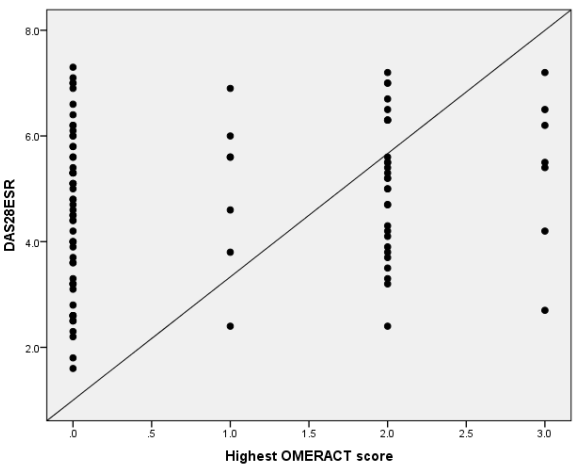


Fig. 2. Correlation between highest OMERACT score and disease activity measured by DAS28-ESR in 100 RA patients

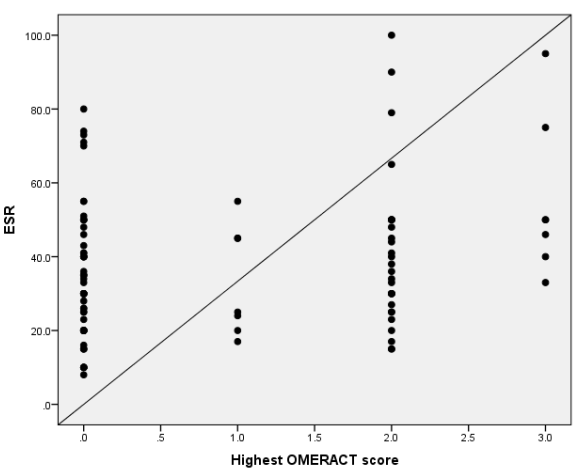


Fig. 3. Correlation between highest OMERACT score and ESR in 100 RA patients

A higher percentage of RA patients with active disease had dry eye, dry mouth, longer SS mean symptoms duration, positive Schirmer's test and abnormal USSFR. However, values did not reach statistical significance,($p>0.05$) (data not shown).

Table 5 and Figures 2 and 3 show correlations between mean of DAS28-ESR score, ESR, CRP and salivary glands US scores (Salaffi and OMERACT) in 100 RA patients.

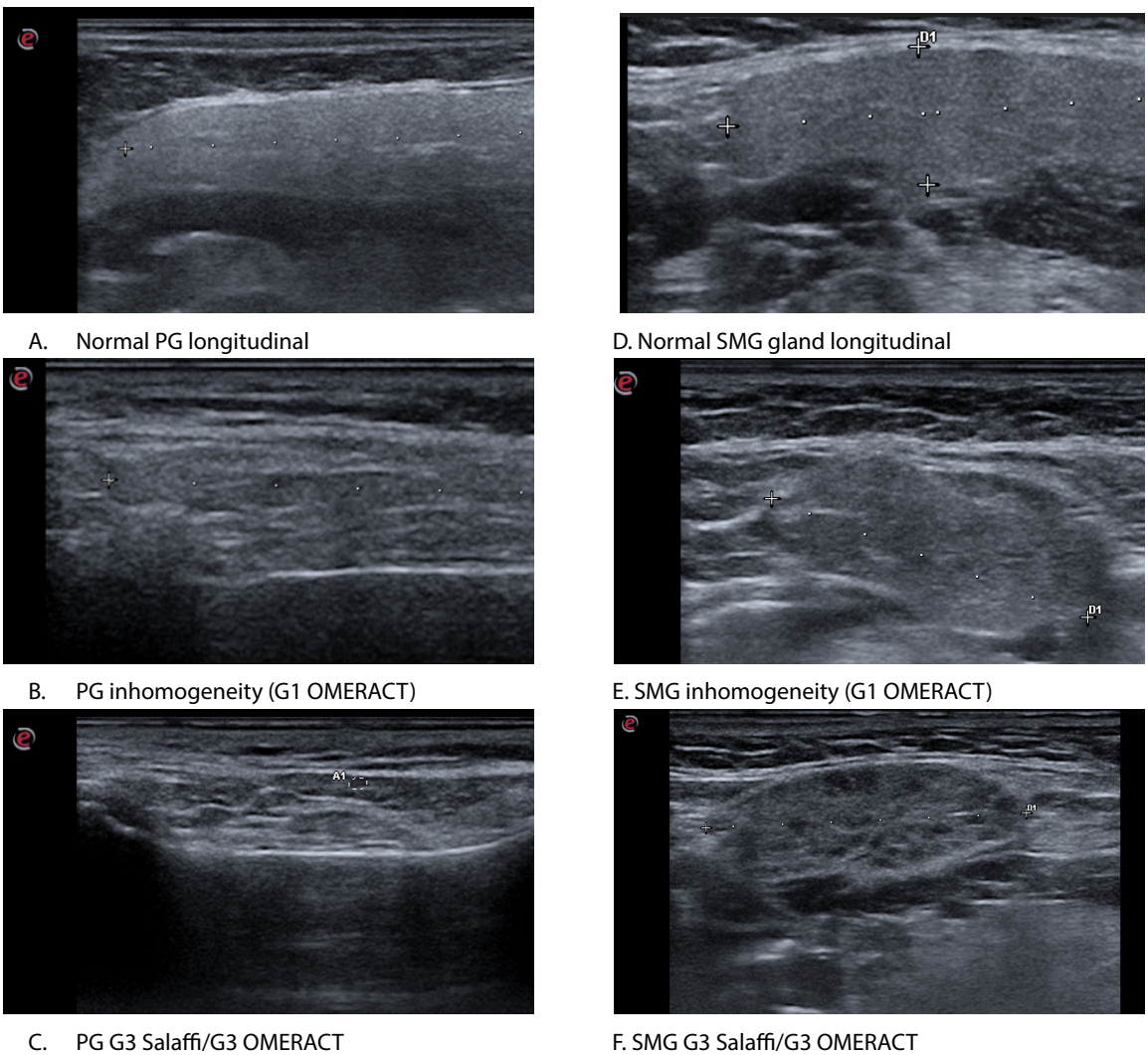
According to major salivary glands ultrasound, Figure 4 shows US images of normal PG, SMG, and abnormal glands by Salaffi and OMERACT scores.

Discussion

Based upon the recent 2016 ACR/EULAR classification criteria for SS, diagnosis of SS depends largely on the presence of anti-Ro antibodies and minor salivary gland biopsy which is invasive and inconvenient.[18] New modalities are required to support SS diagnosis. SGUS of the major salivary glands is very promising given its wide availability, ease, rapidity, reproducibility, and safety being nonivasive with no radiation exposure.[19] Typical SGUS findings in SS are oval anechoic or hypoechoic lesions with hyperechogenic bands.[20]

In the current study there was female predominance (F:M ratio=99:1), their mean ages were 43.64±9.08 years. Similar demographic data were found by Nawata et al.[21] (65.3% females and 34.7% males), Rabault et al.[22] (81% females and 19% males) and Sparks et al. (82% females and 18% males).[23] As for the mean ages, similar results were found by Naseri et al. (49.46±11.81), Hammer et al. (53.3±13.2), and Hajiabbasi et al. (48.3±13).[2,24,25] These findings reflect the already established epidemiological incidence of RA in middle aged females.

Using two US semiquantitative scores; Salaffi and OMERACT to detect the presence and grade the severity of salivary glands involvement by SS, 39% of RA patients were recorded to have at least one abnormal gland by Salaffi versus 43% by OMERACT.[13,14] Furthermore, there was a highly significant agreement between the 2 scoring systems with more abnormal glands detected by OMERACT scoring system, reflecting its better sensi-



A. Normal PG longitudinal

D. Normal SMG gland longitudinal

B. PG inhomogeneity (G1 OMERACT)

E. SMG inhomogeneity (G1 OMERACT)

C. PG G3 Salaffi/G3 OMERACT

F. SMG G3 Salaffi/G3 OMERACT

Fig. 4. Longitudinal U/S images of A: normal PG, and B: SMG, and D: abnormal glands by Salaffi and OMERACT scores (B, C, E, and F)

tivity for early diagnosis of salivary glands abnormality.

In Salaffi et al., among the 77 patients with SS, 66 (85.7%) had abnormal US findings by Salaffi score.[26] The findings of parotid and SMG were in concordance with each other and were equally frequent with sensitivity of 75.3% and specificity of 83.5%. In Rabault et al., among 98 patients in their study with assessment of SGUS using OMERACT score, they found that 22.5% had at least one salivary gland scored grade 1, 7.1% had at least one salivary gland scored grade 2 and only one patient 1% had a parotid gland scored 3.[22] In Robin et al., 88% of the patients with abnormal SGUS by OMERACT score met the ACR/EULAR 2016 and American-European Consensus Group (AECG) 2002 classification criteria for the diagnosis of SS with sensitivity of 52.4% and specificity of 90%.[27] Zabotti et al., found that SGUS OMERACT score was reliable for the major salivary glands evaluation in SS, even if carried out by properly instructed less-expert sonographers.[28]

In our study, before US, the number and percent of RA patients with high likelihood of having SS (≥80% probability) and not having SS (≤20% probability) together were considered certain, they were 39% which increased after SGUS to 90% with high statistically significant difference. On the other hand, the percent of RA patients with undetermined /vague probability of having SS (40-60% probability) decreased from 21% to 1% after SGUS with high statistically significant difference making SGUS a valuable tool for diagnosis and screening of SS.

In agreement, Elghamry et al. stated that SGUS is a very helpful tool in detection of SG abnormalities in patients with RA and SS. Jousse-Joulin et al. found that SGUS had similar weight compared to minor items (anti-Ro, positive Schirmer's test, dry mouth and salivary flow rate<0.1 mL/min) and its addition improves the performance of the 2016 ACR/EULAR classification criteria for SS diagnosis.[29,30]

Zabotti et al. and El-Barbary et al., found SGUS of RA patients to be a reliable, promising tool that could be used as a practical noninvasive and sensitive technique for early detection of pathological changes for sSS in RA patients.[28,31] Theander and Mandl reported that, SGUS using a simplified score for assessment of parenchyma inhomogeneity was highly specific for SS and offered the advantage of identifying patients with severe disease or at risk of lymphoma.[32]

Furthermore, Takagi et al. stated that incorporating the SGUS criteria as an alternative to one of the three ACR classification items achieved 91% sensitivity, 96% specificity, which was comparable to that of the original ACR classification for diagnosing SS.[33]

Several studies have compared SGUS with other established tests for the diagnosis of salivary gland affection in SS. Milic et al., stated that SGUS findings could replace sialoscintigraphy in American European classification criteria (AECC) for the diagnosis of SS. Shimizu et al., found that SGUS could replace sialography for SS screening.[34,35] In addition, El Miedany et al. found that the SGUS findings agreed well with the MRI findings, concluding that SGUS could replace MRI as a routine diagnostic test.[36] In 1013, Ali et al., in their study on 196 patients, reported a significant correlation between US scores of major salivary glands and focal scores of minor salivary glands biopsy in Sjogren's syndrome group and the sialadenitis group suggesting a uniform disease process. This correlation was absent for the non-salivary gland disease group.[37] To our knowledge, no previous studies utilized probability method in diagnosis of SS, however, they used SGUS in comparison to sialography, biopsy or other methods.

In the current study, there was a significant association between presence of SS and longer RA duration, presence of CTS and higher anti-CCP antibody titer. Despite higher percent of RA patients with active disease, higher ESR and CRP in RA patients with SS compared to those without SS, values didn't reach statistical significance. These findings partially agree with El-Barbary et al. who found that the duration of RA in patients with SS was longer than patients without SS but no significant difference between RA patients with and without SS regarding anti-CCP titer, DAS28-ESR score, ESR and CRP levels.[31]

Haga et al. found no significant difference between RA patients with and without SS regarding RA disease duration, anti-CCP titer, DAS28-ESR score, ESR and CRP levels.[38] In Antero et al., they found no relation between the presence of sSS and RA disease activity (p=0.31) or RA duration (p=0.95).[39] In Lafitte et al. only 4 out of 25 SS patients had CTS.[40]

We compared RA patients with active disease with those in remission and compared RA patients with different grades of disease activities, only significantly higher mean right parotid gland OMERACT score was reported in patients with high disease activity. The small number of RA patients in remission (8) may account for this. However, this may reflect an association between RA disease activity and SS severity especially in the right parotid gland.

Similarly, Das et al. found that RA patients with changes on SGUS had higher disease activity scores than those without changes.[41] However, in Haga et al. there were no significant differences between RA patients with and without sSS regarding disease activity measured by DAS28.[38]

In the present study, the association between RA disease activity and SS severity in SG was confirmed by the presence of a significant correlation between right parotid gland scores by both Salaffi and OMERACT (severity of SS) and DAS-28 ESR score and CRP (RA activity). Furthermore, there was a significant positive

correlation between DAS28-ESR and ESR and the highest OMERACT score in RA patients. In agreement, Rabault et al., reported a significant correlation between RA disease activity (by DAS28 score) and SGUS abnormalities according to OMERACT scoring system.[22] However, in Elghamry et al., there was no significant correlation between RA disease activity (by DAS28 score) and SGUS severity of SS in RA patients with sSS that may be explained by low disease activity by DAS28 score in RA patients in their study.[29]

In this work, a higher percentage of RA patients with active disease had dry eye, dry mouth, longer SS mean symptoms duration, positive Schirmer's test and abnormal USSFR. However, values did not reach statistical significance. This agrees with Haga et al., who found no significant correlation between DAS-28 score and sicca symptoms or the presence of SS.[38] Also, Fujita et al. found that RA activity had no significant association with the presence of dry eye.[42] On the other hand, Uhlig et al. found that reduced tear or saliva production were related to RA disease activity.[43]

In the current study, there was no significant relation between RA disease severity (as reflected by positivity and mean RF titer and by presence of and the mean erosion index) and any of SS related parameters. In agreement, Uhlig et al. found no significant relation between RA disease severity (RF positivity or joint erosions and deformity) and sicca symptoms.[43] Haga et al., found that the concentration of RF was higher in RA patients without versus with SS, but not being significant.[38]

Study limitations: Limitations to this work include that: although OMERACT scoring system proved superiority in detection of more abnormal glands with just inhomogeneity, hence helping early diagnosis of SS, its use may result in overestimation and over diagnosis especially in the submandibular gland where it showed confusing images due to similar homogeneity with the surrounding structures.[44] To overcome this, OMERACT scoring system should be included within a diagnostic algorithm in clinical practice. Our proposed diagnostic algorithm for secondary SS in RA includes: RA duration ≥5 years (1 point), dry eye (1 point), dry mouth (1 point), +ve Schirmer test (3 points), abnormal USSFR test (3 points), OMERACT score by ultrasound (sum scores for 4 glands, range 0–12), with total score 0–21, to be tested for validity and the threshold cutoff score for diagnosis of secondary SS in RA. Also, we used probability method to propose the diagnosis of SS in RA patients which although it depends on Clinical judgement (derived from clinician's diagnostic skill and clinical experience, draws from memory of encountered cases, instinctive, fast, always available) and clinical prediction rules (quantifies the relative importance of various clinical data points when evaluating a patient for a specific disease, facilitates identifying which information is important to obtain and helps in the interpretation of the clinical information), however, it has limitations that it is dependent on clinician's diagnostic skill and experience, prone to cognitive biases with high variability of estimates from clinician to clinician compared to deterministic method with gold standard.[45] This was part of our objectives to avoid invasive diagnostic tool (SG biopsy) by adding the value of salivary gland ultrasound.

Conclusion

RA is frequently associated with sSS especially in cases with longer disease duration, higher anti-CCP antibody titer and CTS. SGUS is a useful tool that helps diagnosis and grading of severity of SS in RA. Ultrasound OMERACT score proved superiority in detection of salivary gland abnormality allowing early diagnosis and is better correlated with disease activity compared to Salaffi score.

Declarations

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Author contributions

Conceptualization, M.M.A.G.; Methodology, M.M.A.G. and C.S.M. ; Software, M.M.A.G., C.S.M. and M.A.E.A.; Validation, M.M.A.G.; Formal Analysis, M.M.A.G. and C.S.M.; Investigation, M.M.A.G., C.S.M. and M.A.E.A.; Resources, M.M.A.G. and A.A.M.K.; Data Curation, C.S.M. and R.M.H.; Writing – Original Draft Preparation, M.M.A.G. and C.S.M.; Writing – Review & Editing, M.M.A.G., C.S.M., and S.A.E.B.; Visualization, S.A.E.B. and R.M.H.; Supervision, M.M.A.G.; Project Administration, M.M.A.G.

Conflicts of interest

The authors declare no competing interests.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Ain Shams Univeristy in 2019 (FWA00017585).

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ORIGINAL PAPER

Evaluation of corin and copeptin as novel biomarkers for polycystic ovary syndrome – diagnostic accuracy and associations with cardiometabolics

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ABSTRACT

Introduction and aim. Polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects 5–18% of women of reproductive age and is intricately linked to metabolic dysfunction and cardiovascular risks. This study aimed to evaluate the diagnostic utility of copeptin and corin as potential biomarkers in PCOS and their association with cardiometabolic risk factors.

Material and methods. This case-control study included 60 women diagnosed with PCOS (Rotterdam criteria) and 30 healthy controls. Serum levels of copeptin and corin and metabolic parameters were measured using enzyme-linked immunosorbent assay kits. Statistical analyses included receiver operating characteristic (ROC) curves, logistic regression, and correlation tests.

Results. The results revealed significantly elevated corin (1450.23±264.91 vs. 619.17±159.19 pg/mL, $p<0.001$) and copeptin levels (5.81±1.66 vs. 2.46±0.64 ng/mL, $p<0.001$) in patients with PCOS compared to controls. Both biomarkers were strongly correlated with insulin resistance (HOMA-IR: $r=0.648$ for corin and $r=0.750$ for copeptin) and dyslipidemia. ROC analysis demonstrated exceptional associative biomarker precisions for corin (AUC=1.00) and copeptin (AUC=0.89). Univariate regression identified corin (odds ratio [OR]=1.018) and copeptin (OR=1.344) as independent predictors of PCOS.

Conclusion. This study identified plasma corin and copeptin levels as potential biomarkers for PCOS diagnosis and risk stratification. Elevated corin levels predict infertility, while copeptin levels correlate with metabolic dysfunction, particularly in obese, insulin resistant phenotypes.

Keywords. copeptin, corin, dyslipidemia, insulin resistance

Introduction

Polycystic ovarian syndrome (PCOS) is one of the most prevalent endocrinopathies, affecting 5 to 18% of women worldwide, with manifestations spanning the reproductive, metabolic and psychological domains.[1] PCOS is characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology, and arises from a complex interplay of genetic susceptibility, epigenetic modifications, hypothalamic-pituitary-ovarian axis dysregulation, and metabolic disturbances.[2] The Rotterdam criteria, first published in 2003 and updated in 2023, continue to provide the fundamental diagnostic framework for PCOS. Diagnosis requires the presence of at least two of the following three characteristics: clinical or biochemical hyperandrogenism, oligomenorrhea or amenorrhea (indicating ovulatory dysfunction), and

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polycystic ovarian morphology (PCOM) confirmed by ultrasound or elevated levels of anti-Müllerian hormone (AMH) levels.[3]

The prevalence of PCOS ranges from 6% to 18% and is influenced by diagnostic criteria (NIH, Rotterdam or Androgen Excess-PCOS Society) and population characteristics.[4] The Rotterdam criteria, when applied broadly, identified four phenotypes with distinct metabolic and reproductive risks. Phenotype A (hyperandrogenism + ovulatory dysfunction + polycystic ovaries) represents more than 60% of cases and correlates with severe metabolic dysfunction, while phenotype D (ovulatory dysfunction and polycystic ovaries) exhibits milder manifestations.[5] Geographical disparities exist, with a higher prevalence in South Asian and Middle Eastern populations, likely due to genetic admixture and environmental factors.[1] The prevalence of infertility has been reported to be 70–80% in PCOS.[6]

PCOS is a complex endocrine metabolic disorder characterized by intersecting pathways of insulin resistance, chronic inflammation, and androgen excess with significant clinical heterogeneity in all phenotypes.[7] PCOS exhibits a complex bidirectional relationship with metabolic dysfunction, characterized by insulin resistance in 65–80% of lean and 95% of obese individuals, regardless of body mass index (BMI).[8] Approximately 50-80% of affected women exhibit insulin resistance due to defects in post-receptor signaling defects in skeletal muscle and adipose tissue, characterized by excessive serine phosphorylation of insulin receptor substrate-1 (IRS-1), which affects GLUT4 translocation and glucose uptake.[9] This metabolic dysfunction coexists with dyslipidemia in 40-55% of cases and android obesity in 52–64% of patients, creating a pro-inflammatory environment marked by elevated tumor necrosis factors α, interleukin 6, and oxidative stress, even in lean individuals.[10] Early diagnosis remains critical given the association between PCOS and a 4-fold increased risk of type 2 diabetes and a 2-fold increase in cardiovascular events, compounded by a 28–57% prevalence of anxiety/depression.[11]

Metabolic syndrome (MetS) affects 33–46% of patients, with abdominal obesity and insulin resistance exacerbating ovarian dysfunction through adipose-derived cytokine signaling and altered steroidogenesis.[12] MetS, defined as a group of conditions including insulin resistance, abdominal obesity, hypertension, and dyslipidemia, has become a major health risk in society. This syndrome negatively affects reproductive function and leads to ovarian dysfunction and other hormonal disorders that can increase infertility and menstrual irregularities.[13,14]

Copeptin, a glycosylated 39-amino acid peptide derived from the C-terminal segment of pre-provasopressin (preproAVP), has emerged as a promising biomarker for PCOS. As a cleavage product of preproAVP, copeptin is synthesized along with arginine vasopressin (AVP) and neurophysin II during transport from the hypothalamus to the posterior pituitary. Although AVP plays a central role in fluid homeostasis, vascular tone regulation, and endocrine stress responses, it also serves as a stable surrogate marker for AVP due to its equimolar secretion and stability in circulation.[15]

Insulin resistance is a central feature of PCOS, affecting up to 70% of patients, regardless of obesity status. Copeptin has been implicated in metabolic dysfunction through its association with AVP-mediated pathways that influence glucose homeostasis and lipid metabolism.[16] Serum copeptin levels correlate with systolic and diastolic blood pressure, in addition to various factors associated with metabolic syndrome, including obesity and hypertriglyceridemia. Women with PCOS may exhibit elevated copeptin levels compared to those without the condition. One of the risks of PCOS is an increased likelihood of developing type 2 diabetes mellitus. Copeptin serves as a novel biomarker of clinical significance in patients with PCOS, potentially enhancing the identification of diabetes susceptibility.[17]

Corin is a transmembrane serine proteinase (EC 3.4.21) with a complex molecular architecture essential for its enzymatic function and cellular localization. Human corin is a unique mosaic protein consisting of an N-terminal cytoplasmic tail, a transmembrane domain, and an extensive extracellular region containing two frizzled-like domains (Fz1 and Fz2), eight low-density lipoprotein (LDL) repeats (LDLR), a scavenger receptor (SR) domain, and a C-terminal serine protease domain harboring the catalytic triad of histidine, aspartic acid, and serine residues.[18] This exceptionally large protein comprises 1042 amino acids with 19 predicted N-linked glycosylation sites in its extracellular region, conferring an apparent molecular mass of approximately 150-200 kDa in SDS-PAGE analysis. The human corin gene is located on chromosome 4p12, spanning over 200 kb with 22 exons, making it one of the largest protease genes identified to date.[19] Structurally, the LDLR8 module contains a critical DSSDE motif that regulates specific apical trafficking in polarized epithelial cells, highlighting the sophisticated cellular machinery that governs corin localization and function.[20] The insulin resistance characteristic of PCOS likely influences corin expression and activity through complex endocrine and metabolic pathways. Dysfunctional adipose tissue signaling and altered natriuretic peptide profiles contribute to cardiovascular risk in patients with PCOS, potentially mediated by aberrant corin-ANP interaction.[19]

Aim

To evaluate the diagnostic precision of plasma corin and copeptin as potential new biomarkers of PCOS, we assessed their association with cardiometabolic risk factors (insulin resistance, dyslipidemia, and obesity).

Material and methods

A total of 60 samples were collected from women of reproductive age (20-43 years) during their visits to the gynecological departments of the Al Zahraa Teaching Hospital and the Fertility Center of the Al Sadr Medical City from October 1, 2024, to January 24, 2025. These women were diagnosed with PCOS by gynecologists according to Rotterdam criteria. Demographic data, laboratory results, family history, and blood pressure data were collected using a structured questionnaire. The inclusion criteria required that participants be married and meet Rotterdam's diagnostic criteria, while the exclusion criteria excluded women with a history of smoking, chronic diseases (eg, diabetes, autoimmune diseases, thyroid disorders, hypertension, cardiovascular disease, chronic renal failure, or malignancies), or use of medications such as lipid-lowering agents, ovulation stimulants, corticosteroids, or antidiabetic medications. In addition, a control group of 30 healthy and fertile women aged 20 to 43 years was selected with no history of smoking, regular menstrual cycles, normal ovarian morphology, or underlying medical conditions, as confirmed by gynecological evaluation. Both groups were matched for age and reproductive status to ensure comparability. Before sample collection, all research participants were notified and each gave their verbal consent to be obtained from each participant. On 1 October 2024, document number 5578 stated that the local ethics commission of the College of Health and Medical Techniques of Al-Furat Al-Awsat Technical University, Al-Kufa, Iraq reviewed and approved the research protocol, consent form, and subject data.

Sample collection

Samples were obtained from women diagnosed with PCOS and from healthy controls without endocrine or metabolic disorders. Five milliliters of venous blood was collected on the second day of the menstrual cycle and transferred to gel tubes for serological and immunological tests. An enzyme-linked immunosorbent assay (ELISA) kit was used to conduct the experiment: a human copeptin ELISA kit from BT Laboratory Cat. No E1129Hu and a Human Corin (CRN) ELISA kit from Sunlong Catalog Number: SL1938Hu. ELISA kits (ELabscience, USA) were used to measure fasting insulin (FINS) and a fluorescent immunoassay (Minividas, Biomerieux, France) was used to measure luteinizing hormone (LH) VIDAS®REF30 407-01, follicle stimulating hormone (FSH) VIDAS REF30 40601 H, and total testosterone levels. Insulin resistance (IR) Determination of human insulin level Cat. No: E-EL-H2665 was estimated using the homeostatic model assessment of insulin resistance (HOMA-IR) according to the formula: HOMA-IR=(fasting insulin [μU/mL]×fasting glucose [mg/dL])/405. According to the World Health Organization, overweight and obesity are defined based on body mass index (BMI), with overweight defined as BMI <25 kg / m^2 and obesity as BMI ≥30 kg/m^2.[21]

Blood lipid level analysis: These tests rely on enzyme-based colorimetric methods and are performed using a spectrophotometer. The test uses: enzymatic conversion: specialized enzymes break down lipids in the sample, ultimately producing hydrogen peroxide. Color formation: hydrogen peroxide reacts with special reagents, aided by the enzyme peroxidase, to form a colored dye. Measurement: The optical absorbance meter measures the intensity of this color at a wavelength of 500 nanometers, where the intensity is directly proportional to the lipid concentration. The concentration is calculated by comparing the absorbance to a standard of known concentration. Main analysis methods: total cholesterol (TC) and triglycerides (TG): The serum sample is mixed directly with the enzyme reagents, then incubated, and the light absorbance of the resulting dye is measured. The high-density lipoprotein (HDL): this involves a preliminary step of precipitating other lipoproteins (such as LDL) from the serum. The remaining HDL in the clear fluid is then analyzed using the same enzymatic colorimetric method. LDL cholesterol: This is not measured directly but is calculated using the Friedewald equation.

LDL (mg/dL)=total cholesterol - HDL - (triglycerides/5)which is based on the results of total cholesterol, HDL, and triglyceride (reference values: normal <100 mg/dL, suspected 150 mg/dL, elevated 190 mg/dL).

Statistical analysis

All data were tested for normality. For data comparison, an independent t-test was applied, while the chi-square test was used when appropriate. was analyzed using the receiver ROC curve analysis. The Pearson correlation test was used to evaluate associations between parameters related to PCOS characteristics. Univariate logistic regression was used to assess the predictive capacity of biomarkers. Statistical analyses were performed using SPSS v.28 (IBM, IL, USA), and statistical significance was set at $p<0.05$.

Results

This study compared the demographic and clinical characteristics of PCOS individuals and healthy controls (Table 1). The mean age of PCOS patients (28.15 years) was marginally higher than that of controls (27.6 years), and the difference was not statistically significant ($p=0.663$). However, PCOS patients exhibited a significantly elevated mean BMI ($p = 0.004$), 33.3% classified as obese compared to 10% of controls, highlighting metabolic disparity. Hyperandrogenism-related clinical markers were markedly prevalent in PCOS, including hirsutism (86.7% vs. controls, $p<0.0001$) and menstrual irregularities (80% vs. 6.7% regular cycles in controls, $p<0.0001$), aligning with diagnostic criteria. In partic-

ular, 56.7% of PCOS patients were nulliparous versus none of the controls (p<0.0001).

Table 1. Demographic information on POCOS and the healthy women groups*

Demographic features	Categories	Studied groups: PCOS patients n=60		Healthy control n=30		p
		n	%	n	%	
Age (year)	Mean±SD	28.15±5.96		27.6±4.93		0.663[a]
	≤25 years	22	36.7	10	33.3	X^2=0.097
	>25 years	38	63.3	20	66.7	0.755[b]
BMI (kg/m²)	Mean±SD	28.42±4.44		26.14±2.85		0.004[a]
	Normal weight	13	21.7	10	33.3	X^2=5.883
	Overweight	27	45	17	56.7	0.053[b]
	Obesity	20	33.3	3	10	
Hirsutism	Yes	52	86.7	0	0	X^2=61.579
	No	8	13.3	30	100	0.0001[b]
Menstrual cycle	Regular	12	20	28	93.3	X^2=43.56
	Irregular	48	80	2	6.7	0.0001[b]
No. children	Non	34	56.7	0	0	X^2=28.407
	One	17	28.3	18	60	0.0001[b]
	Two	7	11.7	11	36.7	

* [a] independent T-test, [b] – Chi-square

Table 2 presents a comparison of metabolic and glycemic parameters between individuals diagnosed with PCOS and healthy controls. All measured parameters, including fasting blood sugar (FBS), insulin levels, HOMA-IR, TC, TG, LDL, and HDL, showed statistically significant differences (p=0.001). PCOS patients exhibited elevated levels of FBS (104.32±15.23 vs. 86.47±7.08 mg/dL), insulin (14.91±3.01 vs. 8.22±2.47 µU/mL), HOMA-IR (3.77±1.01 vs. 1.74±0.52), TC, TG, and LDL compared to healthy controls. On the contrary, HDL levels were significantly lower in PCOS patients (28.63±2.44 vs. 36.23±2.06 mg/dL).

Table 2. Comparative analysis of metabolic and glycemic parameters in PCOS patients versus healthy controls (independent T-test, mean±SD)

Parameters	PCOS patients n=60	Healthy control n=30	p
FBS (mg/dL)	104.32±15.23	86.47±7.08	<0.001
Insulin (µU/mL)	14.91±3.01	8.22±2.47	<0.001
HOMA-IR	3.77±1.01	1.74±0.52	<0.001
TC (mg/dL)	226.57±27.47	178.67±7.93	<0.001
TG (mg/dL)	168.15±16.25	106.63±12.02	<0.001
HDL (mg/dL)	28.63±2.44	36.23±2.06	<0.00
LDL (mg/dL)	152.22±11.81	128.27±2.73	<0.001

Table 3 provides a comparative analysis of serum corin and copeptin levels between women diagnosed with PCOS and healthy controls. Serum corin concentrations were significantly elevated in the PCOS cohort (1450.23±264.91 pg/mL) compared to the control group (619.17±159.19 pg/mL), demonstrating a statistically significant difference (p<0.001). Similarly, copeptin levels were substantially higher in the PCOS group (5.81±1.66 ng/mL) than in healthy controls (2.46±0.64 ng/mL), with a significant disparity (p<0.001).

Table 3. Comparison of serum corin and copeptin levels between women with PCOS and healthy controls (independent T-test, mean±SD)

Parameters	PCOS patients n=60	Healthy control n=30	p
Corin (pg/ml)	1450.23±264.91	619.17±159.19	<0.001
Copeptin (ng/ml)	5.81±1.66	2.46±0.64	<0.001

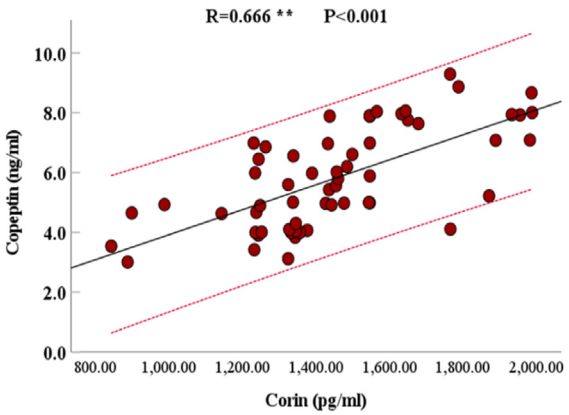

Fig. 1. Correlations between corin and copeptin in PCOS patients

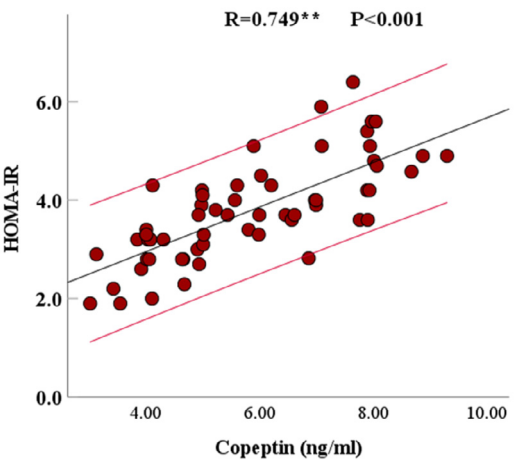

Fig. 2. Correlations between copeptin and insulin resistance in PCOS patients

Correlation coefficients (R) and the corresponding p-values for associations between circulating corin (pg/mL) and copeptin (ng/mL) levels and various clinical and biochemical parameters. For corin, significant negative correlations with FSH were observed (R = -0.359, p=0.005), while strong positive correlations were identified with FBS (R=0.515, p<0.001) and insulin levels (R=0.648, p<0.001). Similarly, copeptin was significantly and positively associated with FBS (R=0.375, p=0.003)

and insulin (R=0.750, p<0.001). Age, BMI, testosterone, lipid profiles (TC, TG, HDL, LDL) and LH levels showed no statistically significant correlations with either biomarker. The figure shows a strong correlation between corin and copeptin levels, where increasing chlorine levels are associated with increasing copeptin levels, and vice versa. There was a strong positive correlation between copeptin levels and HOMA-IR. As copeptin levels increased, HOMA-IR levels also increased, indicating increased insulin resistance (Fig. 1 and 2).

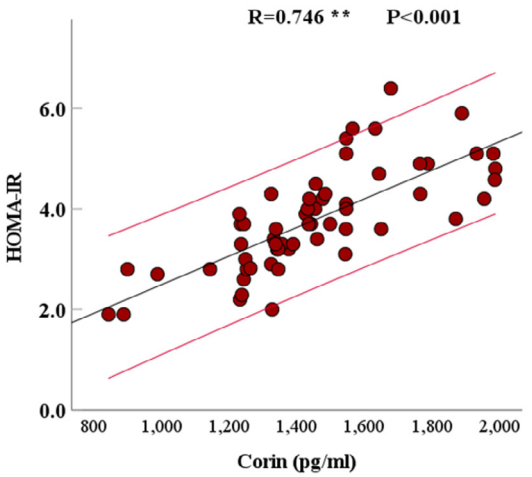

Fig. 3. Correlation of circulating corin levels with insulin resistance in a PCOS patient

Table 4. Univariate logistic regression analysis of risk predictors in PCOS patients*

PCOS patients[a]	p	OR	95% CI	
Age (year)	0.394	0.964	0.886	1.049
BMI (kg/m^2)	0.197	1.115	0.945	1.316
LH (mIU/mL)	0.0001	2.730	1.566	4.759
FSH (mIU/mL)	0.0001	0.238	0.118	0.482
Total testosterone (ng/mL)	0.040	3.318	1.055	10.436
FBS (mg/dL)	0.005	1.165	1.046	1.298
Insulin (µU/mL)	0.0001	2.064	1.434	2.971
HOMA-IR	0.0001	1.53	1.293	1.819
TC (mg/dL)	0.0001	1.004	1.002	1.007
TG (mg/dL)	0.0001	1.008	1.004	1.011
HDL (mg/dL)	0.036	1.015	1.001	1.028
LDL (mg/dL)	0.0001	1.006	1.003	1.009
Corin (pg/mL)	0.015	1.018	1.003	1.033
Copeptin (ng/mL)	0.0001	1.344	1.196	1.511

* [a] The reference category was healthy controls, CI confidence interval, OR – odds ratio

Table 4 presents the results of the univariate logistic regression analysis that evaluates the risk predictors for PCOS compared to healthy controls. Key hormonal and metabolic biomarkers, including LH, FSH, testosterone, HOMA-IR, and lipid profiles, were analyzed. Significant predictors of PCOS included elevated LH (OR=2.730, 95% CI: 1.566–4.759, p<0.01), reduced FSH (OR=0.233, 95% CI: 0.118–0.482, p<0.01), and higher testosterone levels (OR=3.328, 95% CI: 1.055–10.436, p<0.05). Metabolic markers such as insulin resistance (HOMA-IR: OR=1.533, p<0.01) and dyslipidemia (LDL: OR=1.006, p<0.01) were strongly associated with PCOS. The novel biomarkers corin (OR=1.018, p<0.05) and copeptin (OR=1.344, p<0.01) also showed significant positive associations.

Table 5 summarizes the diagnostic performance of corin and copeptin evaluated using AUC. All biomarkers demonstrated statistically significant discriminative power (p<0.0001). Corin and copeptin achieved near-perfect AUC values (1.00 and 0.99, respectively), indicating exceptional potential diagnostic accuracy. Corin exhibited the highest sensitivity (0.93 and 0.95), while copeptin showed balanced sensitivity (0.98) and marginally higher specificity (0.03) compared to other markers.

Table 5. Diagnostic accuracy of serum biomarkers based on ROC analysis

Markers	Area	p	95% CI		Cutoff	Sensitivity	1-Specificity
			Lower bound	Upper bound			
Corin (pg/mL)	1.00	<0.001	0.99	1.00	>987.35	0.95	0.00
Copeptin (ng/mL)	0.99	<0.001	0.98	1.00	>3.11	0.98	0.03

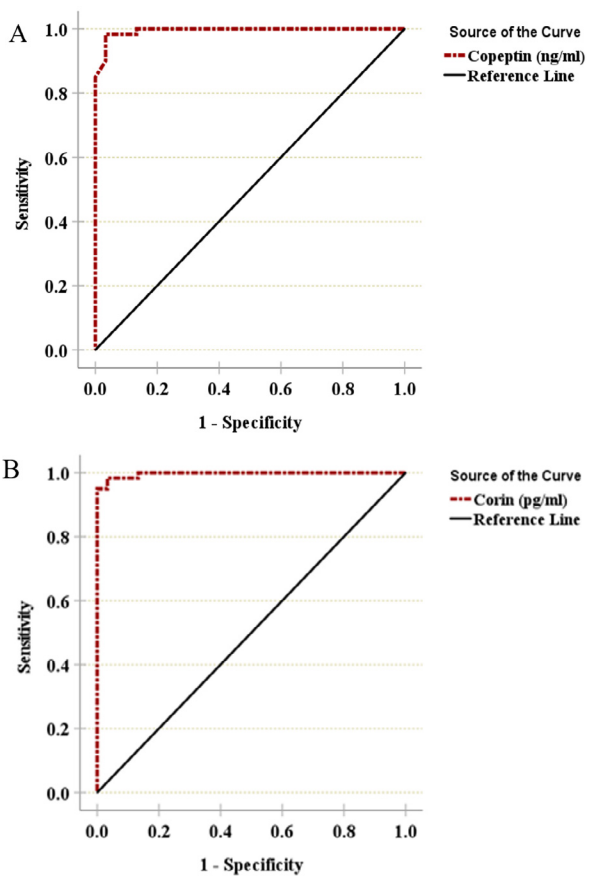

Fig. 4. Analysis of the ROC curve of A: copeptin and B: corin

Discussion

PCOS is a major health problem in women.[22] The pathological mechanisms underlying PCOS are complex and have not yet been completely elucidated. Dysregulation of adipose tissue metabolism in PCOS indicates the significant role of regulatory factors of adipose tissue in disease pathogenesis. The findings of the current study of elevated HOMA-IR levels in patients with PCOS corroborate the well-documented centrality of insulin resistance in the pathophysiology of PCOS. A meta-analytical synthesis of prior research indicates that approximately 75% of individuals with PCOS exhibit insulin resistance, highlighting its predominance as a metabolic hallmark of the disorder.[23] This metabolic aberration is further evidenced by consistent reports of elevated fasting insulin concentrations in PCOS cohorts compared to controls matched in age and BMI, strengthening the association of the syndrome with a hyperinsulinemic state.[24] In particular, the interplay between insulin resistance and PCOS appears to be intrinsic rather than secondary to adiposity, although obesity may enhance its severity. A longitudinal investigation carried out in a university cohort demonstrated that women with PCOS exhibited significantly higher insulin resistance indices than controls in all BMI strata, independent of body weight variations.[24,25]

Corin, a transmembrane serine protease integral to the natriuretic peptide system, facilitates proteolytic activation of ANP, a key regulator of cardiovascular and metabolic homeostasis. Despite its established role in ANP processing, the broad physiological and pathological functions of corin remain unclear. Unlike other membrane-bound proteases, corin undergoes proteolytic cleavage, generating a soluble form that is detectable in circulation.[26] Notably, this circulating corin retains its ability to activate ANP, mirroring the activity of its membrane-bound counterpart.[27] Given this functional equivalence, soluble corin has been hypothesized to contribute to the pathogenesis of PCOS.

The present study confirms this hypothesis, demonstrating significantly elevated plasma corin levels in PCOS patients compared to healthy controls. A 2024 case-control investigation comprising 70 women diagnosed with PCOS and 70 age-matched healthy controls revealed a marked elevation in median plasma corin concentrations (optimal diagnostic threshold: 1186 pg/mL) in the PCOS cohort. This cutoff exhibited robust diagnostic accuracy, achieving 100% sensitivity and 97.1% specificity for discriminating PCOS cases from controls. Furthermore, elevated plasma corin concentration was independently associated with infertility in the PCOS group (odds ratio, 5.9), underscoring its potential clinical utility as a biomarker. The observed elevation may reflect compensatory cardiovascular and metabolic adaptations linked to PCOS pathophysiology, potentially mediated by the role of corin in ANP processing and cardiovascular homeostasis.[19] In contrast, corin levels in other biological compartments showed divergent trends. For example, endometrial flushing fluid collected during the implantation window exhibited marginally reduced mean corin concentrations in women with PCOS; a comparison with controls revealed a difference that did not achieve statistical significance.[28] The findings presented here demonstrate concordance with the results of the current study, thereby corroborating the proposed hypotheses.

The present study corroborated that individuals diagnosed with PCOS exhibit significantly elevated serum copeptin concentrations compared to healthy controls. In particular, this observation was particularly pronounced in specific PCOS subgroups. Previous studies have consistently reported higher mean copeptin levels in PCOS cohorts than in healthy controls,[29] with obese PCOS patients demonstrating a marked increase in copeptin concentrations compared to BMI-matched controls. These levels were positively correlated with insulin concentrations and HOMA-IR.[30] Furthermore, copeptin has been inversely associated with HDL cholesterol and positively associated with elevated insulin levels, BMI, HOMA-IR, and waist circumference.[31] A case-control study involving 158 women with PCOS identified copeptin as the strongest predictor of HOMA-IR, underscoring its potential role in the pathophysiology of IR within this population.[32] These findings align with cross-sectional analyses indicating significantly higher copeptin levels in PCOS patients, particularly those with obesity, compared to the control groups.[33] Researchers have postulated that copeptin may contribute to metabolic dysregulation and atherogenesis in hyperandrogenemic, insulin-resistant PCOS patients.[29]

On the contrary, the relationship between PCOS and copeptin appears to depend on metabolic status. A 2024 case-control study demonstrated that normoglycemic, normal weight PCOS patients without IR exhibited lower copeptin levels than healthy volunteers.[34] Similarly, comparative analyses of non-obese PCOS patients and non-obese controls revealed no statistically significant differences in copeptin concentrations, suggesting that metabolic status, rather than PCOS itself, may be the primary driver of copeptin elevation.[30] Clinically, elevated copeptin levels in obese PCOS patients correlate with cardiometabolic markers, including total testosterone, HOMA-IR, waist-hip ratio (WHR), BMI, and hirsutism scores, positioning copeptin as a potential biomarker for cardiovascular risk stratification in this population.[35] Although both corin and copeptin show promise as biomarkers in PCOS, their associations appear distinct: corin is more closely related to cardiovascular sequelae, while copeptin demonstrates stronger associations with met-

abolic dysfunction and stress response pathways.[36] Recent evidence indicates that increased circulating copeptin levels correlate with various components of metabolic syndrome, such as dyslipidemia, insulin resistance, glucose intolerance, hyperinsulinemia, hypertension, and abdominal obesity.[37]

Study limitations and strengths

This study has significant strengths, including the potential investigation of corin and copeptin as possible potential diagnostic candidates for PCOS, supported by solid ROC analyses (AUC=1.00 and 0.99, respectively), complete metabolic profiling and adherence to standardized diagnostic criteria. Its phenotype-aware design and stringent statistical methods further improve its clinical relevance by relating biomarkers to infertility and cardiometabolic risks. Despite these strengths, the moderate sample size and lack of a priori power analysis can limit the generalizability of the findings. Future studies should incorporate power calculations to ensure adequate sample sizes to detect clinically meaningful differences, which may allow larger, longer-term studies to validate discoveries and clarify molecular pathways. The lack of phenotype-specific analyzes (e.g., phenotype A vs. D) restricts our understanding of biomarker variability between PCOS subtypes.

Conclusion

This study highlights the potential of plasma corin and copeptin as biomarkers for the diagnosis and risk stratification. Corin levels increase, are highly diagnostic of PCOS, and serve as a potential predictor of infertility in these women. Copetin, which is classified as an endocrine marker, is also significantly elevated in patients with PCOS, particularly those with obesity and insulin resistance, which attests to its correlation with metabolic dysregulation. Analysis of both markers will provide a thorough perspective on the metabolic and reproductive risks associated with PCOS, which could lead to a better diagnosis and personalized therapy. Furthermore, the nature of the relationship between copeptin and PCOS is context-dependent: elevation is most pronounced in metabolically compromised phenotypes, whereas non-obese, insulin-sensitive PCOS is associated with lower levels than controls. This highlights the diversity of the PCOS phenotype and where biomarker profiles are concerned, and the importance of a common assessment of metabolic status.

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Declarations

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Author contributions

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Conflicts of interest

The authors have nothing to disclose.

Data availability

Study data is available from the corresponding author upon reasonable request.

Ethics approval

On 1 October 2024, document number 5578 stated that the local ethics commission reviewed and approved the research protocol, consent form, and subject data.

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ORIGINAL PAPER

Impact of diabetes on dengue – a comparative study of clinical and inflammatory variables in patients with and without diabetes

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ABSTRACT

Introduction and aim. Dengue fever is a mosquito-borne viral disease and its severity may be influenced by comorbid conditions such as diabetes mellitus, which can alter the inflammatory and clinical response.
This study aimed to evaluate and compare clinical characteristics, laboratory findings, and inflammatory markers between patients with and without diabetes who were diagnosed with dengue infection.

Material and methods. The retrospective observational study included 100 patients with confirmed dengue infection, divided equally into 50 with diabetes and 50 without. It examined the distribution of age, HbA1C levels, clinical symptoms, bleeding events, liver enzymes, and inflammatory markers. Correlation analyzes were conducted to assess the relationship between HbA1C levels and inflammatory markers within each group. In addition, inflammatory markers were compared in different age categories (<50 years and ≥50 years) and by diabetic status.

Results. Laboratory findings, including liver enzymes and inflammatory markers, were markedly elevated in the diabetic cohort ($p<0.001$). The correlation analysis revealed a strong positive relationship between HbA1c and inflammatory markers in the diabetic group ($r>0.8$, $p<0.001$), while weaker correlations were observed in the non-diabetic group ($r=0.4–0.6$, $p<0.001$). Inflammatory markers were significantly higher in diabetic patients, particularly those 50 and older.

Conclusion. Diabetes may contribute to a more intense inflammatory response in dengue, highlighting it as an independent risk factor for severe clinical outcomes in dengue infection.

Keywords. C-reactive protein, dengue and diabetes, dengue fever, ferritin, interleukin 6, triglyceride

Introduction

Dengue fever is one of the most common arthropod-borne viral infections in the world; it is caused by four different serotypes of the dengue virus (DENV). DENV is a positive-stranded RNA virus of the Flaviviridae family that primarily infects humans through the bite of infected Aedes mosquitoes. When infected mosquitoes feed on human blood, they inject saliva containing infectious virus particles into the skin. According to current estimates, more than 390 million DENV infections occur yearly.[1,2] Among the known aedes-borne viruses, DENV is the most common, deadly and widespread virus.[3] Currently, there are no targeted or specific therapies available for diseases caused

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by Aedes-borne viruses. However, medical treatment is available for symptoms such as headache, seizure, and fever. Several techniques, such as those targeting human hosts, human-vector interactions, and vectors specifically, can be utilized to prevent the spread of Aedes-borne illnesses.[4] Vector control techniques are mostly used because they provide direct or biological reduction/elimination of vectors while causing no major impact on human hosts. The Wolbachia-based control method is one of the biological vector control methods that involves replacing wild mosquito populations with mosquitoes infected with Wolbachia. This bacterium inhibits viral replication in the mosquito's midgut, thereby reducing the mosquito's ability to transmit viruses.[5,6] Hyperglycemia on a molecular level increases the severity of DENV infection by potentially inducing the poly A binding protein (PABP) to facilitate viral translation. Hyperglycemia affects the structural and functional integrity of the endothelium, resulting in a persistent inflammatory condition caused by the activation of T-lymphocytes and the secretion of pro-inflammatory cytokines. Furthermore, hyperglycemia adversely impacts the immune system by reducing chemotaxis, leukocyte adhesion, and the phagocytosis process.[7] Microvascular and macro-vascular damage, which compromises circulation, is a histopathologic hallmark of patients with diabetes.[8]

Dengue viruses can cause a diverse array of clinical manifestations, ranging from asymptomatic infections to mild febrile illnesses, and extending to severe dengue, characterized by heightened capillary permeability and the potential for shock.[9] Increased levels of inflammatory markers are pathologically associated with chronic inflammatory conditions. Therefore, people with underlying inflammatory conditions, such as diabetes, may exhibit a heightened vulnerability to mortality associated with dengue. This association, which is related to immunological and metabolic dysregulation, is frequently cited as a significant predictor of the clinical progression of dengue infection in diabetes.[10]

Diabetic patients with dengue are at increased risk of developing severe complications, highlighting the role of comorbidities and poor glycemic control in the worsening of disease outcomes.[11] Compromised immune systems and fragile blood vessels, as well as increased vulnerability to bleeding, can contribute to a worsening of dengue symptoms in these patients.

Aim

Consequently, this study aims to improve the understanding of the relationship between inflammatory mediators and the clinical manifestations observed in both diabetic and nondiabetic patients suffering from dengue infection.

Material and methods

Study design and setting

This retrospective observational study was conducted at SUM Ultimate Medicare, Bhubaneswar. Due to the retrospective nature of the study, the requirement of informed consent was waived. Ethical approval was obtained from the Institutional Ethics Committee Clinical Research & Studies, SUM Ultimate Medicare, Bhubaneswar (Registration No. ECR/1604/Inst/OD/2021).

Participants

A total of 100 patients with confirmed dengue infection were included in the analysis, comprising 50 individuals with diabetes and 50 without diabetes. The sample size was determined based on a power analysis assuming a median value of medium to large effect size (Cohen's d=0.65), 80% power and a significance level of 0.05 required at least 39 patients in each of the two groups. The sample size was calculated using software power analysis and sample size version 3.1.9.3.

Inclusion and exclusion criteria

Participants aged between 15 and 70 years with a confirmed diagnosis of dengue infection were eligible for inclusion. In the diabetic group, a documented history of diabetes mellitus was required for a minimum duration of five years. Exclusion criteria included individuals with chronic kidney disease, autoimmune disorders, chronic obstructive airway disease, chronic steroid use, hemoglobinopathies, pregnancy, and other chronic diseases such as tuberculosis

Data collection

Data were collected for each patient to ensure a complete clinical and laboratory profile. Demographic information included age and sex. A detailed medical history was documented, with particular emphasis on the presence of diabetes mellitus, its duration, and any additional comorbidities. The clinical features evaluated at the time of presentation included fever, arthralgia, myalgia, abdominal pain, diarrhea, bleeding manifestations such as petechiae, melena, or epistaxis, and signs of serositis. Laboratory investigations included complete blood count (CBC) and liver function tests, specifically serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). Inflammatory markers measured included interleukin-6 (IL-6), ferritin, triglycerides (TG), and C-reactive protein (CRP). Glycemic control was evaluated using the level of glycated hemoglobin (HbA1c).

Dengue diagnosis

Dengue infection was confirmed using both NS1 antigen testing and IgM antibody detection. NS1 antigen testing was performed using a rapid immunochromato-

graphic test, while IgM antibodies were detected using an enzyme-linked immunosorbent assay (ELISA). A positive result in either test, along with compatible clinical characteristics, was considered a diagnostic of dengue infection. Dengue virus-specific IgM antibodies were detected using the PanbioTM Dengue IgM Capture ELISA kit (Catalog No. 01PE20, Abbott Laboratories, Brisbane, Australia). This qualitative enzyme-linked immunosorbent assay (ELISA) is intended to support the diagnosis of dengue infection in patients presenting clinically consistent symptoms. According to the manufacturer's data, the assay has a serological sensitivity of 94.7% for primary dengue infections and 55.7% for secondary infections, with a serological specificity of 100% in negative samples. Although the assay does not report a numerical detection limit, it is optimized for identifying IgM antibodies during the acute phase of infection. The reported intraassay variability is <10%, and the interassay variability is <15%.

Diabetes classification

Patients were classified as having diabetes according to their medical history and current use of antidiabetic medications. Furthermore, an HbA1c level ≥6.5% was used to confirm the diagnosis in patients with a known history of diabetes.

Blood sample collection and analysis

Blood samples were taken from all patients within 24 hours of hospital admission. The samples were processed according to standard laboratory protocols. CBC was performed using an automated hematology analyzer. Liver function tests and inflammatory markers were measured using standard biochemical assays. HbA1c was measured using high performance liquid chromatography (HPLC). Blood samples were collected in EDTA anticoagulant tubes and analyzed within 24 hours. HbA1c was separated from other hemoglobin fractions on charge differences using a cation-exchange HPLC column. The percentage of HbA1c was determined by calculating the ratio of glycated hemoglobin to total hemoglobin. This method is certified by the National Glycohemoglobin Standardization Program (NGSP) and is standardized according to the reference of the Diabetes Control and Complications Trial (DCCT), ensuring high accuracy and reproducibility.

Outcome measures

Primary outcome measures included the levels of inflammatory markers (CRP, ferritin, triglycerides, and IL-6) and the severity of clinical manifestations in diabetic versus nondiabetic patients with dengue infection. Secondary outcomes involved evaluating the correlation between HbA1c levels and levels of inflammatory markers.

Statistical analysis

The normality of continuous variables was assessed using Z scores, with values falling within±3.29 considered normally distributed. Continuous variables are presented as mean±standard deviation, while categorical variables are reported as frequencies and percentages. Independent sample t tests were used to compare the mean between the two groups. Associations between categorical variables or comparisons of proportions were analyzed using Chi-square tests or Fisher's exact tests, as appropriate. Pearson's correlation coefficients were calculated to evaluate the linear relationships between continuous variables. Generalized linear regression models were used to investigate the associations between continuous outcome variables and mixed-type independent variables. All statistical analyzes were conducted using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA). A p-value of <0.05 was considered statistically significant for all analyzes.

Results

A total of 100 patients with confirmed dengue infection were included in the study, comprising 50 patients with diabetes and 50 patients without diabetes. Diabetic patients had significantly higher mean age and HbA1c levels compared to non-diabetic patients. While the proportions of men and individuals with hypertension were higher in the diabetic group, these differences were not statistically significant. The clinical characteristics and manifestations of bleeding were also compared between the two groups, with no significant differences observed. However, laboratory parameters, including liver enzymes (SGPT, SGOT) and inflammatory markers (CRP, ferritin, triglycerides and IL-6) were significantly elevated in diabetic patients compared to nondiabetic patients ($p<0.001$). The baseline characteristics, clinical characteristics, bleeding manifestations, and laboratory findings are summarized in Table 1.

Correlation analysis

A correlation analysis was performed to assess the relationship between HbA1c levels and inflammatory markers within each group. Among diabetic patients, all inflammatory markers demonstrated a strong positive correlation with HbA1c levels ($r>0.8$, $p<0.001$). On the contrary, the non-diabetic group showed moderate correlations ($r=0.4–0.6$, $p<0.001$). All results were statistically significant, indicating a significant association between HbA1c and inflammatory markers. Detailed findings are presented in Table 2.

Subgroup analysis

The association of inflammatory variables was assessed with age groups (<50 years and ≥50 years) and diabetic status (diabetic and non-diabetic) was evaluated. There

was a significant difference in each of the inflammatory variables between the two age groups in diabetic patients, while in non-diabetic patients, except in TG values, the rest inflammatory variables were also significantly different between the two age groups. For TG, there were no significant differences between diabetic and nondiabetic patients for the age group of <50 years, while the differences were significant in the age group of ≥50 years. In other inflammatory variables, significant differences were found between diabetic and non-diabetic patients. In the above results, the values of the inflammatory variables were higher in older patients compared to younger patients. A similar result was also found, where the values of the inflammatory variables were higher in diabetic patients compared to nondiabetic patients. These results suggest that the association between diabetes, which is usually common in older age, elevated inflammatory markers in dengue infection, supporting the hypothesis that diabetes is an independent risk factor for a more severe inflammatory response in dengue infection. The results are summarized in Table 3.

Table 1. Baseline characteristics and clinical characteristics of study patients (n=100), data are presented as mean±standard deviation and compared using independent samples t-tests, categorical variables are expressed as number (%) and compared using Chi-square test

Demographic characteristic	Diabetic (n=50)	Non-diabetic (n=50)	p
Age (years), mean±SD	56.48±8.48	39.28±13.228	<0.001
Male n (%)	36 (72%)	33 (66%)	0.523
Female n (%)	14 (28%)	17 (34%)	
Hypertension, n (%)	9 (18%)	5 (10%)	0.249
HbA1c (%), mean±SD	8.38±1.16	4.44±0.619	<0.001
Clinical feature			
Fever, n (%)	39 (78%)	41 (82%)	0.617
Arthralgia, n (%)	37 (74%)	35 (70%)	0.658
Myalgia, n (%)	21 (42%)	18 (36%)	0.539
Abdominal pain, n (%)	41 (82%)	38 (76%)	0.461
Diarrhoea, n (%)	9 (18%)	7 (14%)	0.585
Bleeding manifestations			
Petechiae, n (%)	19 (38%)	17 (34%)	0.675
Melena, n (%)	6 (12%)	4 (8%)	0.505
Epistaxis, n (%)	5 (10%)	7 (14%)	0.538
Polyserositis, n (%)	34 (68%)	27 (54%)	0.151
Platelet transfusion requirement, n (%)	24 (48%)	21 (42%)	0.548
Laboratory parameters			
SGPT (units/L)	296.89±119.36	154.49±112.37	<0.0001
SGOT (units/L)	250.41±108.97	118.16±100.19	<0.0001
CRP (mg/dL)	21.045±5.28	8.96±3.98	<0.0001
Ferritin (ng/mL)	9141.311±2860.75	2264.29±2686.413	<0.0001
Triglycerides (mg/dL)	328.96±86.44	233.26±82.354	<0.0001
IL-6 (pg/mL)	12.27±2.83	5.324±2.49	<0.0001

Table 2. Correlation between HbA1c and inflammatory markers in diabetic and nondiabetic patients (n=100), Pearson correlation coefficients were used to assess the relationships

Inflammatory marker	Diabetic Group (n=50)		Diabetic Group (n=50)	
	r value	p	r value	p
HbA1c	--	--	--	--
CRP	0.856	<0.001	0.529	<0.001
IL-6	0.893	<0.001	0.568	<0.001
Ferritin	0.869	<0.001	0.291	<0.001
Triglycerides	0.877	<0.001	0.466	<0.001

Table 3. Association between age groups and diabetes status with inflammatory markers (n=100), data are expressed as mean±standard deviation, comparisons were performed using independent samples t test: between age groups within diabetic patients (#), within non-diabetic patients (##) and between diabetic and non-diabetic patients within the same age group ($)

Variable's	Age group	Diabetic (11/39)	p #	Non-diabetic (43/7)	p ##	p $
CRP (mg/dL)	<50 years (n=54)	17.8±3.43	0.019	8.36±3.45	0.06	<0.001
	≥50 years (n=46)	21.96±5.38		12.72±5.21		<0.001
IL-6 (pg/mL)	<50 years (n=54)	10.07±1.07	0.02	4.98±1.88	0.013	<0.002
	≥50 years (n=46)	12.9±2.87		7.46±4.48		<0.002
Ferritin (ng/mL)	<50 years (n=54)	7241.45±1193.01	0.011	1406.22±908.96	<0.001	<0.003
	≥50 years (n=46)	9677.17±2973.02		7535.29±3932.34		0.018
TG (mg/dL)	<50 years (n=54)	253.85±58.46	0.001	228.63±73.72	0.329	0.298
	≥50 years (n=46)	350.15±81.51		261.73±127.48		0.02

Table 4. Association of sex, age, and diabetes status with inflammatory markers (n=100), a generalized linear regression analysis was performed to assess the relationship between inflammatory markers and sex, age, and diabetes status

Dependent variables		Regression coefficient (β)		
		β	95% confidence interval	p
CRP	Sex	-0.082	(-3.27–0.57)	0.166
	Age	0.231	(0.05–0.21)	0.003
	Diabetes	-0.646	(-12.07– -7.59)	<0.001
IL-6	Sex	-0.017	(-1.28–0.95)	0.774
	Age	0.221	(0.02–0.12)	0.004
	Diabetes	-0.659	(-7.06– -4.45)	<0.001
Ferritin	Sex	-0.136	(-2348.47– -231.21)	0.017
	Age	0.329	(59.24–148.08)	<0.001
	Diabetes	-0.570	(-6251.64– -3781.63)	<0.001
TG	Sex	0.099	(-11.74–53.09)	0.209
	Age	0.521	(2.23–4.95)	<0.001
	Diabetes	-0.183	(-72.99–2.64)	0.068

Multivariate analysis

Given the association between inflammatory markers and HbA1c, further analysis was conducted to assess individual relationships of these inflammatory markers with sex, age and diabetes status using a generalized linear regression model. The results revealed that age and diabetes status were significant independent predictors of CRP, IL-6, and triglyceride levels. On the contrary, ferritin levels were significantly influenced by sex, age, and diabetes status. A summary of these findings is presented in Table 4.

Discussion

Research on the role of increased inflammatory markers in diabetic patients with dengue remains limited. Growing research indicates a connection between endothelial dysfunction and insulin resistance, suggesting a close relationship between the endothelium and insulin action.[12] In this study, patients with diabetes had more complications and a higher percentage of elevated inflammatory markers, suggesting that diabetes may be a risk factor for severe dengue infection. The clinical presentation of dengue can vary widely from mild symptoms to severe forms such as hemorrhagic fever, shock syndrome, and even death. This spectrum of severity is often influenced by underlying chronic diseases such as diabetes, which can alter immune responses.[13,14] Although several studies have demonstrated that diabetes mellitus can result in immunological and endothelial dysfunction, the exact mechanism by which diabetes causes severe dengue manifestation remains unclear.[15] Recent meta-analyses that examine diabetes mellitus as a risk factor for severe dengue reported that hyperglycemia in patients with diabetes may impair immune function by altering cytokine production. These changes can potentially improve dengue virus replication and contribute to greater disease severity and mortality.[16,17] Therefore, diabetic patients with dengue are at increased risk of developing severe complications, highlighting the role of comorbidities and poor glycemic control in worsening disease outcomes.[11]

Multiple studies have highlighted the increased severity of dengue in the context of poor glycemic control. For example, Lee et al. found that diabetic patients with well-managed blood glucose levels and no additional comorbidities were less likely to develop severe dengue compared to those with poor glycemic control, regardless of the presence of other health conditions. Similarly, Raj et al. concluded that hyperglycemia, independent of other clinical symptoms, is associated with worse outcomes in diabetic dengue patients. Marques-Vidal et al. also observed that elevated inflammatory markers are related to poor glycemic control and increased insulin resistance.[18] Understanding the interaction between inflammatory markers and demographic factors such as age, sex, and diabetes status can provide deeper insight into how these variables influence disease progression and severity

Elimam et al. established that CRP and IL-6 serve as important inflammatory markers, with elevated levels often associated with insulin resistance and poor glycemic control in individuals with diabetes.[19] These markers are produced by adipose tissue and the liver during systemic inflammation and demonstrate a relationship with insulin resistance. Hotamisligil noted in his research that increased concentrations of CRP and IL-6 can interfere with insulin signaling and glucose metabolism.[20] Furthermore, Masenga indicated that although triglycerides are primarily classified as a lipid marker, they are also associated with inflammatory processes and have been associated with increased oxidative stress and insulin resistance in diabetic patients.[21] Similarly, Zheng et al. reported a strong association between elevated triglyceride levels and both insulin resistance and poor glycemic control.[22]

Ferritin, a protein that stores iron, has been increasingly recognized as a marker of chronic inflammation. Increased levels of ferritin are frequently found in individuals with diabetes, and it has been proposed that ferritin may play a role in the promotion of oxidative stress and insulin resistance.[23] Ferritin concentrations can increase as part of the acute phase response, which occurs in reaction to systemic inflammation, and such elevations can be observed in both diabetic and non-diabetic individuals experiencing inflammatory states.

The relationship between CRP and HbA1c has been extensively studied, revealing a positive association, particularly among individuals diagnosed with diabetes. CRP, an acute-phase protein, tends to increase during episodes of systemic inflammation. According to Pradhan et al., elevated CRP levels in diabetic patients correlate with inadequate glycemic control, suggesting that inflammation plays a role in the disruption of glucose metabolism.[24,25] A recent study demonstrated that patients with diabetes and dengue had significantly higher CRP levels compared to patients without diabetes. Additionally, older patients with dengue, particularly those with diabetes, were more likely to show a marked rise in peak CRP levels during hospitalization and had an increased risk of progressing to severe dengue, findings consistent with our study.[26]

Research shows that both age and gender significantly influence inflammatory responses. For example, Marcos-Pérez et al. found that older adults tend to exhibit elevated levels of CRP, IL-6, and ferritin, which may contribute to an increased risk of type 2 diabetes with advancing age.[27] Gender-related differences in inflammation have also been observed, reporting that women often display higher levels of IL-6 and CRP than men, likely due to hormonal factors. Furthermore, diabetes status is

a critical factor, as individuals with diabetes frequently show elevated inflammatory markers compared to non-diabetic individuals, underscoring the role of inflammation in the pathophysiology of the disease. In line with these findings, our study identified a significant correlation between inflammatory markers and glycemic levels. This observation is further supported by evidence from a separate investigation, which revealed a strong association between serum inflammatory cytokine levels and both disease severity and clinical outcomes in dengue patients.[28] These insights highlight the importance of closely monitoring glycemic control in diabetic individuals to reduce the risk of severe dengue outcomes.

Latt et al. found that people with diabetes had a higher probability of experiencing severe manifestations of dengue, indicating that diabetes is an important risk factor for poor clinical outcomes.[29] Similarly, a study conducted by Figueiredo MAA and colleagues in Brazil found a strong association between dengue severity and diabetes mellitus, further supporting our findings.[30] Diabetes is known to nearly double the risk of a wide range of vascular complications, independent of other conventional risk factors.[31] The clinical presentation of dengue can vary widely from mild symptoms to severe forms such as hemorrhagic fever, shock syndrome, and even death. This spectrum of severity is often influenced by underlying chronic conditions like diabetes, which can impair immune responses. Recent meta-analyses have identified diabetes as a significant contributor to an increased risk of developing severe dengue and mortality associated with dengue.

This study compared the levels of the inflammatory mediators CRP, TG, ferritin, and IL-6 between dengue patients with and without diabetes. The observed association between these inflammatory markers and HbA1c levels is consistent with the findings reported by Singh et al.[32] These results confirm that individuals with diabetes who contract dengue tend to exhibit higher levels of inflammatory variables and experience more severe disease outcomes compared to non-diabetic patients. These findings highlight the importance of routine HbA1c testing in all diabetic patients diagnosed with dengue. Effective management of blood glucose levels is crucial to improving clinical outcomes. In addition, more extensive public health efforts, including comprehensive national dengue control and elimination campaigns, are essential to reduce the burden of the disease.

This study presents a novel comparative analysis of inflammatory and clinical profiles in patients with and without diabetes with dengue, offering a unique perspective on the immunometabolic interactions that may exacerbate the severity of the disease. Unlike previous studies that often focus on dengue or diabetes in isolation, this research bridges the gap by investigating how chronic hyperglycemia and long-standing diabetes impact the inflammatory environment during dengue infection. By integrating biochemical data (CRP, IL-6, ferritin, triglycerides) with glycemic control and age-related differences, the study uncovers a compelling pattern of amplified inflammatory responses in diabetics, particularly those aged ≥50 years. Furthermore, the robust correlation between HbA1c and pro-inflammatory markers provides new evidence supporting the role of poor glycemic control as a modifiable risk factor for adverse outcomes in dengue. This nuanced stratification of patients not only contributes to the limited body of literature on dengue-diabetes interactions, but also underscores the importance of metabolic comorbidities in shaping infectious disease trajectories. To our knowledge, this is one of the few studies in the country that quantitatively evaluate and contrast inflammatory markers in dengue patients stratified by diabetes status and age, making a significant contribution to tropical medicine and chronic disease research.

This study has several limitations that should be taken into account. Firstly, its retrospective design limits the ability to establish baseline equivalence between the diabetic and non-diabetic groups, introducing the possibility of selection bias. A significant proportion of diabetic participants were older than 50 years, with a mean age of 56 years, and severe dengue cases were predominantly observed in this older age group. This introduces age as a possible confounding factor in the association between diabetes and dengue severity. Additionally, the relatively small sample size may reduce statistical power and limit the generalizability of the findings to larger or more diverse populations. Another important limitation is the lack of serotype-specific data, which is critical, as different dengue virus serotypes can have varying impacts on disease progression and outcomes. In addition, the study did not distinguish between primary and secondary dengue infections, which could further increase the immune response. The scope of the inflammatory markers evaluated was also limited due to the unavailability and high cost of advanced immunological assays. As a result, key immune mediators such as interferons, interleukins, and other cytokines could not be evaluated.

Future research should address these limitations by including larger and more heterogeneous study populations, incorporating serotype-specific analysis, and expanding the range of inflammatory and immunological markers. Such studies would be instrumental in deepening our understanding of the immunopathogenesis of dengue, particularly in patients with diabetes, and in clarifying the mechanistic role of inflammatory mediators in shaping disease severity and outcomes.

Conclusion

Diabetes is an independent risk factor for progressing to severe dengue infection. Inflammatory marker levels

were significantly higher in dengue patients with diabetes compared to those without the disease, suggesting their potential role in the progression of severe disease and their utility as a possible predictor of disease severity. Consequently, dengue infections in people with diabetes were linked to increased inflammation and a greater probability of experiencing severe manifestations of dengue.

Declarations

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Author contributions
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Conflicts of interest
The author(s) declared that they have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability
All data generated or analyzed during this study are included in this published article.

Ethics approval
This study was approved by the Institutional Ethics Committee Clinical Research & Studies, SUM Ultimate Medicare, Bhubaneswar (Registration No. ECR/1604/Inst/OD/2021).

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ORIGINAL PAPER

Phytochemical composition, antioxidant, antihyperlipidemic, and hepatoprotective effects of phenolic components of Iraqi sumac (*Rhus coriaria*)

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ABSTRACT

Introduction and aim. Hyperlipidemia is a pathogenic disease associated with significant cardiovascular complications. *Rhus coriaria,* traditionally recognized as sumac, is abundant in numerous phenolic constituents that enhance its antioxidant, antibacterial, and anti-inflammatory characteristics. The aim was to investigate the phytochemical and pharmacological attributes of phenolic constituents of *R. coriaria*,

Material and methods. 32 male albino mice were assigned at random into 4 groups (n=8). Group 1 (control), group 2 (induced), group 3 (atorvastatin) and group 4 (phenolic). All groups received a diet that was rich in fat for a duration of 28 days, except the control group, which instead consumed a standard diet. Group 2 received no treatment, while group 3 and group 4 received atorvastatin 10 mg/kg and phenolic fractions of *R. coriaria* 500 mg/kg, respectively, for a further 28 days. Lipid profiles, oxidative indicators, biochemical parameters, and liver histopathological examination were estimated.

Results. Phenolic fractions substantially improved total cholesterol (167.5±2.4 vs. 280.4±17.6 mg/dL), triglycerides (181.1±12.5 vs. 238.6±11.05 mg/dL), low-density lipoprotein (109.0±1.6 vs. 209.2±16.8 mg/dL), and very low-density lipoprotein (36.2±2.5 vs. 47.7±2.21), while raising high-density lipoprotein levels (42.3±1.8 vs. 23.5±2.3 mg/dL) as opposed to the induced group ($p<0.05$). Furthermore, the phenolic constituents significantly reduced liver enzyme activities like alanine transaminase (27.4±1.8 vs. 45.2±2.8 U/L), aspartate aminotransferase (31.7±2.1 vs. 44.9±2.0 U/L), and alkaline phosphatase (28.0±2.1 vs. 50.9±1.9 U/L), and decreased total blood bilirubin (0.6±0.08 vs. 1.7±0.1 mg/dL) and albumin (4.7±0.7 vs. 6.6±0.3 g/dL) when compared to the induced nontreated group ($p<0.05$). Phenolic treatment also alleviated tissue malondialdehyde (221.09±3.2 vs. 475.98±44.02 nmol/mL) and increased reduced glutathione (35.48±1.86 vs. 11.65±0.78 µg/mL) as compared to the group without induced non-treated group ($p<0.05$) and restored liver histopathological changes.

Conclusion. Phenolic compounds have the potential to treat hyperlipidemia due to their anti-oxidative and anti-inflammatory properties.

Keywords. hyperlipidemia, phenolic constituents, *R. coriaria*

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Introduction

A medical condition known as hyperlipidemia is defined by an increase in the blood levels of lipoproteins and/or one or more components of the lipid profile, which encompass a variety of inherited and acquired illnesses.[1,2] Hyperlipidemia may also be specifically defined as triglycerides (TG), low-density lipoprotein (LDL) and overall cholesterol, or lipoprotein values that exceed 90% of the population average or fall below 10% of the average for high-density lipoprotein (HDL).[3,4] The term "primary prevention" refers to controlling risky variables, such hyperlipidemia, in order to minimize the likelihood of the development of atherosclerosis.[5,6] Additionally, hyperlipidemia is related to substantial generation of ROS, or reactive oxygen species, which play a critical part in the development and progression of cardiovascular diseases, including atherosclerosis.[7,8]

A pharmacological intervention must be selected based on the specific lipid abnormalities. Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, resins, ezetimibe, hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase blockers (statins), and niacin are the most effective drugs to lower LDL cholesterol. Niacin, marine omega-3, and fibric acid derivatives are most effective in lowering TG levels with very-low-density lipoprotein (VLDL) while increasing HDL cholesterol level.[9,10] Nonetheless, the majority of these drugs are associated with various troubles, including myalgia, myopathy, rhabdomyolysis, altered liver function, gallstones, stomach irritation, and possible renal failure. Hence, the advancement of innovative lipid-lowering medicines as alternatives to allopathic medications is of vital importance.[11]

Natural ingredients and their bioactive constituents are widely explored as potential effective remedies for various types of ailments.[12-16] *Rhus coriaria (R. coriaria)*, the Mediterranean herb named sumac, is an element of the Anacardiaceae clan. Historically, it has been used as spices and in the cosmetic, veterinary, and pharmaceutical industries.[17,18] The primary phenolic molecules isolated from sumac include gallic acid, ellagic acid, tannins, quercetin, rutin, catechins and anthocyanins.[19,20] The combination of these phenolic substances imparts *R. coriaria* its distinctive medicinal characteristics, making it useful in traditional medicine for multiple diseases.[21] These phytochemicals have been proven to be effective in avoiding cardiovascular diseases and tumors, diabetes, wounds, also skin aging.[22-26] Phenolics function principally by detoxifying ROS and strengthening endogenous antioxidant defenses.[27-29] Furthermore, they exert remarkable anti-inflammatory benefits by regulating cell activity and modulating cytokine secretion.[30-34] These constituents are currently used to provide hepatoprotection in a variety of settings, including acute liver damage and prevention of chronic liver pathology.[35,36]

Aim

This research was planned to explore the phytochemical composition of the phenolic fractions obtained from *R. coriaria* and to examine their antioxidant, antihyperlipidemic, and hepatoprotective activities in a mouse model of hyperlipidemia evoked by the high-fat diet (HFD).

Material and methods

Plant materials

In April 2020, the fruitful plant *R. coriaria* was acquired from Rawendos, which is located in northern Iraq. After being cleaned, it was ground using mechanized grinding equipment, allowed to dry at ambient temperatures in a shaded area, and then weighed.

Separation and fractionation of distinct active ingredients

400 g of coarsely ground, shade-dried powder of R. coriaria plant was stripped of fat with hexane for 24 hours and subsequently dried at room temperature (25°C) in order to extract and fractionate the active ingredients. Two liters of ethanol with a concentration of 85% were used in the Soxhlet apparatus to extract the plant components until they were completely gone. A deep greenish residual that represents the original portion was produced when the ethanolic extracts were dried at lower pressures and temperatures no greater than 40°C[37] and acidified to a pH of 2 using 300 milliliters of 5% HCl and then split three occasions using the same amount of ethyl acetate to create two distinct phases (ethyl acetate and watery acidic).[38,39]

Qualitative preliminary analyses of phytochemicals

Chemical investigations were carried out using the plant's ethanolic extract; we used recognized techniques to identify the active components.[40]

Test for flavonoids

NaOH test: liquid solutions of sodium hydroxide and hydrogen chloride were added to roughly two milliliters of extraction and portions, and the formation of an orange-yellow color was monitored.[25] Flavonoids typically absorb UV light, particularly in the range of 300-400 nm.

Separation of phenolic elements via high-performance liquid chromatography

The phenolic fraction is identified using high performance liquid chromatography. After the combination has been isolated using the principles of column-based chromatography, it is identified and quantified employing spectroscopy.[41] At the end of the column is a meter that counts the quantity of the split constituents.

The result of this detection is known as a liquid chromatogram.

HPLC condition model SYKAMN (Germany)

The C18-OSD column (25 cm, 4.6 mm) was used at 30°C with eluent A (methanol) and B (1% formic acid in water (v / v)) in particular circumstances: first 0 4 min at 40% B; 4–10 min at 50% B; at a flow velocity of 0.7 mL / min. The spectra were acquired at 280 n.

Preparation of drugs

A dose of 10 mg/kg of the usual medication, atorvastatin, was administered after 0.3 mg of 0.3 mg of the solution had been disintegrated in a 2% ethanolic mixture and adjusted to the appropriate proportion.[42] The phenolic fraction was prepared in distilled water by dissolving 17.5 mg in 300 units and administered at a concentration of 500 mg/kg. This dose is selected based on latest literature indicating the advantages of phenolic fractions as anti-inflammatory properties and antioxidants in the treatment of hyperlipidemia.[43-45]

Induction of hyperlipidemia

The typical nutrition (vegetables, cereals, fruits like apples and grapes, seeds like sunflower and groundnut, and vitamins A, E and D3) was supplemented with HFD (2% cholesterol and 1% peanut butter) to create elevated lipid levels for a period of 28 days. Every week, the body weight was determined.

Extract administrant

An intragastric tube was used to give a phenolic component of R. coriari for a period of 28 days.[45]

Experimental design

This work was implanted in the Faculty of Medicine's Department of Pharmacology at AL Nahrain University from the second of September 2022, until July 1, 2023. The Ethics Review Board of Al-Nahrain University School of Medicine (approved number 168) ensured that all procedures adhered to moral principles. From AL Nahrain University, 32 male albino mice, weighing between twenty and thirty grams, were acquired at the age of two to three months. A week before the start of the trial, the animals were acclimated to normal settings. The mice were randomly assigned to 4 groups, 8 mice in each group. In preclinical studies, a group size of 8 animals may be statistically acceptable due to several factors. First, power analysis typically demonstrates that, for large effect sizes, this number could indicate significant differences with sufficient statistical power. Furthermore, the use of homogeneous mouse strains and standardized conditions reduces variability, thus improving the accuracy of findings. Ethical guidelines prioritize minimizing animal use, and a sample size of 8 per group achieves a balance between statistical reliability and ethical considerations. Repeated measures can further diminish the need for larger sample sizes. In general, 8 animals are often a suitable number when justified by the size of the effect and experimental design. Animal grouping using a randomization approach reduces stress and ensures appropriate control of the environment. Reducing variability and variations in behavior can be achieved by keeping mice in distinct cages that are the right size and that use the same bedding, enhancement and food for every group. Proper experimental procedures for handling and treatment might also reduce external influences that may provide contradictory findings. After randomization, we compared the baseline characteristics to ensure that the groups were balanced. The age and weight of animals that underwent histopathology or treatment administration were comparable in all studied animal groups. The study groups were separated as follows: group 1 (normal) was fed a typical diet. The second group (induced) received HFD for a duration of 28 days. After 28-every-day HFD, Group 3 (atorvastatin) received 10 mg/kg of atorvastatin for an additional 28 days.[43] After 28 days of HFD.[43] Group 4 (phenolic fraction) received 500 mg / kg of phenolic fraction of R. coriaria for an additional 28 days.

Blood collections

Mice were fasted for 24 hours before having their hearts punctured to draw blood. Centrifugation at ambient temperature for fifteen minutes at three thousand revolutions per minute followed. Lipid profile indices and liver function enzymes were biochemically analyzed using acquired serum.[46-48]

Tissue homogenization

At the end of the trial, the animals were anesthetized and sacrificed, and the liver organ was separated into two halves to create tissue homogenate for histology and biomarker evaluation. Malondialdehyde (also known as MDA) and reduced glutathione (GSH) samples were extracted by homogenizing the initial portion of liver tissue, which was then centrifuged at 5000 rpm for 15 minutes.[49-51]

Biochemical analysis

Measurement of liver enzymes and indicators of lipid profile

The covenantal detecting kits were employed for the estimation of serum concentrations of total cholesterol and TG (Catalog No.: 303113050, ELITech Group, France, assay method: enzymatic calorimetric, intra-assay CV: ≤3%, inter-assay CV: not specified,), LDL, VLDL, and HDL (Catalog No.: 0599, Stanbio Laboratory, USA, assay method: enzymatic calorimetric, intra-assay CV: ≤3%, inter-assay CV: not specified) absorbance reader: AutoAnalyzer, SEAL Analytical GmbH, Germany.

The alanine transaminase (ALT), aspartate amino-

transferase (AST), alkaline phosphatase (ALP), and total circulating bilirubin (TSB) values were measured in serum using standard test kits and an automated analyzer (ARCHITECT c4000 chemistry analyzer, Abbott Diagnostics, USA) and a pertinent kit (ALP2 REF: 04S87R, ALT2 REF: 04S88R, AST2 REF: B4S900, and Total Bilirubin Kit REF: 04V510) donated by Abbott Diagnostics and designed for use with the ARCHITECT analyzer as directed in the manufacturer's guide.

The bubbles of air divide the continuous passage of the substance into distinct sections where chemical reactions take place. After that, the reagents and specimens are combined and transported through mixture coils and pipes. The samples are transferred by tubing between various devices, each of which performs a different set of operations, including the distillation process, dialysis, extraction procedure, ion exchange, heating, incubation, and output monitoring.[52-55] ISE, flame photometry, ICAP, fluorometry, and other techniques have been developed, but a continuous flow analyzer is based on color responses utilizing a flow by photometer.[56,57] Three repetitions of biochemical analysis experiments were performed, and the results did not reveal any significant differences.

Measurement of oxidative stress parameters

The oxidative stress parameters were measured in three separate experiments and no major variations were noticed in the findings were observed. The standard competitive enzyme-linked immunosorbent assay (ELISA) was adopted for the determination of tissue MDA and GSH levels (MDA Invitrogen: Catalogue No.: EEL160; Sensitivity (LOD): 18.75 pg/ml; GSH Invitrogen: Catalogue No.: EEL155; Sensitivity (LOD): 0.94 µg/ml). The microtiter plate was precoated with antibodies selective for MDA or GSH. Standards or specimens are introduced into designated plate wells with biotin-conjugated antibodies. After the washing procedure, Horseradish peroxidase (HRP) coupled with avidin was added to the holes.[58,59] The color development resulting from the addition of substrate solution was inversely corresponding to the concentration of MDA or GSH in the sample. At 450 nm, the generated color strength was determined.[60,61]

Histopathological examination

The isolated liver was immediately preserved, encased in paraffin after being fixed in a 10% formalin solution. Subsequently, after fixation, the samples were dehydrated by passing through ascending grades of ethanol concentrations: 70%, 80%, 95% and 100%. The samples were treated with xylol to remove the dehydrating agent (alcohol).[62-64] Next, the tissue samples were embedded by pouring out paraffin that had melted in metallic templates. A rotating microtome set to 5 µm thickness was used to cut liver slices.[65-67] Hematoxylin and eosin (H&E) were used to stain tissue biopsies. Background and excess stain were removed with 1% acid alcohol (1% HCl in 70% alcohol). Afterwards, DPX, or di-n-butyl phthalate in xylene, was applied as droplets. Covering sheets were placed on the slides, and they were maintained at room temperature overnight to dry.[68-70]

Statistical analysis

Microsoft Excel 2010 and SPSS version 26 (IBM, Armonk, NY, USA) were used for data entry and analysis. The mean SD was used to express continuous variables. A nonparametric test (Mann-Whitney) was used in place of parametric examinations (independent t test and one-way ANOVA) when the Shapiro-Wilk test of Normality showed that the data did not have typical distributions. When 0.05 was the p-value, the significance level was taken into account.[71,72]

Results

Qualitative and quantitative estimation of phenolic compounds by HPLC

A homogenized plant sample (3 g) was treated with a water and ethanol (70/30) to extract the phenolic components. An ambient temperature ultrasound bath was used for the extraction procedure. A rotating evaporator operating under vacuum extracted the solvent, which was subsequently dried to a constant mass at 40°C. Several bands representing distinct phenolic components of R. coriaria are shown in the chromatogram. As indicated in Table 1, Figure 1, and Figure 2, HPLC was used to identify quercetin, vanillic acid, p-coumaric acid, gallic acid, ferulic acid, and caffeineic acid.

Table 1. Retention times (Rt) in the fraction of minutes of phenolics in *R. coriaria*

Constituent	Rt of *R. coriaria* fraction	Rt of Standard
Caffeic acid	9.20	9.25
Gallic acid	3.22	3.25
Ferulic acid	7.85	7.85
p-coumaric acid	2.18	2.15
Quercetin	5.95	5.92
Vanillic acid	4.00	4.08

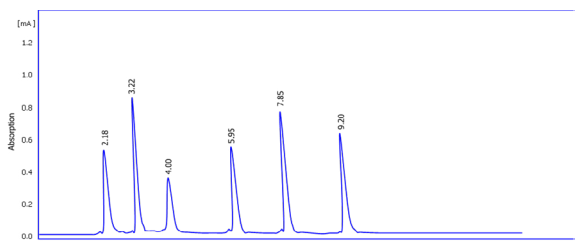

Fig. 1. HPLC chromatogram presenting the peaks of different constituents of the phenolic fraction of *R. coriaria*

Effects of tested agents on serum lipid profiles

Table 2 illustrates that HFD-treated mice developed hy-

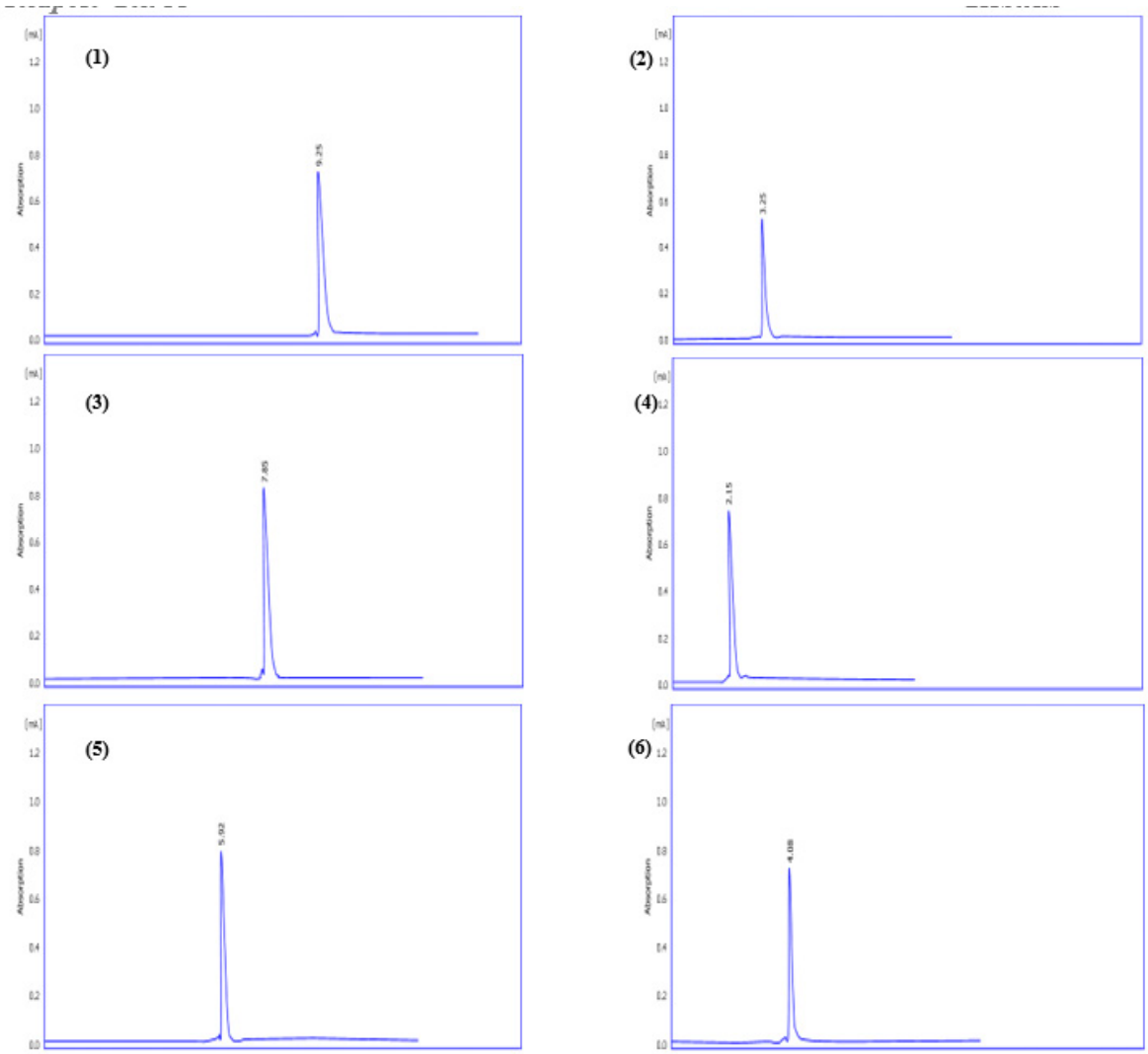

Fig. 2. HPLC chromatograms of each peak of standards for the constituents of the phenolic fraction.
(1) Caffeic acid reference, (2) gallic acid reference, (3) ferulic acid reference, (4) p-coumaric acid reference (5) quercetin acid reference, and (6) vanillic acid reference

perlipidemia, as seen by marked increases in TG, LDL, and VLDL levels and a marked decrease in good cholesterol in the induced nontreated group matched to the control group. Additionally, compared to the induced untreated group, the groups obtaining atorvastatin (10 mg/kg) and the phenolic component (500 mg / kg) found a noteworthy reduction in serum amounts of overall cholesterol, TG, LDL, and VLDL, with a notable increase in the amount of HDL ($p<0.05$). LDL values were markedly lower in the atorvastatin group compared to the phenolic group ($p<0.05$). However, in contrast to the atorvastatin group, the phenolic group showed nearly identical outcomes in terms of overall cholesterol, TG, and VLDL levels ($p>0.05$), with a notable increase in HDL relative to the atorvastatin group ($p<0.05$).

Effects of tested agents on liver function parameters

The result in Table 3 shows a remarkable up-regulation of serum ALP, ALT, AST, albumin, and TSB amounts within the induced untreated group compared to the seemingly healthy controls ($p<0.05$). Additionally, the groups given atorvastatin and the phenolic portion showed notable decreases in the serum levels of ALP, ALT, AST, albumin, and TSB in opposition to the induced untreated group ($p<0.05$). It is noteworthy that the phenolic fraction group revealed a significant difference in ALT, AST and TSB levels juxtaposed to the atorvastatin group ($p<0.05$).

Effects of tested agents on oxidative stress parameters

The concentration of MDA and a considerable decline ($p<0.05$) in GSH activity compared to the healthy control group. Compared to the induced untreated group, the groups receiving atorvastatin and phenolic components exhibited a considerable increase in GSH and a substantial reduction in MDA ($p<0.05$). Table 4 shows a considerable increase in GSH and a substantial reduction in MDA in the phenolic group compared to the

atorvastatin group (p<0.05).

Table 2. Impact of agents tested on serum lipid profiles[a]

Groups	Serum lipid levels (mg/dL)				
	Total cholesterol	TG	LDL	VLDL	HDL
Control (apparently healthy)	128.7±17.1	173.3±19.3	56.1±17.8	34.6±3.8	38±1.2
Induced (non-treated)	280.4±17.6*	238.6±11.05*	209.2±16.8*	47.7±2.21*	23.5±2.3*
Atorvastatin (10 mg/kg/day)	171.6±3.5**	189.9±14.9**	99.7±6.2**ϕ	38.35±3.3**	29.82±2.3**
Phenolic (500 mg/kg/day)	167.5±2.4**	181.1±12.5**	109.0±1.6**	36.2±2.5**	42.3±1.8**#

[a] The results are indicated as Mean±SD, n=8, *– marked change (p<0.05) vs. control group, ** marked change (p<0.05) opposed to induced group (nontreated), # marked change (p<0.05) opposed to atorvastatin group, marked change (p<0.05) opposed to phenolic fraction group. TG triglycerides, VLDL – very low-density lipoprotein, LDL low-density lipoprotein, HDL high-density lipoprotein, SD standard deviation

Table 3. Impact of agents tested on liver function parameters[a]

Groups	Liver marker levels				
	ALT (U/L)	AST (U/L)	ALP (U/L)	Albumin (g/L)	TSB (mg/dL)
Control (apparently healthy)	37.6±2.2	38.0±1.2	21.5±3.0	5.4±0.2	0.7±0.0
Induced (non-treated)	45.2±2.8*	44.9±2.0*	50.9±1.9*	6.6±0.3*	1.7±0.1*
Atorvastatin (10 mg/kg/day)	37.0±2.0**	38.2±1.7**	31.3±5.2**	5.5±0.2**	0.8±0.07**
Phenolic (500 mg/kg/day)	27.4±1.8**#	31.7±2.1**#	28.0±2.1**	4.7±0.7**	0.6±0.08**#

[a] results are indicated as Mean±SD, n=8, *– marked change (p<0.05) opposed to control group, ** marked change (p<0.05) opposed to induced group (nontreated), # marked change (p<0.05) opposed to atorvastatin group, AST aspartate aminotransferase, ALT alanine transaminase, TSB – total serum bilirubin, ALP alkaline phosphatase, SD – standard deviation

Impact of the drugs examined on changes in liver histopathology

Analysis of histological sections of the mouse liver in the seemingly healthy control group demonstrated a normal hepatic architecture without any histopathological alterations. The induced untreated group had notable alterations in the liver region, including significant steatosis and inflammation, compared to the normal controls. Furthermore, liver tissue of mice administered atorvastatin (10 mg/kg/day) exhibited mild lipid steatosis and inflammation compared to the induced untreated group. Histopathological evaluation of the mouse liver treated with the phenolic portion (500 mg/kg/day) revealed almost normal liver tissue, devoid of steatosis and inflammation, in contrast to the induced non-treated control group. The marked indicators (arrows) represent changes in specific areas within the histological section as depicted in Figure 3.

Table 4. Effects of tested agents on oxidative stress parameters[a]

Groups	Levels of oxidative indicators	
	MDA (nmol/mL)	GSH (µg/mL)
Control (apparently healthy)	229.95±9.61	48.62±5.11
Induced (non-treated)	475.98±44.02*	11.65±0.78*
Atorvastatin (10 mg/kg/day)	334.41±11.15**	26.51±2.85**
Phenolic (500 mg/kg/day)	221.09±3.2**#	35.48±1.86**#

[a] Results are indicated as Mean±SD, n=8, *– marked change (p<0.05) opposed to the control group, ** marked change (p<0.05) opposed to the induced (nontreated) group, #– marked change (p<0.05) opposed to the atorvastatin group, GSH reduced glutathione peroxidase, MDA malondialdehyde, SD – standard deviation

Discussion

Hyperlipidemia is a notable contributing cause of heart problems and often occurs in people with coronary artery disease.[73] Existing therapies to manage hyperlipidemia have a history of recurring adverse effects, particularly a higher likelihood of gallstone development, myopathy, and rhabdomyolysis. Therefore, there is a dire need to discover new and successful anti-hyperlipidemic medicines with minimal complications.[74,75] Recently, herbal remedies have become a popular way of treating dyslipidemia due to their better pharmacologic profile and fewer undesirable reactions.[76] In this study, the changes observed in lipid profiles in the non-treated induced group may be attributed to hyperlipidemia caused by a HFD, which disrupts lipid metabolism primarily via diminishing β-oxidation and improving cholesterol biosynthesis and oxidative injury by down-regulating the expression of free radical scavenging enzymes.[77] Cholesterol uptake increases in tandem with the rate of bile acid re-absorption, which in turn enhances the liver's ability to utilize cholesterol. Furthermore, higher blood concentrations of overall cholesterol, TG, and LDL, together with decreased lipoprotein lipase function, are associated with a compromised anti-oxidative defensive mechanism.[78]

However, alterations in serum lipid profiles were reversed by treatments with atorvastatin and phenolic fractions, suggesting a hypolipidemic impact. Atorvastatin is the main HMG-CoA blocker prescribed for the treatment of dyslipidemia. It substantially lowers blood lipid concentrations and may diminish hepatic fat deformity.[43] While various statins exhibit lipid-lowering results,

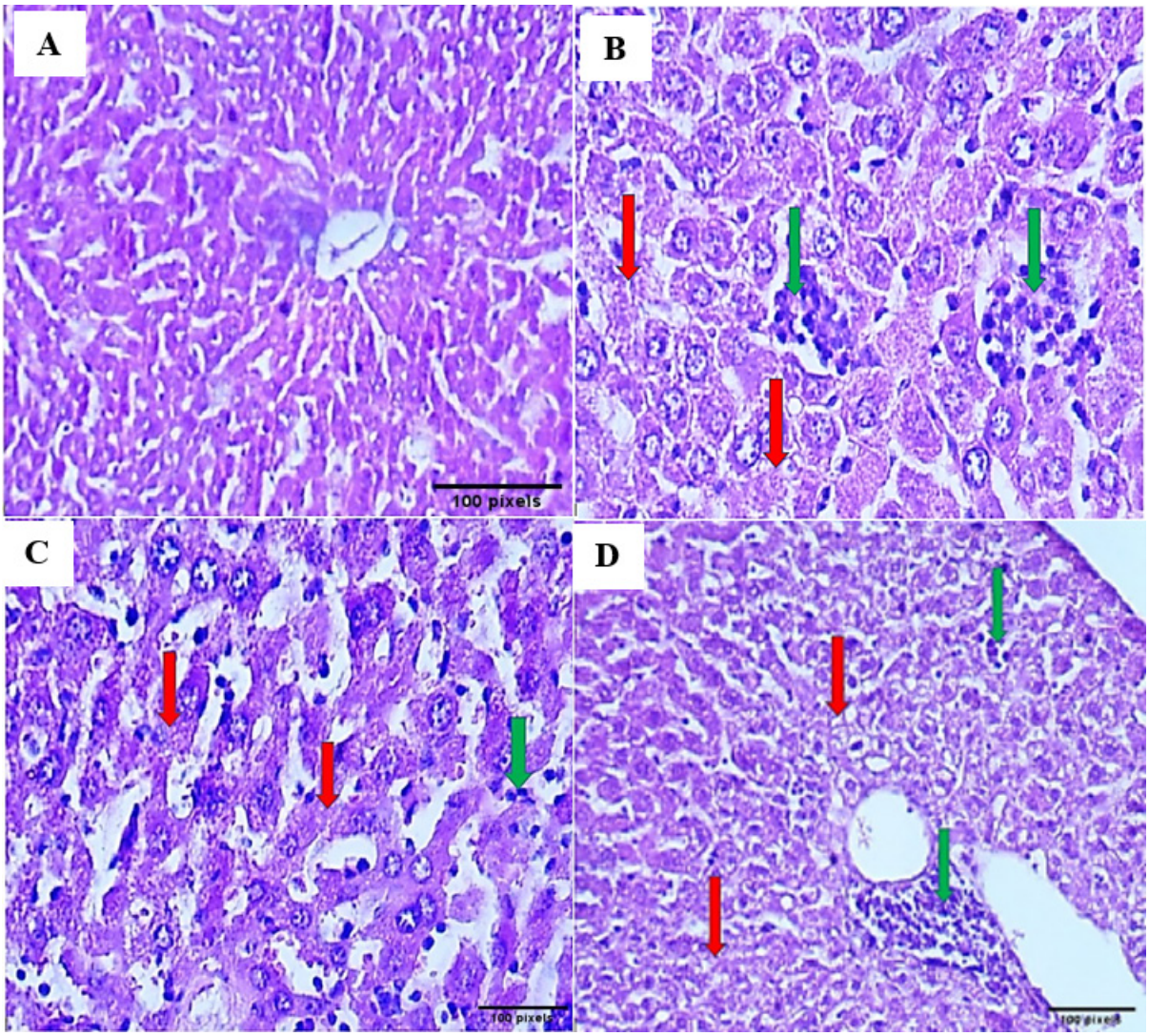

Fig. 3. Effect of the agents tested on histological examination of mouse liver, red arrow: steatosis level, green arrow indicates the degree of inflammation. A: standard control group (scale bars = 100 mm; H&E 20×), B: caused (group not treated, scale bars = 100 mm, H&E 20×), C: group treated with atorvastatin (H&E 20×, scale bars=100 mm), D: group received with phenolic component (H&E 20×, scale bars=100 mm)

atorvastatin displays superior declines in serum LDL and TG than other statins, likely attributable to its prolonged action, which probably reflects a prolonged retention of atorvastatin and its main metabolites in the liver.[79]

Oxidative stress stems from ROS overproduction and / or diminished antioxidant defenses.[80-82] MDA is a product of lipid peroxidation, while glutathione serves as the primary intrinsic antioxidative molecule against oxidative harm.[83-88] Phenolic compounds can reduce lipid levels and modulate redox pathways through several interconnected mechanisms. They act as potent antioxidants by donating hydrogen atoms or electrons to neutralize ROS, thus reducing oxidative stress, which is a major contributor to lipid peroxidation and atherosclerosis.[89,90] Phenolics can also up-regulate natural detoxifying enzymes such as catalase, superoxide dismutase, and glutathione peroxidase through activation of Nrf2 signaling.[91,92] In lipid metabolism, they inhibit enzymes such as HMG-CoA reductase, hindering cholesterol synthesis, and promote LDL receptor transcription, enhancing lipid clearance. Furthermore, they can suppress inflammation by downregulating NF-κB signaling, which indirectly affects lipid metabolism and oxidative balance.[93] Phenolics can also modulate gut microbiota, influencing bile acid metabolism and lipid absorption.[94] Their interaction with peroxisome proliferator activated receptors (PPARs) helps regulate fatty acid oxidation and storage.[95] Through these combined actions, phenolic fractions exert lipid-lowering and antioxidant effects.

Liver diagnostic enzymes, including AST, ALT, and ALP, assess the internal stability and deterioration.[96] The present investigation revealed that the administration of HFD gave rise to elevated amounts of ALP, ALT, AST, albumin, and TSB amounts. The breakdown of cellular integrity contributed to the leakage of liver enzymes into the bloodstream. Circulatory ALT level is

liver-specific and serves as a crucial indication of liver dysfunction or potential injury. Furthermore, increased serum amounts of AST and ALP may suggest enhanced biliary congestion.[97] In general, significant elevation in AST, ALT, and ALP amounts in the induced nontreated group implies hepatic injury and steatosis perhaps resulting from deposits of lipid intermediates [98] The use of atorvastatin and phenolic fraction reduced the levels of liver enzymes, TSB, and albumin amounts, perhaps related to their hypolipidemic activities. The hepatic histological analysis verifies the mitigative impact of the examined agents.[99,100]

Furthermore, a double-blinded randomized study involving 41 type 2 diabetics found that daily intake of 3 g of sumac extract optimized paraoxonase activities and lessened insulin resistance, MDA and C-reactive protein.[101,102] Supplementing sumac to patents with fatty liver ailment resulted in profound decrement in liver fibrosis, hepatic enzymes, and insulin resistance after 12 weeks of consumption.[103] The impacts of R. coriaria can depend on a multitude of bioactive polyphenols like gallic acid and flavonoids such as kaempferol, quercetin, myricetin and rutin, as they are established for their antioxidant, anti-inflammatory, and liver-protective properties.[104-106]

Study limitations

To the greatest extent of our ability, this is a novel investigation to analyze the phytochemical compositions of phenolic elements derived from *R. coriaria*, together with their antioxidant defense, hyperlipidemia prevention, and hepatoprotection. Even so, it is important to emphasize the shortcomings of the work. Initially, more information from clinical protocols is essential to estimate the established pharmacological benefits of phenolic compounds extracted from *R. coriaria*. Furthermore, measurement of inflammatory mediators is recommended because hyperlipidemia sufferers are associated with exceptionally high amounts of inflammatory cytokines, namely interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α).

Conclusion

*R. coriaria*is an *R. coriaria* is enriched in a variety of phenolic constituents, namely gallic acid, ferulic acid, quercetin, p-coumaric acid, and vanillic acid. These phenolic fractions confer a beneficial impact on hyperlipidemia provoked via a fatty diet, completely normalizing lipid profiles, biochemical indicators, and oxidative markers while dramatically preventing the majority of liver histopathological alterations. The findings suggest that the phenolic ingredients appear to be equivalent to or even more effective than the standard drug, atorvastatin.

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Declarations

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Author contributions

Conceptualization, S.F.H., A.R.A.R. and E.J.K.; Methodology, S.F.H. and E.J.K.; Software, H.R.S.; Validation, S.F.H., A.R.A.R. and E.J.K.; Formal Analysis, A.H.A.; Investigation, S.F.H. and A.H.A.; Resources, A.H.A.; Data Curation, S.F.H.; Writing – Original Draft Preparation, H.R.S.; Writing – Review & Editing, H.R.S. and A.H.A.; Visualization, A.R.A.R..; Supervision, A.R.A.R. and E.J.K.; Project Administration, H.R.S.; Funding Acquisition, S.F.H.

Conflicts of interest

The authors declare that they have no conflict of interest.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval

The study adhered rigorously to the Declaration of Helsinki's ethical norms. The Ethics Review Committee of the College of Medicine (approval no. 168) ensured that all treatments followed ethical standards on 22 March 2024.

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ORIGINAL PAPER

Evaluation of nutritional knowledge in relation to primary prevention of doctors and nurses in Morocco

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ABSTRACT

Introduction and aim. Currently, Morocco suffers the burden of non-communicable diseases. Healthcare professionals play a crucial role in guiding the population toward healthy dietary choices, which can reduce the risk of these types of diseases. This study aims to assess nutritional knowledge in relation to primary prevention of doctors and nurses working in the hospital network and primary health care facilities in Morocco.

Material and methods. The study population consists of 472 nurses and 185 physicians. A self-administered questionnaire composed of four main sections (nutrition by the Mediterranean diet; nutrition for children; nutrition for pregnant and breastfeeding women; and nutrition for the elderly) was used for data collection.

Results. The Mediterranean diet obtained the highest score of 0.46 (IQR [0.30, 0.53]) for physicians. But for nurses, the highest score was for child nutrition 0.33 (IQR [0, 0.333]). Our results reveal a statistically significant association of the median total score of answers of health professionals with basic training ($p<0.001$), receiving information on nutrition ($p<0.001$), their degree (doctors or nurses) ($p<0.001$) and the workplace ($p<0.001$).

Conclusion. The training programs of the medical faculties and nursing training institutes in Morocco should be revised in favor of a more in-depth training in nutrition.

Keywords. doctor, health professionals, nurse, nutritional knowledge, primary prevention

Introduction

According to the World Health Organization (WHO), the prevention of non-communicable diseases (NCDs) is possible through the identification of the main common risk factors, their prevention, and their control. An unhealthy diet is considered one of these risk factors.[1]

The prevention of metabolic and cardiovascular diseases is strongly correlated with diet quality.[2] Diet is considered the greatest modulator of the immune system and is of great interest where new challenges appear in the field of prevention and treatment.[3]

The Mediterranean diet (MD) is considered one of the healthiest diets. The main characteristics of the

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Mediterranean diet, such as high consumption of fruits, vegetables, legumes and whole grains, as well as the use of olive oil as the main source of fat, and the limitation of the consumption of red meat and dairy products, are associated with a lower incidence of coronary heart disease and a 30% reduction in the risk of type 2 diabetes, as well as protection against cardiovascular disease.[4,5]

Certain categories of population are in the most nutritionally vulnerable stages of the life cycle, such as pregnant and breastfeeding women, children, and the elderly. They need to pay particular attention to their diet.

A healthy and balanced diet during pregnancy is crucial to meet the energy and nutrient needs of both the mother and the growing fetus. Nutritional deficiencies can lead to negative outcomes such as anemia, premature delivery, and low birth weight.[6]

During lactation, energy and micronutrient needs are higher than in adulthood and pregnancy. Infants should be exclusively breastfed for the first 6 months to support optimal growth and development, with micronutrient levels based entirely on the mother's nutrition.[7,8]

Elderly individuals face a higher risk of malnutrition due to physiological and psychological factors, such as a diminished sense of taste and difficulty chewing or swallowing. The preference for soft, easy-to-eat foods, which are often high in sugar and fat, can increase the risk of comorbidities such as metabolic syndrome and cardiovascular disease.[9,10]

Morocco is experiencing an epidemiological and health transition characterized by lifestyle changes and increased risk factors for non-communicable diseases (NCDs), such as sedentary behavior and unhealthy eating habits.[11] Only 28.2% of the urban population adheres to a Mediterranean diet, with excessive carbohydrate (43%) and lipid (18%) intake.[12] Salt and sugar consumption also exceeds WHO recommendations, at 11g and 95.25g per person daily, respectively.[13] Non-communicable diseases are the leading cause of mortality, accounting for 84% of deaths in 2022.[1] The prevalence of hypertension is 29.3%, diabetes is 10.6% and high cholesterol is 10.5%, with 33% of the population overweight and 20% obese.[14] This underscores the crucial importance of having healthcare professionals who are well versed in nutritional knowledge. These experts play a vital role in educating the public about healthy and balanced diet choices, thereby helping to prevent the increasing prevalence of various health conditions. By incorporating nutritional advice tailored to the cultural and socioeconomic needs of Moroccans, these professionals can not only raise awareness about the risks associated with an unbalanced diet, but also promote healthy lifestyle habits.

In Morocco, doctors and nurses play a vital role in nutritional education by assessing the dietary needs and providing personalized advice. Medical training includes fundamental nutrition education, while nursing programs focus on patient counseling techniques. This collaborative approach ensures comprehensive nutritional support, although there may be variations between different regions and institutions. However, training programs in Moroccan medical schools lack well-developed nutrition modules, offering only basic concepts related to certain diseases and some recommended practices. Similarly, health professional training institutes typically include only one nutrition module, which consists of a total of 40 hours.

The training of health workers must provide an adequate and up-to-date level of knowledge of nutrition for prevention. Recognizing this need, decision-makers are aware of this, and a dynamic of reforms of health professionals training curricula is being incubated in all medical faculties as well as nursing training institutes. Furthermore, the orientation and adaptation of continuing education programs also requires knowledge of the needs of health professionals in terms of nutrition.

Aim

This study aims to evaluate the knowledge of health workers in the north region of Morocco about nutrition for the primary prevention of non-communicable diseases.

No previous research has examined the nutritional knowledge of healthcare professionals in Morocco. The results of this study could have significant implications on the review of training programs for physicians and nurses in the country.

Material and methods

Study design

This cross-sectional exploratory study was conducted in northern Morocco between June and December 2022, involving 146 health centers and 9 public hospitals in the Tangier-Tetouan-Al Hoceima region. The target population included a sample of 1,692 health professionals, including 253 doctors and 1,439 nurses, who met the inclusion criteria: having completed basic training in public nursing institutes and public medical schools, working in health centers or public hospitals, and participating voluntarily, in order to control for possible confounder variables. However, laboratory and radiology technicians, physiotherapists, specialist physicians, private sector professionals, and those who refused to participate were not included. Furthermore, dietitians were excluded because their presence could distort results by influencing responses on nutritional knowledge compared to other health professionals. The study examined the effect of basic training, continuing education, workplace, and access to nutritional information, including Ministry of Health guides, on the level of knowledge of these professionals on primary prevention. Taking into account the similarities between pub-

lic health facilities and the uniformity of basic training at the national level, the sample was randomly selected by clusters, including 146 health centers and 9 public hospitals in the region, with all eligible professionals working in these facilities. This random selection method reduced bias and improved the generalizability of the study results. The planned sample size was 657 participants (185 doctors and 472 nurses), calculated from Cochrane equations and determined by power analysis.[15] The sample was then stratified into four strata: 238 nurses and 131 physicians working in primary health care centers and 234 nurses and 54 doctors working in public hospitals.

Data collection

Data collection was carried out using a self-administered questionnaire to collect sociodemographic information and training information (initial, continuing, and other sources of nutritional information). A multiple choice questionnaire assessed participants' knowledge of participants about nutrition, including the Mediterranean diet, infant nutrition, nutrition for pregnant and breastfeeding women, and nutrition for the elderly. The addition of a section on the Mediterranean diet highlights the knowledge and ability to advise on healthy food choices, reflecting their ability to integrate evidence-based dietary recommendations into their practice.

The quiz was developed based on similar studies inspired by literature and standardized nutritional guidelines (Moroccan Nutrition Guide (2016)[16]; Nutritional Guide Version 2 (2016)[17]; "Nutrition and Breastfeeding" Manual (2018)[18]; Nutrition Guide for Individuals Aged 55 and Over (2019)[19]; Integrated Child Care Guide: Algorithm for Health Professionals (2018)[20]; Nutrition Guide for Caregivers of the Elderly (2015)[21]; Nutrition for Children and Adolescents for All Parents (2015))[22] and was reviewed by experienced dietitians. Before the study, it was piloted with public sector physicians and nurses, who were not included in the final survey, to avoid potential misunderstandings. Participants received oral information about the study and participation was voluntary. The questionnaires were anonymous and took approximately 20 minutes to complete.

Statistical analyses

Statistical analyzes were performed using SPSS version 21.0 software (IBM, Armonk, NY, USA), with statistical significance evaluated using a threshold of $p<0.05$. The results of the Mediterranean diet knowledge scores were compared with other categories to identify specific gaps in the training of health professionals.

Descriptive statistics, including mean scores and medians, were presented to provide a concise summary of the variables studied. For each question, we assign a value of 1 to the correct answer and 0 to the incorrect answer (True=1; False=0). The score is the average of all participants' responses to each question. A score close to 1 indicates that healthcare professionals have good nutritional knowledge. Subsequently, we calculated the median of the scores for each axis and then determined the median of the response scores for all questions related to primary prevention. We chose response scores to directly measure each participant's correct responses, facilitating interpretation of performance, identification of areas for improvement, and comparison between doctors and nurses, while avoiding sample size bias.

A Kolmogorov-Smirnov normality test showed that the data did not follow a normal distribution ($p<0.05$), justifying the use of non-parametric tests. The response scores were described by the median and interquartile range. The Mann-Whitney test was used to assess the association between the mean score and variables such as gender, workplace, basic training, and continuing education. This test allows comparisons between two independent groups and the analyzes are bilateral with a significance level of $p<0.05$.

We used Spearman's rank correlation to analyze the relationship between physicians' and nurses' nutritional knowledge scores and their age. Then, multiple linear regression analysis was performed to identify significant sociodemographic variables associated with good nutritional knowledge (score close to 1). This approach allows us to examine the simultaneous effect of multiple independent variables on a continuous dependent variable.

In our study, we aim for a power of 80% (0.80) and a precision of 5% (0.05) to ensure the robustness of the results. To estimate the size of the effect, we used the formula $r=z^2/n$ for the Mann-Whitney test and r^2 for the Spearman correlation. This provides an indication of the magnitude of the observed relationships.[23]

Ethical approval

All participants received a written information sheet and signed informed consent. The right to refuse to participate, to refuse to respond to questions asked, or to withdraw at any stage of the process without any penalty or consequence was ensured before encouraging participation. The ethical principles of the Declaration of Helsinki and the Oviedo Convention on Human Rights and Biomedicine were followed.

Results

Information on the sociodemographic characteristics

The study involved 185 physicians (55.7% men, 44.3% women) with a median age of 42±5.32, and a response rate of 98%. Most (70.8%) work in primary care, and 82.2% reported no nutrition training during their education, while 14.6% participated in continuous training. About one third receive Moroccan nutrition guides.

Table 1. Information on the sociodemographic characteristics*

Variable	Doctors n=185	Nurses n=472
Age (year)		
26–36	16.2	44.1
37–47	68.6	40.9
48–55	15.1	15
Median age	42±5.32	38.00±6.83
Sex (% of total)		
Female	44.3	56.4
Male	55.7	43.6
Work place (% of total)		
Primary healthcare center	70.8	50.4
Hospital	29.2	49.6
Courses in nutrition during training (% of total)		
Initial training	17.8	92.4
Continuing education	14.6	7.4
Receiving information[a]	29.7	31.6

* [a] receiving information: reception of information through official documents from the Ministry of health such as Moroccan nutrition guides, national nutrition strategy guide, etc.

Furthermore, 472 nurses (56.4% women, 43.6% men) with a median age of 38.00±6.8 had a response rate of 94%. Half work in primary care and 92.4% stated that nutrition training was part of their basic education, but 92.6% reported that nutrition training. Nearly one-third claimed to have received Moroccan nutrition guides.

The study revealed that 17.8% of doctors received nutrition courses during their basic training and 14.6% during continuing education. Although 92% of nurses reported taking a 40-hour nutrition course in their basic training, and In the study, only 32% of the participants received ministerial nutrition guidelines.

Evaluation of nutritional knowledge

Table 2 presents the scores and percentages of correct answers from physicians and nurses to questions about nutrition for the Mediterranean diet, nutrition for children, nutrition for pregnant and breastfeeding women and nutrition for the elderly.

Regarding nutritional knowledge about Mediterranean diet, the median score of doctors'answers of the questions is 0.46 (IQR [0.30, 0.53]), except for questions on the type of the cereal and the shape of fruit to be preferred in this type of diet, where the majority (85% and 81% respectively) answered correctly. For nurses, the median of answers score to the questions is 0.24 (IQR [0.15, 0.30]), with the exception of the type of cereal to be preferred, two thirds of the participants correctly answered. The median of answers of both health professionals is 0.23 (IQR [0.15, 0.46]).

Regarding child nutrition, the median score of doctors' answers to the questions is 0.33 (IQR [0.33, 0.33]), and almost a quarter had no knowledge of exclusive breast feeding during the first 6 months of life. While the median of score nurses 'answers on child feeding scores is 0.33 (IQR [0, .033]), and more than a third did not know that the adoption of exclusive breastfeeding is recommended from birth to 6 months. The median of answers of both health professionals is 0.33 (IQR [0.33, 0.33]).

Regarding the nutrition of pregnant and breastfeeding women, the median of doctors' knowledge is 0.335 IQR [0.22, 0.44]), however, the median of the score of the nurses' responses is only 0.11(IQR [0, 0.22]). The median of answers of both health professionals is 0.11 (IQR [0, 0.33]).

Concerning the nutrition of the elderly, doctors have a high median score of answers (0.4 IQR [0.2, 0.6]) than nurses (0.2 IQR [0, 0.4]). The median of answers of both health professionals is 0.20 (IQR [0, 0.40]).

The results of the study provide an overview of the level of knowledge of physicians and nurses in relation to primary prevention. We can see from Figure 1 that the median of the response among physicians are optimal compared to those of nurses.

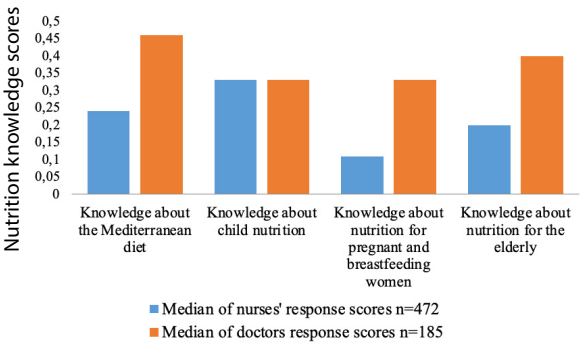

Fig. 1. Distribution of the knowledge of doctors and nurses about Mediterranean diet nutrition, nutrition for children, pregnant and breastfeeding women, and the elderly

Relationship between the median of the total score from answers of health professionals (doctors and nurses) to questions on nutritional knowledge in relation to primary prevention and their sociodemographic variables

To analyze the association between nutritional knowledge and sociodemographic variables, the median of nutritional knowledge in relation to primary prevention of all participants was calculated (Table 3).

The present study identified a significant ($p<0.001$) association between the median of answers of all participants to questions related to primary prevention and their degree (doctors or nurses). Furthermore, the analysis suggests a significant difference between health professionals who received a nutrition course during basic training and those who did not ($p<0.001$). Furthermore, the reception of nutrition information on nutrition

Table 2. Knowledge of physicians and nurses about Mediterranean diet nutrition

Variables	Score of answers (% of correct answers of nurses, n=472)	Score of answers (% of correct answers of doctors, n=185)	Score of total answers (% of total correct answers)	p	Effect size
Knowledge about the Mediterranean diet					
Frequency of fruit and vegetable consumption	0.26 (26.4)	0.38 (38.9)	0.30 (32.65)	0.02	0.015
Preferred form of vegetables	0.24 (23.9)	0.33 (31.9)	0.26 (27.90)	0.037	0.007
Preferred fruit shape	0.46 (45.8)	0.82 (81.1)	0.56 (63.45)	0.000	0.102
Frequency of consumption of (bread, cereals, potatoes legumes)	0.26 (25.4)	0.29 (28.2)	0.26 (26.80)	0.474	0.001
Frequency of pulse consumption	0.15 (14.8)	0.27 (26.9)	0.18 (20.85)	0.000	0.020
Preferred grain type	0.60 (60.0)	0.85 (85.8)	0.67 (72.90)	0.000	0.061
Frequency of consumption of milk and dairy products	0.25 (25.0)	0.39 (38.5)	0.29 (31.75)	0.001	0.018
Frequency of consumption of Meat, poultry, fish, eggs)	0.21 (21.6)	0.49 (47.0)	0.29 (34.30)	0.000	0.064
which food to favor(Meat, poultry, fish and eggs)	0.14 (13.6)	0.24 (21.6)	0.16 (17.60)	0.012	0.010
Frequency of fish consumption	0.26 (26.1)	0.44 (44.9)	0.31 (35.50)	0.000	0.033
Fats to favor daily	0.08 (7.8)	0.26 (24.9)	0.13 (16.35)	0.000	0.053
Sweet products to consume occasionally	0.15 (15.3)	0.45 (44.0)	0.23 (29.65)	0.000	0.093
Preferred drinks	0.13 (12.9)	0.42 (43.6)	0.21 (28.25)	0.000	0.111
Scores' median of answers (% of total correct answers)	0.24 (24.5)	0.46 (42.84)	0.23 (33.67)	0.000	0.196
Interquartile ranges	[0.15, 0.30]	[0.30, 0.53]	[0.15, 0.46]		
Knowledge about nutrition for children					
Exclusive breast feeding from 0 to 6 months	0.63 (62.5)	0.74 (73.5)	0.66 (68.00)	0.008	0.011
Complementary foods to give to the child other than breast milk from 0 to 6 months	0.12 (11.7)	0.23 (23.2)	0.15 (17.45)	0.000	0.021
Complementary foods to give the child other than breast milk from 6 to 12 months	0.16 (16.1)	0.20 (20.0)	0.17 (18.05)	0.234	0.002
Median of answers (% of total correct answers)	0.33 (30.1)	0.33 (38.9)	0.33 (34.50)	0.002	0.014
Interquartile ranges	[0, 0.33]	[0.33, 0.33]	[0.33, 0.33]		
Knowledge of nutrition for pregnant and breastfeeding women					
Priority fruits and vegetables for pregnant women	0.25 (24.6)	0.33 (32.4)	0.27 (28.50)	0.041	0.006
Frequency of consumption of (bread, cereals, potatoes and legumes)	0.14 (14.4)	0.32 (33.0)	0.20 (23.70)	0.000	0.044
Frequency of consumption of milk and dairy products	0.14 (13.6)	0.19 (19.5)	0.15 (16.55)	0.058	0.005
Milk and dairy products authorized for pregnant women	0.14 (14.4)	0.27 (27.2)	0.18 (20.80)	0.000	0.022
Milk and dairy products authorized for breastfeeding women	0.15 (15.3)	0.31 (31.3)	0.20 (23.30)	0.000	0.033
Meats allowed to pregnant women	0.20 (19.7)	0.43 (43.8)	0.26 (31.75)	0.000	0.060
Meats allowed to breastfeeding women	0.14 (14.4)	0.32 (31.4)	0.19 (22.90)	0.000	0.037
Preferred fish for pregnant and breastfeeding women	0.23 (22.9)	0.32 (33.5)	0.26 (28.20)	0.005	0.012
Drinks recommended	0.16 (16.1)	0.36 (36.8)	0.22 (26.45)	0.000	0.050
Median of answers (% of total correct answers)	0. 11 (17.26)	0.33 (32.1)	0.11(24.68)	0.000	0.154
Interquartile ranges	[0, 0.22]	[0.22, 0.44]	[0, 0.33]		
Knowledge about nutrition for the elderly					
Fruit and vegetables preferred by the elderly	0.27 (27.10)	0.35 (34.1)	0.29 (30.6)	0.080	0.005
Frequency of consumption of (bread, cereals, potatoes and legumes)	0.15 (14.60)	0.30 (30.8)	0.19 (22.7)	0.000	0.034
Frequency of consumption of milk and dairy products	0.14 (13.60)	0.32 (31.9)	0.19 (22.75)	0.000	0.044
Which food should limit(meat, poultry, fish, eggs)	0.34 (34.10)	0.49 (47.0)	0.38 (40.55)	0.002	0.014
Drinks recommended	0.23 (22.70)	0.57 (56.8)	0.32 (39.75)	0.000	0.107
Median score of answers (% of total correct answers)	0.2 (22.42)	0.4 (40.12)	0.20 (31.27)	0.000	0.113
Interquartile ranges	[0, 0.4]	[0.2, 0.6]	[0, 0.40]		
Median score of answers of the nutritional knowledge in relation to primary prevention (% of total correct answers)	0.21 (23.57)	0.36 (38.49)	0.24 (31.03)	0.000	0.211
Interquartile ranges	[0.15, 0.32]	[0.27, 0.50]	[0.16, 0.35]		

(p<0.001) and the workplace of physicians and nurses (p<0.001) are significantly associated with the median of -nutritional knowledge.

Table 3. Association between the median of answers of health professionals (doctors and nurses) to questions on nutritional knowledge in relation to primary prevention and their sociodemographic variables *

Variables	Correlation coefficient (r)	Median (Interquartile ranges)	Raw analysis p	Adjusted analysis p	Effect size
		Gender			
Female		0.24 [0.16, 0.35]	0.837		$6.04*10^{-5}$
Male		0.25 [0.16, 0.36]			
Age	0.169		0.00	0.166	0.0028
		Diploma			
Doctors		0.36 [0.27,0.50]	0.00	0.00	
Nurses		0.21 [0.15,0.32]			0.211
		Work place			
Primary healthcare Center		0.31 [0.2, 0.4]	0.00	0.00	0.104
Hospital		0.2 [0.12, 0.3]			
		Basic training			
Yes		0.22 [0.15, 0.33]	0.00	0.00	0.056
No		0.31 [0.23, 0.41]			
		Continuing training			
Yes		0.23 [0.15, 0,5]	0.172		0.003
No		0.25 [0.16, 0.35]			
		Receiving information[a]			
Yes		0.31 [0.2, 0.41]	0.00	0.00	0.045
No		0.24 [0.15, 0.32]			

* [a] receiving information: reception of information through official documents from the Ministry of health such as Moroccan nutrition guides, national nutrition strategy guide, etc.

The results show that the age of the participants is positively correlated with the mean score (r = 0.169, p<0.001). However, no statistically significant differences between the median on based on gender (p=0.83) or on continuing training (0.172) were found. Multivariate analysis confirmed the results of bivariate tests concerning the association between scores' median of nutrition response to the questions on nutrition in relation to primary prevention and sociodemographic dimensions with the exception of age (p=0.166), this variable is not significantly associated with the median of the response scores

The size of the effect of the Mann-Whitney test showed that diploma (r=0.211), continuing training (0.201), and gender (r=0.02) had a small effect. Furthermore, for basic education (r=0.78) and in the workplace it is high (r=0.61), but for receiving nutritional information it is moderate (r=0.38). Regarding age, it appears that the latter has a low explanatory capacity (r=0.028) for the differences observed between the median based on age.

Discussion

The study results reveal that, for both doctors and nurses, response scores to questions regarding the four domains studied are unsatisfactory, remaining well below 1. This raises questions about the nutrition training provided to these groups. A low level of knowledge can lead to inappropriate recommendations, harming patients' health, and reducing the risk of chronic diseases.

Among doctors, the highest scores were obtained for the Mediterranean diet (0.46), while nurses achieved their best results for infant nutrition (0.33). These differences may be explained by the fact that doctors, generally specialized in disease diagnosis and management, tend to attach greater importance to the Mediterranean diet, recognized for its benefits in cardiovascular health and the prevention of chronic diseases. On the contrary, nurses, often on the front lines of care, have more direct experience with infants and children, which explains their higher score (0.33) for infant nutrition. They play a vital role in educating parents about nutrition and child development. These results are suboptimal compared to other studies conducted in Arab countries, Asia, and in Croatia, where participants' (doctors and nurses') response scores to nutritional questions were above 0.5.[24-27]

Concerning the Mediterranean diet, the analysis of the interquartile range (IQR) shows greater variability in knowledge between doctors (IQR of [0.30, 0.53]) compared to nurses (IQR of [0.15, 0.30]), who show scores concentrated in a lower range. Although most doctors and a significant proportion of nurses correctly answered questions about some aspects of the Mediterranean diet. For physicians, this result is lower than that of Spain (0.55), but almost similar to the study in Las-Palmas (0.41).[28,29] Regarding nurses, the scores found in Las Palmas are higher than our result (0.40).[29]

Regarding the elderly, the interquartile range of doctor response scores (IQR [0.2, 0.6]) indicates greater knowledge and diversity of understanding, probably due to their varied clinical experiences, specialized training, or particular interest in nutritional issues related to the elderly. In the literature, although several studies have evaluated the knowledge of physicians in areas of nutrition for adults over 18 years of age, no studies evaluating the knowledge of the specific nutritional needs of the elderly were found. Our study seems to be the first to investigate this interesting aspect. On the contrary, for nurses, the interquartile range (IQR [0, 0.4]) suggests limited knowledge and possibly highlights gaps in their ability to adequately counsel elderly patients. The small number of similar studies reported in the literature a revealed generally higher score of answers than ours.[30-32]

The results on doctors and nurses' knowledge of infant nutrition highlight worrying gaps in parental education about their children's nutrition, which is essential

to prevent malnutrition-related diseases. In fact, nearly a quarter of doctors do not recognize the importance of exclusive breastfeeding during the first six months. This result is similar to a literature study, but lower than a study conducted in Baghdad reported that the score of doctors' responses to questions on child nutrition is 0.50.[25,34,35] This result remains very low in comparison with the Australian health professional population, which reported a score of 0.75.[33]

For nurses, the interquartile range reveals a lower variation (IQR of [0, 0.033]), which reveals a virtual lack of knowledge on this crucial subject. This result is lower than that of Ghana (0.54).[32]

The results on the nutritional knowledge of pregnant and breastfeeding women reveal significant differences between doctors and nurses, as well as a general concern about their level of knowledge. For physicians, the interquartile range (IQR [0.22, 0.44) indicates that the majority of them have low knowledge but some physicians show higher scores, which indicates that they have a better understanding of nutritional issues related to pregnancy and breastfeeding. This result is lower than that reported in Saudi Arabia (0.70) and Croatia (0.86).[27,37] In contrast, the interquartile range (IQR) of [0, 0.22] among nurses indicates a concerning lack of knowledge in this critical area. This situation is particularly alarming given the importance of providing appropriate nutritional guidance for the health of both mothers and their breastfeeding infants. This result is lower than that of a recent study conducted in Australia, which reported a value of 0.42 among all participants, including both physicians and nurses.[36] For nurses specifically, this finding is particularly concerning, as it indicates suboptimal levels compared to the researchers' conclusion of 0.54.[32]

The significant association ($p<0.001$) between the median response scores of healthcare professionals and their qualifications highlights the importance of adequate training and practical experience. Furthermore, the lack of association with demographic factors such as age ($p=0.166$) and sex ($p = 0.83$), indicates that other factors influence nutritional skills. This highlights the need to focus on experience and training; however, it is worth noting, some studies have noted an association with age.[32,38]

The study reveals a lack of adequate nutrition training for doctors and nurses, which is comparable to a study in the United States showing that only 25% of medical schools integrate nutrition into their curricula.[39] Although most nurses attend classes, many feel ill prepared to manage nutritional issues, highlighting gaps in their training.

The significant association ($p<0.001$) between basic training and scores' median nutrition knowledge highlights the importance of solid training in this area. Furthermore, a similar study also highlights the need for continuing training to maintain and update these skills.[41] It is crucial to improve both the basic and continuing training of healthcare professionals to enable them to effectively advise their patients on these issues. Furthermore, a significant relationship was observed between access to official nutritional guidelines and knowledge scores, indicating that regular updating recommendations can improve understanding.[42,44] In New Zealand, some midwives rely on documents from the Ministry of Health (47%), while others consult dietitians (53%).[42] They also use various resources, such as professional journals, websites and social media.[25,43] Regular access to these sources of information is essential for strengthening nutritional knowledge of healthcare professionals.

The significant association between the workplace and the median for doctors and nurses ($p=0.00$) reveals that participants who worked in health centers scored higher than those who worked in hospitals. This suggests that the activities at these centers are more focused on prevention, which encourages health professionals to engage in more self-training. On the contrary, in hospitals, the focus is primarily on curative care rather than preventive care. This finding is consistent with other previous research.[40,45,46]

The small effect of the Mann-Whitney test reveals that the median for doctors and nurses are very similar, indicating that the type of degree has no significant impact on the results. Similarly, the relationship between gender and scores is negligible, suggesting that other factors, such as previous training and professional experience, have a greater influence on nutritional knowledge.

Study limitations

The limitation of this study is that private sector professionals were not included in our study; in addition, the study was conducted in the northern region of Morocco.

Although basic training is unified, continuing training depends on the continuing training unit of the regions. Young doctors and nurses with less experience were relatively overrepresented in the sample. This could be due to selection bias, as it was difficult to obtain consent from relatively older doctors and nurses, who only concerned 26 professionals in the public sector.

Conclusion

In conclusion, this study revealed significant gaps in the nutritional knowledge of physicians and healthcare professionals on the primary prevention of non-communicable diseases. It appears that primary care health workers show better skills in nutrition compared to those working in hospitals, although this knowledge remains below expectations. As primary care physicians and nurses are on the front lines of providing care, it is

crucial that they receive continuing education in nutrition to provide appropriate and reliable advice to both patients and the general public. However, our results indicate that there is an urgent need to improve the nutritional knowledge of physicians and nurses in Morocco. Therefore, policy makers must actively engage in promoting educational initiatives in nutrition, both at the level of basic education and at the level of continuing education in faculties of medicine and nursing, to ensure that health professionals have the necessary skills to effectively advise the population.

Declarations

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Author contributions
Conceptualization, N.E. and A.N.; Methodology, Z.B.; Software, Z.B.; Validation, N.E., A.N. and Z.B.; Formal Analysis, A.N.; Investigation, Z.B.; Resources, Z.B.; Data Curation, Z.B.; Writing – Z.B and N.E Original Draft Preparation, Z.B.; Writing – Review & Editing, N.E. and A.N; Visualization, N.E.; Supervision, N.E.

Conflicts of interest
Authors declared they have no conflicts of interest.

Data availability
All the data generated or analyzed during this study are included in this published article.

Ethics approval
Informed consent was obtained from all participants.

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REVIEW PAPER

A brief review of the cardiovascular complication of COVID-19 – what is the pathophysiology of arrhythmia during infection?

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ABSTRACT

Introduction and aim. COVID-19, caused by SARS-CoV-2, significantly affects the cardiovascular system beyond its respiratory manifestations. This review examines the intricate relationship between COVID-19 and cardiovascular complications, focusing on cardiac arrhythmias and their underlying pathophysiology.

Material and methods. A comprehensive literature search was conducted in PubMed and Google Scholar from its inception to December 2024, including peer-reviewed articles published in English.

Analysis of literature. In COVID-19, a spectrum of cardiovascular complications is observed, including acute myocardial infarction, arrhythmias, myocarditis, venous thromboembolism, and heart failure/cardiac shock. The pathophysiology of cardiovascular damage in COVID-19 involves multiple mechanisms, primarily including direct viral cardiotoxicity, systemic inflammation, and hypercoagulability. Arrhythmias are a common cardiac complication in COVID-19, encompassing a range of disturbances, from bradycardia to ventricular fibrillation. The mechanisms underlying arrhythmias in COVID-19 are multifaceted, including direct viral injury to cardiomyocytes, hypoxia, systemic inflammation, hyperthermia, autonomic imbalance, electrolyte imbalances, side effects of medications, and drug-drug interactions.

Conclusion. Understanding the complex interplay of these factors is crucial for the early diagnosis and appropriate management of cardiac complications in patients with COVID-19. To mitigate cardiovascular morbidity and mortality in individuals with COVID-19, cardiovascular monitoring and the development of targeted therapeutic strategies are highly recommended.

Keywords. arrhythmias, cardiovascular diseases, coronavirus, COVID-19, pathophysiology

Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has presented an unprecedented global health crisis, requiring a comprehensive understanding of its multifaceted impact on human physiology.[1] While the virus primarily targets the respiratory system, a growing body of evidence reveals a complex and significant interplay between SARS-CoV-2 and the cardiovascular system, echoing observations from previous coronavirus outbreaks such as SARS and MERS.[2,3] This cardiovascular involvement extends beyond the

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acute phase of the disease, raising concerns about long-term cardiac sequelae and contributes significantly to both morbidity and mortality.

The pathophysiology of COVID-19-related cardiovascular complications is complex and multifactorial. The proposed mechanisms include direct viral cardiotoxicity, systemic inflammation ("cytokine storm"), endothelial dysfunction, and a hypercoagulable state.[4] These processes can contribute to myocardial injury, plaque instability, microvascular dysfunction, and ultimately, a variety of cardiovascular events.[5] Preexisting cardiovascular conditions, such as hypertension, coronary artery disease, and heart failure, significantly increase the risk of severe COVID-19 and the development of cardiovascular complications.[6,7] Furthermore, established cardiovascular risk factors, including advanced age, diabetes mellitus, and obesity, also play a crucial role in modulating the severity of COVID-19 and its cardiovascular manifestations.[8,9] This complex interaction is further underscored by laboratory findings in patients with COVID-19, which frequently reveal elevated cardiac biomarkers, including troponin and brain natriuretic peptide (BNP).[10,11] These elevated biomarkers serve as indicators of cardiac stress and injury, offering critical insights into the pathophysiological mechanisms linking COVID-19 and cardiovascular health. The interplay of these factors creates a complex clinical picture, necessitating a deeper understanding of the underlying mechanisms to guide effective management strategies.

Cardiovascular complications associated with COVID-19 are diverse, encompassing a spectrum of conditions including myocardial injury, heart failure, vascular dysfunction, and thromboembolic events. 3 These complications can manifest acutely, during convalescence, and may persist for months or years after initial infection, posing a significant challenge for long-term health management.[12] In particular, cardiac arrhythmias, ranging from benign palpitations to life-threatening ventricular fibrillation, have emerged as a particularly prevalent and concerning manifestation of COVID-19 in the cardiovascular system.[13] Studies have reported a prevalence as high as 17% in hospitalized non-ICU patients and exceeding 44% in critically ill ICU patients.[3,14] This high prevalence underscores the importance of understanding the mechanisms underlying these rhythm disturbances.

While the clinical manifestations of COVID-19-related arrhythmias are increasingly recognized, the precise pathophysiological mechanisms that drive these rhythm disturbances remain incompletely elucidated. Several contributing factors have been proposed, including direct viral infection of cardiomyocytes, hypoxia secondary to respiratory involvement, systemic inflammation, autonomic dysfunction, electrolyte imbalances, and the pro-arrhythmic effects of certain medications used to treat COVID-19.[13,15] However, the relative contribution of each of these factors and their complex interactions require further investigation. This knowledge gap hinders the development of targeted therapies and preventive strategies to mitigate the risk of arrhythmias in patients with COVID-19.

This review aims to address this critical need by providing an update and comprehensive overview of current understanding of COVID-19-associated cardiovascular complications associated with COVID-19, with a particular focus on arrhythmia. This review explores the various proposed mechanisms that contribute to arrhythmogenesis in the context of COVID-19, including direct viral effects, the role of inflammation and the immune system, and the influence of other contributing factors such as hypoxia, autonomic dysfunction, and drug-induced prolongation of QT. By synthesizing the existing evidence, this review seeks to inform clinicians, researchers, and policymakers, ultimately contributing to the improved diagnosis, treatment, and prevention of this significant cardiovascular complication of COVID-19. In addition, this review offers a unique contribution by focusing specifically on the pathophysiological mechanisms of arrhythmias, providing a more detailed and mechanistic perspective. This focus is crucial to developing targeted interventions and mitigating the burden of chronic cardiovascular sequelae in COVID-19 survivors.

Aim

This review aims to explore the multifaceted impact of COVID-19 on the cardiovascular system, with a particular focus on the clinical complication and underlying pathophysiology of cardiac arrhythmias. The various types of arrhythmias in COVID-19 and their underlying mechanisms are also elaborated.

Material and methods

A comprehensive literature search was conducted in PubMed and Google Scholar from its inception to December 2024, including peer-reviewed articles published in English. For searching articles, the authors used the following keywords: "cardiovascular", "myocardial injury", "cardiac injury" "COVID-19", "coronavirus", "heart failure", "coagulable", "thrombosis", "embolism", "arrhythmia", "dysrhythmia", "myocardial infarction", "acute coronary syndrome", "myocarditis". The search included systematic reviews, meta-analyses, randomized controlled trials, observational studies, narrative reviews, case series, case reports, and clinical guidelines focusing on cardiovascular complications in COVID-19. Pre-print articles were excluded. The reference lists of identified articles were meticulously reviewed. Two authors were independently assigned to search for articles relevant to their respective topics. Any discrepancies be-

tween the two authors were resolved through discussion and consensus with a third author.

Analysis of the literature

Pathophysiology of cardiovascular damage in COVID-19

Patients with myocardial damage exhibit increased morbidity and mortality from COVID-19.[16] The precise mechanisms underlying cardiovascular damage in COVID-19 remain under investigation and likely involve multiple factors. Although some studies suggest direct viral cardiotoxicity, the virus can also induce a hyperinflammatory state through the release of inflammatory cytokines.[4] This can lead to vascular inflammation, plaque instability, myocardial inflammation, hypercoagulability, and direct myocardial depression. The systemic complications of COVID-19, such as sepsis and disseminated intravascular coagulation (DIC), can also contribute to cardiac damage.[5] The pathophysiological processes involved in cardiovascular damage during COVID-19 and their manifestation are illustrated in Figure 1.

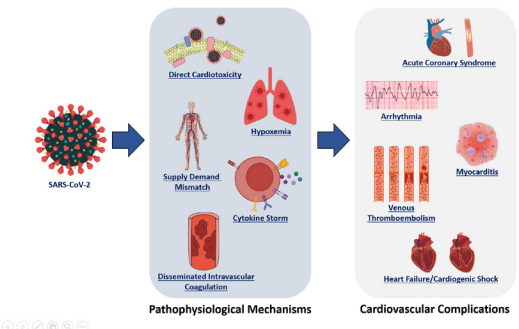

Fig. 1. Pathophysiological mechanisms underlying cardiovascular damage and their clinical manifestation in COVID-19. SARS-CoV-2 infection can induce direct cardiotoxicity, respiratory distress leading to hypoxemia, hemodynamic instability leading to a mismatch of oxygen and nutrient supply-demand, high levels of inflammatory molecules ('cytokine storm') and damage to the endothelium that contributes to hypercoagulable state such as disseminated intravascular coagulation (DIC), the pathological process during the infection period can cause a spectrum of cardiovascular complications, including acute myocardial infarction, arrhythmias, myocarditis, venous thromboembolism, and heart failure/cardiogenic shock

Cardiac biomarker in COVID-19

A modest increase in cardiac troponin levels (<2-3 times the upper limit of normal) can be attributed to pre-existing cardiac conditions or acute myocardial injury induced by COVID-19.[10] In critically ill patients with COVID-19 and deceased, significantly higher cardiac troponin levels (>5 times the upper limit of normal) are associated with severe respiratory failure, tachycardia, systemic hypoxemia, myocardial injury, endothelial dysfunction (which can lead to plaque rupture and acute coronary syndrome), Takotsubo cardiomyopathy, or multiorgan failure.[10,17] Severe inflammatory or respiratory conditions can lead to elevated levels of brain natriuretic peptide (BNP) or N-terminal pro-BNP (NT-proBNP).[11,18] These biomarkers are closely correlated with cardiac ventricular stress. Furthermore, elevated D-dimer levels are associated with an unfavorable prognosis for COVID-19.19-21. The D-dimer reflects an activated coagulation state, a key characteristic of the disease. Serial D-dimer measurements can help guide prophylactic anticoagulation strategies for venous thromboembolism (VTE).[22]

Cardiovascular complication of COVID-19

Acute myocardial infarction

COVID-19 significantly increases the risk of acute myocardial infarction (AMI).[23] Systemic inflammations, a key feature of the disease, destabilize atherosclerotic plaques, making them more prone to rupture.[24] This, combined with a hypercoagulable state induced by the virus, increases the risk of blood clot formation within the coronary arteries.[25] Treatment of AMI in COVID-19 patients presents unique challenges. Although percutaneous coronary intervention (PCI) remains the preferred treatment for many STEMI cases, careful consideration is crucial, especially in patients with suspected or confirmed COVID-19.[26] Healthcare providers must balance the need for prompt revascularization with the potential for viral transmission and the need for adequate personal protective equipment.[27] In select cases, a conservative treatment approach can be considered, particularly in stable patients with non-ST elevation myocardial infarction.[26] This highlights the critical importance of a multidisciplinary approach and careful risk-benefit assessment when managing AMI in the context of COVID-19.

Arrhythmia

COVID-19 has been shown to significantly affect cardiac rhythm.[15] Although palpitations were reported in 7.3% of patients in one study, the true prevalence of arrhythmias remains uncertain due to limitations in data collection and the potential for under-reporting.[28] The mechanisms underlying COVID-19-associated arrhythmias are multifaceted. Direct viral injury to cardiomyocytes, pericardial inflammation, microvascular dysfunction, myocardial fibrosis, and the influence of pro-inflammatory cytokines can all contribute to the development of arrhythmias.[13] Viral interaction with the renin-angiotensin-aldosterone system can lead to electrolyte imbalances, potentially increasing arrhythmia susceptibility.[29] Furthermore, certain COVID-19 therapeutic agents of COVID-19, particularly chloroquine phosphate and hydroxychloroquine sulfate, can increase arrhythmia risk through QT interval.30 In the management of stable patients with arrhythmias in

the context of COVID-19, clinical focus should initially be directed toward addressing the underlying respiratory distress and fever, rather than immediately implementing strategies to reduce heart rate.[31] Adherence to established treatment protocols is paramount, with amiodarone considered the preferred antiarrhythmic medication for the management of unstable patients or those experiencing heart failure.[31] For cases involving unstable atrial arrhythmias or dangerous ventricular arrhythmias, immediate cardiology consultation and strict adherence to advanced cardiac life support guidelines are essential.[28] The management of arrhythmias in COVID-19 requires a cautious and individualized approach, considering factors such as hemodynamic stability, potential drug interactions, and the presence of underlying conditions. Early recognition and appropriate management of these cardiac complications are crucial to optimize patient outcomes.

Myocarditis

Myocarditis, an inflammation of the heart muscle, is a concerning complication of COVID-19.[32] These findings echo observations from previous coronavirus outbreaks such as MERS-CoV, highlighting the potential for cardiac involvement in viral infections.[33] Myocarditis is associated with worse outcomes in patients with COVID-19, including an increased risk of death and long-term cardiac complications.[34] The clinical presentation of myocarditis in COVID-19 can range from mild symptoms to severe heart failure. Common symptoms include chest pain, shortness of breath, palpitations, and fatigue.[32] However, myocarditis can be asymptomatic in some cases, making early detection crucial. Studies have shown that a significant proportion of hospitalized COVID-19 patients exhibit elevated troponin levels, indicating myocardial injury.[35] Differentiating myocarditis from other conditions, such as acute coronary syndrome, can be challenging. Accurate diagnosis is based on a combination of clinical evaluation, electrocardiograms, echocardiography, and cardiac biomarker analysis. Early recognition and appropriate management are essential to improve patient outcomes.[3]

VTE

VTE is a significant complication of COVID-19.[36] COVID-19 presents a significant risk of VTE through multiple pathophysiological mechanisms, including systemic inflammatory response, coagulopathy, and multiple organ dysfunction syndrome.[37] Prospective studies have documented consistent abnormalities in coagulation parameters among patients with COVID-19, with elevated D-dimer levels emerging as a particularly significant biomarker.[38-40] Clinical research has established that D-dimer elevations exceeding 1 μg/mL correlate with an 18.4 times increased risk of in-hospital mortality among COVID-19 patients.[40] Clinical evidence supports the implementation of prophylactic anticoagulation therapy, with low molecular weight heparin demonstrating particular efficacy in reducing mortality rates among cases of severe COVID-19 cases.[41] The therapeutic benefits of anticoagulation are most evident in patients with D-dimer levels exceeding six times the established upper limit of normal.[42] These clinical observations underscore the critical importance of vigilant monitoring of coagulation parameters and the timely initiation of appropriate anticoagulation therapy, particularly in patients with severe COVID-19 manifestations or marked D-dimer elevations. Regular evaluation of thrombotic risk and proactive therapeutic intervention can significantly improve clinical outcomes in this high-risk patient population.[43]

Heart failure/cardiogenic shock

Heart failure (HF) and cardiogenic shock have been identified as the primary contributors to morbidity and mortality in COVID-19, and clinical studies documenting HF in approximately 23-33% of cases.[40,44] While the precise pathophysiological mechanisms of ventricular dysfunction in COVID-19 have not yet been fully elucidated, a subset of patients develops dilated cardiomyopathy, manifesting as diminished left ventricular systolic function and cardiogenic shock, despite no previous cardiac dysfunction.[45] The pathogenesis of COVID-19-associated HF encompasses multiple mechanisms, including direct myocardial injury, systemic inflammation, pulmonary hypertension, acute respiratory distress syndrome, renal dysfunction, fluid overload, and myocardial oxygen supply-demand mismatch.[28,46] Current therapeutic guidelines advocate for diuresis and standard heart failure protocols, maintaining angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) administration when no acute contraindications exist.[47] Patients presenting with new-onset systolic dysfunction require comprehensive cardiac evaluation, including cardiology consultation, echocardiographic assessment, and biomarker analysis.[31] In cases refractory to conventional therapies, extracorporeal membrane oxygenation (ECMO) may be considered, although preliminary data indicate poor outcomes with mortality rates over 80%.[48] The Extracorporeal Life Support Organization has issued guidelines restricting ECMO implementation to specialized centers for severe ARDS cases, emphasizing the necessity of multidisciplinary evaluation and recommending discontinuation if no recovery is evident after three weeks of support.[49]

Epidemiology of arrhythmias in COVID-19

Arrhythmias constitute the most common cardiac manifestation in patients with COVID-19, with clinical presentations ranging from palpitations to life-threatening

arrhythmias.[50] Patients with COVID-19 exhibit an increased risk of arrhythmias due to a confluence of factors, including pre-existing comorbidities, exposure to medication and the disease's progressive nature of the disease.[51] In a study encompassing 137 patients, 7.3% reported palpitations as a symptom.[52] Cardiac arrhythmias are highly prevalent in critically ill patients with COVID-19, occurring in approximately 19% to 36% of cases, depending on the presence of pre-existing cardiac abnormalities.[53] The most common tachyarrhythmias observed in patients with COVID-19 include atrial fibrillation, atrial flutter, and ventricular tachycardia/fibrillation.[54] The risk of arrhythmias in COVID-19 is multifactorial, encompassing hypoxia, myocarditis, myocardial strain, myocardial ischemia, medication effects, fluid and electrolyte imbalances, and systemic inflammatory responses.[13]

Pathophysiology of arrhythmia in COVID-19

SARS-CoV-2 can directly induce cardiomyocyte damage. The virus enters cells by binding to the ACE-2 receptor on the cell surface, a process facilitated by transmembrane serine protease 2 (TMPRSS2) through the cleavage of the viral spike protein.[55] At entry, the virus utilizes the NF-κB signaling pathway for replication. Activation of NF-κB can influence the expression of the pore-forming subunit of the fast transient outward potassium current, which can contribute to the development of arrhythmias.[56] Furthermore, activation of protein kinase C and oxidation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) can also contribute to arrhythmia in COVID-19. Histopathological findings in the myocardium may include irregularity, cytoplasmic darkening, minimal fibrosis, and mild hypertrophy.[57] These findings suggest ongoing myocarditis and can contribute to the development by inducing electrical abnormalities.[56]

Hypoxemia, a consequence of respiratory dysfunction in COVID-19, creates a relatively hypoxic environment within the myocardium.[58] Hypoxia can induce cardiomyocyte cell death and disrupt ion channel function, leading to alterations in the duration and / or repolarization of the action potential, increasing the risk of arrhythmias.[59]

Pro-inflammatory cytokines are significantly elevated in COVID-19 patients.[60] In particular, interleukin-6 (IL-6) plays a crucial role.[61] Acute exposure to IL-6 significantly increases the current density of L-type calcium channels and improves the amplitude and duration of calcium transients in ventricular cardiomyocytes. Chronic exposure to IL-6 can significantly disrupt intracellular calcium homeostasis. Furthermore, IL-6 can degrade the proteins that connect atrial myocytes, leading to atrial electrical remodeling of the atrium. Elevated IL-6 levels in patients with COVID-19 can therefore contribute to the development by altering cardiac electrophysiology.[56]

Hyperthermic states such as fever, a common symptom of COVID-19, can exacerbate the risk of arrhythmias.62 In individuals with preexisting cardiac conditions (e.g. dilated cardiomyopathy), fever may precipitate ventricular fibrillation.[63] Even in a structurally normal heart, fever can trigger tachyarrhythmias.[64] This may be partly attributed to the temperature-dependent effects on sodium channel function, potentially disrupting the ionic current migration of cardiomyocytes and attenuating the efficacy of antiarrhythmic medications.[56,64,65]

Table 1. Factors contributing to cardiac arrhythmias in COVID-19[26,28,69]

Contributing factor	Mechanism
Direct viral cardiotoxicity	Viral infection of cardiomyocytes leading to myocardial injury can contribute to the development by inducing electrical abnormalities
Drug-induced side effects and drug-drug interactions	Medications used to treat COVID-19, particularly chloroquine phosphate and hydroxychloroquine sulfate, may increase the risk of arrhythmias by prolonging the QT interval. This proarrhythmic effect is further amplified when coadministered with Azithromycin or Lopinavir/ Ritonavir
Hypoxia and pulmonary disease	Respiratory insufficiency can induce cardiomyocyte cell death and disrupt ion channel function, leading to alterations in the duration of the action potential and / or repolarization, predisposing to arrhythmias
Systemic inflammation	Chronic exposure to inflammatory cytokines during infection can degrade atrial myocytes, leading to atrial electrical remodelling and arrhythmias
Hyperthermia	Hyperthermic conditions (eg, fever) may have temperature-dependent effects on sodium channel function, potentially attenuating the efficacy of antiarrhythmic medications and inducing ventricular fibrillation
Autonomic imbalance	Sympathetic nervous system overactivity and vagal nerve dysfunction, potentially induced by the virus after acute stress, can disrupt cardiac rhythm
Electrolyte abnormalities	Virus interaction with the renin-angiotensin-aldosterone system can lead to electrolyte imbalances, potentially increasing arrhythmia susceptibility
Preexisting cardiovascular comorbidities	Individuals with underlying heart conditions, such as hypertension, coronary artery disease, and heart failure, are at a significantly higher risk of severe COVID-19 and may experience a higher incidence of arrhythmias

Treatment of COVID-19 involves the use of various medications, some of which can have adverse effects on cardiac function. The prolongation of the QT interval and Torsades de Pointes are potential side effects of chloroquine, hydroxychloroquine, favipiravir, lopinavir/ritonavir, azithromycin, moxifloxacin and, in certain cases, piperacillin-tazobactam.[66]

Other factors that contribute to cardiac arrhythmias in COVID-19 include autonomic imbalance (due to sympathetic nervous system overactivity and vagal nerve dysfunction), electrolyte abnormalities (due to virus interaction with the renin-angiotensin-aldosterone

system), and preexisting cardiovascular comorbidities (eg hypertension, coronary artery disease, and heart failure).[67] All of the contributing factors for arrhythmias in COVID-19 and their underlying mechanisms are summarized in Table 1.

Type of arrhythmias in COVID-19

Understanding arrhythmic complications in COVID-19 patients is constantly evolving. Observed cardiac arrhythmias in patients with COVID-19 include sinus tachycardia, sinus bradycardia, atrioventricular block, bundle branch block, atrial premature complexes, atrial fibrillation, supraventricular tachycardia, ventricular premature complexes, non-sustained ventricular tachycardia, polymorphic ventricular tachycardia (eg Torsades de Pointes), sustained ventricular tachycardia, ventricular fibrillation, and pulseless electrical activity.[29,67] The schematic picture of the various arrhythmias encountered in patients with COVID-19 is depicted in Figure 2.

Fig. 2. This schema depicts the diverse spectrum of arrhythmias observed in patients with COVID-19 (adapted from Manolis et al., Trends Cardiovasc Med 2020;30:451-60).[69] These arrhythmias arise from a complex interplay of factors, including direct cardiac effects of the virus, such as myocarditis and myocardial injury, systemic inflammation triggered by infection, the adverse (proarrhythmic) effects of COVID-19 therapies, drug-drug interactions, respiratory complications such as hypoxemia and hypercapnia, autonomic nervous system dysfunction, and electrolyte imbalances, the schema includes atrial fibrillation, atrioventricular block, long QT syndrome, pulseless electrical activity, sinus bradycardia, sudden cardiac death, sinus tachycardia, Torsades de Pointes, ventricular arrhythmias, ventricular fibrillation, and ventricular tachycardia

Bradycardia

Bradycardia can serve as a marker for the onset of a severe cytokine storm. In a retrospective case series of four patients, transient bradycardia was observed to persist for 1-14 days in patients with COVID-19, necessitating close monitoring.[68] The most common etiologies include severe hypoxia, inflammatory injury to the sinus node mediated by circulating cytokines, and an exaggerated response to medications.[67]

Sinus tachycardia

Sinus tachycardia, a supraventricular tachycardia, is a frequently reported rhythm disturbance in patients with COVID-19, with an incidence of approximately 72%.[56] This arrhythmia may be triggered by fever, hypoxemia/respiratory insufficiency, hemodynamic disturbances, anxiety/fear, pain, and various other physical and emotional stressors.[3,67]

Postural orthostatic tachycardia syndrome

Postural orthostatic tachycardia syndrome is a condition arising from autonomic dysfunction. The underlying mechanisms can involve peripheral neuropathy, elevated serum norepinephrine levels, baroreceptor dysfunction, or hypovolemia.[69] This syndrome has been reported to develop after acute stress, including viral illnesses, and has been observed in some patients recovering from COVID-19.[70] Symptoms typically include palpitations, dizziness, weakness, resting tachycardia, and worsening of symptoms with activity.[71]

Atrial fibrillation

Atrial fibrillation is the most common cardiac arrhythmia observed in patients with COVID-19, with a reported prevalence of up to 27%.[72] Potential causative mechanisms include systemic infection, direct viral injury to cardiomyocytes leading to peri-myocarditis and subsequent hypoxemia, increased susceptibility in the elderly and those with comorbidities, and sympathetic nervous system overactivity.[15,67]

Ventricular arrhythmias

In COVID-19 patients experiencing acute myocardial injury or myocarditis, serious ventricular arrhythmias can occur.[73-76] These arrhythmias may arise from a confluence of factors, including severe respiratory insufficiency, the systemic inflammatory response associated with the infection, the proarrhythmic effects of COVID-19 therapies, and potential drug interactions.[67] Furthermore, autonomic imbalance, hypoxemia, and electrolyte disturbances can exacerbate the risk of arrhythmia development.[77] Observed ventricular arrhythmias in this context range from premature ventricular complexes and non-sustained ventricular tachycardia to potentially life-threatening sustained ventricular tachycardia and ventricular fibrillation.[67]

Drug-induced QT prolongation and Torsades de Pointes
Ventricular arrhythmias, including Torsades de Pointes, have been reported in association with the use of certain medications that prolong the QT interval, particularly hydroxychloroquine.[15] Hydroxychloroquine exerts its proarrhythmic effect through the blockade of the potassium channel, thereby prolonging the QT interval and increasing susceptibility to Torsades de Pointes.[78] Individuals with congenital long QT syndrome are particularly susceptible to this effect, which can potentially lead to sudden cardiac arrest.[79,80] This risk is further amplified by the concomitant use of azithromycin or lopinavir/ritonavir due to drug-drug interactions.[66]

Role of vaccination in mitigating cardiovascular complications
COVID-19 vaccination has demonstrably reduced global morbidity and mortality. A modeling study estimated that vaccination prevented 14.4 to 19.8 million deaths worldwide within the first year, representing a 63% reduction in COVID-19 mortality.[81] These findings are corroborated by clinical data showing lower rates of acute hospitalization,[82] and reduced incidence of persistent symptoms in vaccinated individuals.[83-85] Furthermore, vaccination has proven effective in mitigating the risk of various cardiovascular complication, including myocardial injury, arrhythmias, and thromboembolic events.[86] Although initial concerns were raised regarding vaccine-associated myocarditis,[87,88] and the overall evidence supports a cardioprotective benefit.[89] Despite the benefit of vaccination, the emergence of novel SARS-CoV-2 variants, which may increase cardiovascular risk via reinfection, necessitates continued post-infection cardiovascular surveillance.[12,84,90] This surveillance is crucial to inform the need and timing of future booster vaccination campaigns. Although vaccination does not entirely eliminate the risk of cardiovascular events, it significantly reduces morbidity and mortality, underscoring its crucial role in public health strategies aimed at alleviating the burden of post-COVID-19 cardiac disease.

Study limitations
Current studies that examine the cardiovascular complications of COVID-19 face several methodological challenges. Studies show significant heterogeneity in patient selection, outcome measures, comparison groups, and research methodologies. Furthermore, many studies have limited sample sizes, which could introduce bias and limiting the generalizability of their findings. This underscores the need for further robust research with standardized methodologies to fully understand the complex relationship between COVID-19 and its cardiovascular sequelae.

Conclusion
COVID-19 is associated with a spectrum of cardiovascular complications. Among these, arrhythmias are the most common, ranging from palpitations to life-threatening arrhythmias. The causes of these arrhythmias in COVID-19 are complex and can include direct viral effects, systemic inflammatory responses, and proarrhythmic effects of certain medications. Treatment strategies for arrhythmias in COVID-19 patients, tailored to their underlying etiology, are highly advisable.

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Conflicts of interest
The authors declare that they have no conflict of interest.

Data availability
No data sets were generated or analyzed during the current study.

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Not applicable.

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REVIEW PAPER

Comparative therapeutic role of ascorbic acid, α-tocopherol and riboflavin in mitigating hepatotoxicity induced by drugs and chemical toxins – a review

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ABSTRACT

Introduction and aim. The liver plays a central role in the metabolism of drugs, xenobiotics, and nutrients, making it highly susceptible to exposure to toxicity due to drugs and chemical toxins (DCT). DCT-induced hepatotoxicity (DIH), remains one of the most common causes of acute liver failure, and potential therapeutic agents such as ascorbic acid, α-tocopherol and riboflavin have been explored to mitigate DIH. This review summarizes the current knowledge in the experimental model.

Material and methods. This review was based in publications available on scientific databases such as PubMed, Scopus, Web of Science, and Google Scholar. After the abstract evaluation, the relevant articles were selected for further analysis.

Analysis of the literature. The vital role of oxidative stress and inflammation in mediating DIH has been demonstrated. Hence, the most effective therapeutic intervention includes agents that exhibit potent antioxidant and anti-inflammatory properties such as ascorbic acid, α-tocopherol and riboflavin.

Conclusion. The comparative therapeutic role of ascorbic acid, α-tocopherol and riboflavin against DIH involves the reparation of hepatic histomorphological impairments and modulation of biochemical and molecular alterations that characterized the onset and progression of DIH.

Keywords. hepatotoxicity, hepatotoxins, vitamins hepatoprotection

Introduction

The liver is considered the largest visceral organ which performs various functions and produces various physiological secretions.[1] It also plays a vital role in the metabolism of nutrients, drugs, and xenobiotics, which makes it prone to toxicity due to exposure to drugs and chemical toxins (DCT). Furthermore, exposure to DCT can cause liver damage and disrupt physiological function leading to organ failure through a direct deleterious effect or through alteration of the hepatic vasculature.[2] Basically, liver damage can be categorized into acute and chronic categories, with the acute damage easily ameliorated through rapid elimination of hepatotoxins, leading to restoration of liver physiological function. However, untreated acute damage could lead to chronic liver pathologies such as cirrhosis, fibrosis, encephalopathy, and liver cancer.[3,4]

DCT-induced hepatotoxicity (DIH), which results from the abuse of drugs and chemical substances, is a common cause of acute liver failure, thereby constituting a public health challenge globally.[5] DCT such as acetaminophen, carbon tetrachloride (CCl_4), isoniazid, aflatoxin B1, methotrexate, and isotretinoin possess different molecular structures (Fig. 1) that form the basis of their distinct mechanisms of action.[6] Essentially, DIH is a major cause of acute liver failure which often results

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in the issuance of warnings about the application or the complete withdrawal of some drugs and chemical substances by a regulatory agency such as Food and Drug Administration (FDA).[4,7]

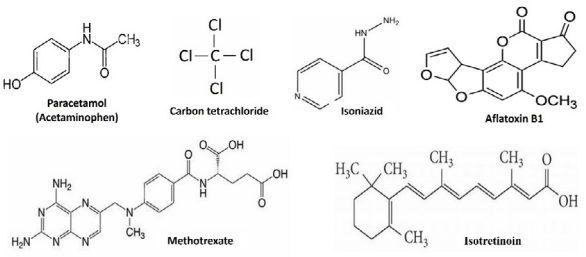

Fig. 1. Molecular structures of the selected DCT

Hepatotoxicity induced by DCT occurs as intrinsic or idiosyncratic with intrinsic hepatotoxicity usually results from the activity of DCT or its metabolites, especially due to excessive dose exposure, thereby making it highly reproducible.[8] On the idiosyncratic hepatotoxicity represents a subset of the broader idiosyncratic adverse drug reactions, which usually occurs at recommended therapeutic doses and is relatively rare.[8,9]

Furthermore, the pathogenesis of DIH have been described to involve multiple phases with the initial phase of tissue injury occurring due to direct cellular stress, mitochondrial dysfunction, activation of inflammatory and immune responses. Initial tissue injury initiates downstream events that include the death receptor-mediated pathways that are characterized by mitochondrial permeability disruption and result in apoptosis or necrosis in the liver tissue.[10] Experimental studies and a model have been explored to exploit the potential of therapeutic agents in modulating the associated pathways, thus mitigating DIH. In this regard, experimental studies have demonstrated the role of essential vitamins (including ascorbic acid, α-tocopherol and riboflavin) as prophylactic or therapeutic agents with potency in mitigating the onset and pathogenesis of DIH.

Aim

This review aimed at summarizing the current findings on the therapeutic activity of ascorbic acid, α-tocopherol and riboflavin in mitigating DIH using an experimental model. The review also explored the mechanisms of hepatoprotective activity of the selected essential vitamins.

Material and methods

Research articles that are relevant to the review were searched across scientific databases including Google Scholar, PubMed, Scopus, and Web of Science. Search keywords included 'hepatoprotective effect of ascorbic acid, 'anti-inflammatory effect of ascorbic acid,' anti-apoptotic effect of ascorbic acid, 'hepatoprotective effect of -tocopherol, 'anti-inflammatory effect of α-tocopherol, 'anti-inflammatory effect of α-tocopherol, 'anti-apoptotic effect of α-tocopherol, 'hepatoprotective effect of riboflavin', 'anti-inflammatory effect of riboflavin', 'anti-apoptotic effect of riboflavin'. The critical assessment of the search results was conducted to identify articles that contain relevant information regarding therapeutic potential of ascorbic acid, α-tocopherol and riboflavin against DIH in the experimental model. The eligibility criteria included articles with findings that are relevant to the purpose of the review, articles published in English, articles published in peer-reviewed journals, while the criteria for exclusion included duplicate articles and articles containing irrelevant information with regard to the aim of the review.

Analysis of the literature

The pivotal role of oxidative stress and inflammation, as anchor cellular events that drive the onset and progression of DIH, has made antioxidant and anti-inflammatory agents the most effective therapeutic intervention. Therefore, the antioxidant and anti-inflammatory properties of essential vitamins such as ascorbic acid, α-tocopherol and riboflavin could delineate their role as potent hepatoprotective agents against DIH.

Hepatotoxic effect of selected DCT and associated mechanisms in the experimental model

The exposure to acetaminophen (a known analgesic) could induced hepatotoxicity, characterized by prominent hepatic morphological impairments such as necrosis, sinusoidal congestion, inflammatory cell infiltration.[11] Other indicators of acetaminophen-induced hepatotoxicity included significantly elevated serum level of liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST).[11-13] Furthermore, the hepatic and serum levels of oxidative stress, inflammatory and apoptotic factors such as malondialdehyde (MDA), tumor necrosis factor-alpha (TNF-α), nuclear factor kappa B (NF-kB), interleukin-6 (IL-6), IL-1β,), interferon-gamma (INF-γ), inducible nitric oxide synthase (iNOS), myeloperoxidase, caspase-3 and Bax were significantly elevated.[11-14] Conversely, the hepatic levels of antioxidant, anti-inflammatory and anti-apoptotic markers such as glutathione (GSH), IL-10, Bcl-2 were significantly decreased.[11-15]

Furthermore, the hepatotoxicity induced by CCl_4 exposure was characterized with impaired liver function indicated by increased levels of markers such as ALT, AST, and alkaline phosphatase (ALP), induction of oxidative damage due to generation and accumulation of reactive oxygen species (ROS), induction of autophagy in hepatic tissue, and observable hepatic histopathological changes which include necrosis, steatosis, and inflammation.[16-18] CCl_4-induced hepatotoxicity was fur-

Table 1. Characterization of selected hepatotoxic drugs and chemical toxins

Selected DCT (other name)	IUPAC NAME (Structural formula)	Molecular weight	Pharmacological/ general properties	Mechanisms of hepatotoxic effect of selected DCT	References
Acetaminophen (paracetamol)	N-(4-hydroxyphenyl) acetamide ($C_8H_9NO_2$)	151.165 g/mol	- used as an analgesic and antipyretic agent	- hepatic morphological impairments - increased serum levels of liver enzymes - elevated inflammatory and apoptotic markers - reduced antioxidant and anti-apoptotic markers	Chariyakornkul et al.[11] Koshak et al.[12] Akgun et al.[13] Ahmed Mohammed and Fadheel[14] Mohamed Kamel et al.[15]
Carbon tetrachloride	Tetrachloromethane (CCL_4)	153.823 g/mol	- used as industrial solvent in dry cleaning, insecticide dispersant, fumigant, extinguisher, and production of chlorofluorocarbons	- increased serum levels of liver enzymes - elevated inflammatory and apoptotic markers - induction of oxidative stress and autophagy in hepatic tissue - hepatic histopathological changes	Shaban et al.[16] Omotoso and Amakhabi[17] Sharma et al.[18] Abdelgalil et al.[19] Rabey et al.[20]
Isoniaziad (isonicotinic acid hydrazide)	Pyridine-4-carbohydrazide ($C_6H_7N_3O$)	137.142 g/mol	- used as antibiotic singly or with other drugs to treat tuberculosis	- elevated serum levels of liver enzymes - reduction of antioxidant enzymes - hepatic histopathological changes - elevated levels of inflammatory and apoptotic markers	Ruan et al.[21] Patel et al.[22] Metushi et al.[23]
Aflatoxin B1	(3S,7R)-11-methoxy-6,8,19-trioxapentacyclo[10.7.0.02,9.03,7.013,17] nonadeca-1,4,9,11,13(17)-pentaene-16,18-dione ($C_{17}H_{12}O_6$)	312.27 g/mol	- a carcinogenic mycotoxin that contaminates food, and crops	- decreased level of oxidative stress and apoptotic markers in hepatic tissue - down-regulation of antioxidant enzymes and anti-inflammatory markers	Moloi et al.[24] Xu et al.[25] Xu et al.[26] Wang et al.[27] Yılmaz et al.[28]
Methotrexate	(2S)-2-[[4-[(2,4-diaminopteridin-6-yl)methyl-methylamino]benzoyl]amino] pentanedioic acid ($C_{20}H_{22}N_8O_5$)	454.447 g/mol	- used as an immuno-suppressant to treat psoriasis, rheumatoid arthritis, Crohn's disease; used as anti-metabolite during chemotherapy for various tissue cancers	- elevated serum levels of liver enzymes - elevated oxidative stress and apoptotic markers - decline in antioxidant enzymes - hepatic histopathological changes - depletion of hepatic folate leading to hepatocyte death	Alorabi et al.[3] Ezhilarasan et al.[6] Mahmoud et al.[29] Kalantari et al.[30] Yanasoglu et al.[31] Almalki et al.[32]
Isotretinoin (13-cis-retinoic acid or Accutane)	(2Z,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexenyl)nona-2,4,6,8-tetraenoic acid ($C_{20}H_{28}O_2$)	300.44 g/mol	- retinoic acid used to treat skin disease such as acne vulgaris and ichthyosis	- increased serum levels of liver enzymes - elevated oxidative stress markers - decline in antioxidant enzymes	Saied et al.[34] Taziki et al.[35] Daye et al.[36] Xu.[37]

ther marked by significantly elevated levels of inflammatory, apoptotic and autophagy-related proteins such as TNF-α, transforming growth factor-beta (TGF-β), IL-6, Bax, Beclin-1 and LC38.[16,19,20]

Furthermore, exposure of isoniazid (a common antituberculosis agent) usually resulted into oxidative stress, energy metabolism, and osmotic imbalance which culminated in toxicity of body tissues including liver tissue.[21] In particular, isoniazid-induced hepatotoxicity was characterized by impairment of hepatic tissue function highlighted by elevated serum levels of ALT, AST, ALP and decreased antioxidant enzyme such as superoxide dismutase (SOD) and catalase (CAT). Further profiling of isoniazid-induced hepatotoxicity revealed hepatic histopathological changes including necrosis and steatosis, as well as up-regulation of markers of oxidative damage and inflammation such as MDA, NO, TNF-α, IL-6, and IL-1β.[22,23]

Regarding aflatoxin B1-induced hepatotoxicity, the primary cellular events included mitochondrial dysfunction, oxidative stress, activation of inflammatory signaling, apoptosis and pyroptosis.[24,25] These events were further characterized by reduced mitochondrial membrane potential (MMP), elevated expression of cytoplasmic cytochrome c, Bax, p53, caspase-3 and caspase-9 proteins.[26] Aflatoxin B1-induced pyroptosis in hepatic tissue occurred via upregulation of NOD-like receptor protein 3 (NLRP3), caspase-1 and GSDMD, IL-1β and IL-18.[27] Aflatoxin B1 exposure further caused oxidative stress which was characterized with increased MDA levels and reduced levels of nuclear factor erythroid 2-related factor 2 (Nrf2) and antioxidant markers including SOD, CAT, glutathione peroxidase (GPx), heme oxygenase-1 (HO-1), NAD (P)H quinone oxidoreductase 1 (NQO1) in hepatic tissue.[27,28]

Hepatotoxicity that resulted from methotrexate treatment was characterized with endoplasmic reticulum (ER) stress, oxidative stress and induction of inflammation and apoptosis. The treatment further resulted into impairment of physiological function of liver characterized with elevated serum levels of liver enzymes such as ALT, AST, and ALP; induction of oxidative stress indicated by elevated hepatic MDA and decline in activity of anti-oxidative enzyme such as SOD, CAT, GSH and GPx.[3,29,30] This was further characterized by prominent derangement of hepatic tissue histomorphology as indicated by necrosis and inflammation.[32] Other cellular markers of methotrexate-induced hepatotoxicity included significant elevation of apoptosis and inflammatory factors including caspase-3, NF-kB, TNF-α, IL-1β, IL-6, IL-12 and depletion of hepatic folate leading to hepatocyte death.[29,32,33]

Furthermore, isotretinoin which is a widely used to treat skin diseases especially acne has been reported to cause oxidative damage of body tissues including the liver. Its mechanisms of hepatotoxicity included increased serum levels of liver enzymes (AST, ALT, AST), cholesterol, triglycerides and MDA with a concurrent decline in hepatic antioxidant markers (GSH, SOD, CAT).[34-36] Furthermore, isotretinoin-induced hepatotoxicity was characterized by a significant elevation of NO and protein carbonyl (PC).[37,38]

Therapeutic effect of ascorbic acid against DIH

Ascorbic acid (or vitamin C) is a water-soluble solid compound, composed of a five-member near-planar ring having two chiral centers that resolve into four stereoisomers (Fig. 2).[38] It is abundantly available in many natural sources, including fresh fruits such as oranges, lemons, grape and leafy green vegetables and exists in the form of ascorbate within the body.[39-41] It acts as an antioxidant which scavenges free radicals to abrogate oxidative stress and also mitigate the inflammatory response that follows exposure to tissue toxins.[41-44] Therefore, the therapeutic potential of ascorbic acid against DIH has been explored in experimental animal model with satisfactory outcomes.

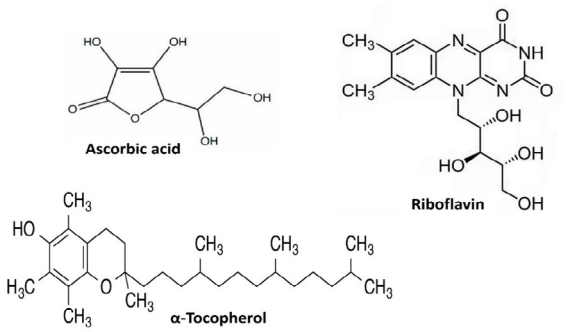

Fig. 2. Molecular structures of ascorbic acid, α-tocopherol and riboflavin

Treatment with ascorbic acid has been shown to exhibit protective effect against isoniazid-mediated hepatotoxicity through its radical scavenging and cytokine suppression activity, which was characterized with increased levels of SOD and CAT, as well as concurrent decreased TNF-α, IL-1β, IL-6, MDA, and NO levels.[22] Moreover, pretreatment with ascorbic acid has been reported to mitigate acetaminophen-induced hepatotoxicity with amplification of the hepatoprotective effect demonstrated when combined with silymarin and alpha lipoic acid.[45] Previous study has reported hepatoprotective activity of ascorbic acid alone or in combination with omega-3 against methotrexate toxicity that was characterized with improvement of liver enzymes and oxidative stress markers such as ALT, ALP, MDA, and lactate dehydrogenase (LDH) as well as liver histomorphology.[3] Based on similar mechanisms, ascorbic acid alone or in combination with natural product (curcumin) or niclosamide have exhibited potent therapeutic effect.

In a previous comparative study, hepatoprotective role of ascorbic acid against acetaminophen (paracetamol)-induced toxicity has been used to assess the therapeutic potential of medicinal plant – *Phoenix dactylifera* L. based on the effect on liver function parameters (AST, ALT, ALP, LDH, total bilirubin, and total protein), antioxidants (SOD, CAT and GPx), and GSH.[48] The antioxidative stress activity of ascorbic acid or in combination with other essential vitamins like α-tocopherol (vitamin E) and cobolamin (vitamin B_{12}) have been reported to drive the hepatoprotective effect against acetaminophen-induced hepatotoxicity through attenuation of oxidative stress and lipid peroxidation, and reduction of serum levels of liver enzymes (ALP, ALP, AST).[49]

Therapeutic effect of α-tocopherol against DIH

α-tocopherol is a lipophilic vitamin, one of the four homologs of tocopherols which together with the four tocotrienols constitute the eight essential compounds of vitamin E. Essentially, all eight compounds contain one chromane ring with one hydroxyl group and side chain; with tocopherols having saturated side chain (Fig. 2) while tocotrienols possess unsaturated isoprenoid side chain with double bonds at 3rd, 7th, and 11th carbon.[50,51] α-tocopherol functions as a potent antioxidant that protects cellular membranes and lipoproteins from peroxidation thereby preventing tissue damage due to free radicals.[52-54]

α-Tocopherol has demonstrated a protective effect against aflatoxin B1-induced toxicities, including hepatotoxicity.[28] The mechanisms of hepatoprotective activity of α-tocopherol against aflatoxin B1 included significantly decreased serum levels of ALP, AST, ALT and LDH, amelioration of associated hepatic histopathological changes, and suppression of oxidative stress makers in haptic tissue.[28] Furthermore, α-tocopherol has shown a therapeutic effect against hepatotoxicity due to methotrexate exposure through mitigation of oxidative stress and reparation of associated hepatic histopathological changes such as hepatocyte degeneration, sinusoidal enlargement, mononuclear cell infiltration, vacuolization and pyknotic nucleus.[52] Moreover, α-tocopherol exhibited potent ameliorative effect against acetaminophen-induced hepatotoxicity based on its antioxidant activity and characterized by reduction of ROS and lipid peroxidation indicated by reduced levels of MD.[55]

Therapeutic effect of riboflavin against DIH

Riboflavin (or vitamin B_2) is a member of B-vitamin family composed of isoalloxazin ring, that possesses three six-carbon rings: benzoic, pyrazine and pyrimidine (Fig. 2). As a water-soluble vitamin, it plays vital

Table 2. General and therapeutic properties of the ascorbic acid, α-tocopherol and riboflavin

Selected Vitamins	IUPAC NAME (Structural formula)	Molecular weight	Common sources	General properties and uses	Mechanisms of therapeutic effect against DIH	References
Ascorbic acid (vitamin C)	(2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one ($C_6H_8O_6$)	176.124 g/mol	orange, tomato, guava, potato, mango, lemon, pepper, strawberries, grape fruits	- water soluble and heat-stable. - used to treat scurvy, acne, gum infection, stomach ulcers caused by *Helicobacter pylori*; plays vital role in formation of collagen fibres and to prevent gallbladder disease	- decreased oxidative stress, inflammatory, apoptotic markers - increase in antioxidant enzymes - decreased serum levels of liver enzymes - reparation of hepatic histopathological changes	Alorabi et al.[3] Metushi et al.[23] Abdulrazzaq et al.[45] Khudair and Al-Gareeb[46] Zeki and Al-Gareeb[47] Bouhlali et al.[48] Abdulkhaleq et al.[49]
α-Tocopherol (vitamin E)	(2R)-2,5,7,8-tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydrochromen-6-ol ($C_{29}H_{50}O_2$)	430.71 g/mol	almonds, nuts, butter, spinach, vegetable oils, cereals, meat, egg, avocado, fruits, wheat	- fat-soluble, viscous pale yellow liquid, insoluble in water; - used to prevent skin or hair damage and reduce risk of eye disorder; used as immune booster and for treatment of atherosclerosis, eczema	- reduction of serum levels of liver enzymes - amelioration of hepatic histopathological changes - suppression of oxidative stress makers	Yilmaz et al.[28] Akman et al.[52] Torquato et al.[53] Sudheesh et al.[55]
Riboflavin (vitamin B_2)	7,8-dimethyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl] benzo[g]pteridine-2,4-dione ($C_{17}H_{20}N_4O_6$)	376.369 g/mol	meat, eggs, almonds, nuts, spinach, apple, kidney bean, spinach, banana, potato, avocado, tomato, oatmeal	- orange-yellow crystals, soluble in water, heat-stable. - used for development of lining of digestive tract, blood cells and normal body growth; used to treat migraine and to lower blood level of homocysteine	- restoration of serum levels of liver enzymes - increased tissue antioxidants - declined oxidative stress markers - amelioration of hepatic histopathological changes	Saedisomeolia and Ashoori[58] Wang et al.[59] Al-Harbi et al.[60]

roles in different biological pathways or processes and functions as a precursor to flavin mononucleotide and flavin adenine dinucleotide (which regulates the activity of flavoprotein enzymes).[56,57] Riboflavin is also an essential nutrient for normal health status; therefore, dietary intake is essential. However, it is present in sources such as milk, calf liver, eggs, fish, fruits, nuts, and legumes, green leafy vegetables, wild rice, mushrooms, yeast, cheese, and dietary products.[56,58]

Furthermore, riboflavin exhibits antioxidant properties and plays a vital role in lipid metabolism by preventing lipid peroxidation.[58,59] The therapeutic role of riboflavin against CCl_4-induced hepatotoxicity has been reported and associated with its antioxidant or anti-inflammatory effects.[60] In essence, the hepatoprotective effect of riboflavin against DCT was characterized by restoration of serum levels of liver enzymes (ALT, AST, ALP), oxidant parameter (MDA).[60] Other indicators of hepatoprotective effect of riboflavin included amelioration of liver histopathological changes due to exposure to CCl_4 exposure and down-regulation of inflammatory makers such as TNF-α.[60]

Conclusion

Generally, the imbalance in the cellular redox status in hepatic tissue that leads to hepatotoxicity can be caused by different mechanisms depending on the specific DCT. However, the induction of oxidative damage and inflammation in liver tissue are regarded as the main mechanisms that trigger the onset and progression of DIH. In contrast, the counteracting potential of essential vitamins such as ascorbic acid, α-tocopherol and riboflavin, against the mechanisms that drive the onset and progression of DIH, highlighted their comparative prophylactic and therapeutic effect against the deleterious impact of DCT on liver tissue.

Declarations

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Author contributions

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Conflicts of interest

Authors do not declare any conflict of interest.

Data availability

The data that support the findings of this study have been included in the article.

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REVIEW PAPER

Detection of gastrointestinal cancers using salivary biomarkers – a systematic review

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ABSTRACT

Introduction and aim. Cancers of the gastrointestinal tract are highly prevalent worldwide, with usually symptomless presentations making diagnosis at an early stage challenging. At the same time, salivary biomarkers are a promising method of early diagnosis in different malignancies. To this end, the present systematic review was carried out to investigate if salivary biomarkers can help with the early detection of gastrointestinal cancer and ascertain their diagnostic value.

Material and methods. Major electronic databases were searched using a combination of keywords and Boolean operators to retrieve all existing literature on the topic from April 2024 until inception. Clinical studies with relevant information were included in the quantitative synthesis.

Analysis of the literature. A total of 48 studies exploring the use of potential salivary biomarkers in esophageal, gastric, colorectal, pancreatic, hepatocellular and biomarkers were included in the present review. All studies retrieved statistically significant correlations between the presence of certain markers in the saliva and development of gastrointestinal cancers.

Conclusion. Salivary biomarkers can help detect different gastrointestinal cancers. However, more studies are required to determine their diagnostic value. The use of artificial intelligence might help clinicians in exploiting these biomarkers.

Keywords. cancer diagnosis, gastrointestinal cancers, salivary biomarkers

Introduction

Malignancies of the gastrointestinal tract and the accessory organs of digestion, known as gastrointestinal cancers, account for more than 25% of cancer cases and 30% of cancer-related deaths worldwide.[1] Gastrointestinal cancers are classified into the following major categories: esophageal, gastric, colorectal, hepatobiliary, pancreatic and bile duct cancers.[2] Most of these malignancies are symptomless at their onset and present symptoms at relatively late stages, indicating the need for continuous screening for detection in early stages.[3-5] The gold standard for screening malignancies of the gastrointestinal tract is through gastroscopy and colonoscopy which can be inconvenient for patients and costly, whereas cancers of the accessory organs are even more difficult to screen and require techniques of imaging

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with high doses of radiation which bear high risks of developing other malignancies throughout time.[6,7]

The use of salivary biomarkers for screening diseases is a relatively novel screening and diagnostic method which is fully non-invasive and bears low-costs, requiring only a sample of a patient's saliva and is reliable in detecting gastrointestinal disease since it contains many different biomolecules like the blood and the histological composition of the gastrointestinal tract is similar to that of the oral cavity.[8,9]

Aim

Since tumors of the gastrointestinal tract and accessory organs of digestion can release different molecules into the gastrointestinal tract and henceforth into saliva, the use of salivary biomarkers for screening them is a very possible rationale, probably revolutionizing the future of cancer diagnostics. Nonetheless, the available evidence on the use of such markers is scattered in different literature records. To this end, the present systematic review was performed to determine whether and which gastrointestinal cancers are detectable using salivary biomarkers, as described in the existing literature. An observed correlation would indicate the need for further research and possible future clinical use of these biomarkers.

Material and methods

The present systematic review was conducted in full compliance with PRISMA 2020 guidelines. The questions guiding the review study were as follows:

1. 'Can salivary biomarkers be used to detect gastrointestinal cancers?',
2. "What gastrointestinal cancers are detectable using salivary biomarkers and what are their diagnostic values?".

Search strategy

The online databases PubMed, Scopus and EMBASE were searched systematically for articles dating from inception until 30 April 2024, using the keywords "saliva", "salivary", "anal", "bile duct", "colon", "colorectal", "rectal", "bowel", "esophageal", "oesophageal", "gallbladder", "liver", "hepatocellular", "pancreas", "pancreatic", "stomach", "gastric", cancer", "carcinoma", "tumor", "cholangiocarcinoma", "marker" and "biomarker" combined with Boolean operators. The search was limited to articles written in the English language.

Using the citation manager EndNote release version 20.6 (Clarivate Plc, Philadelphia, USA), duplicates were removed and subsequently citations were screened based on their titles and abstracts. The inclusion criteria were cohort and case-control studies, including a total of more than 20 participants who evaluated salivary biomarkers for gastric cancer. The final selection was made after the remaining studies were evaluated on their full text. The selection process was performed by two independent reviewers (AA and DK). Any discrepancy that arose was resolved by a third researcher (CC).

Data extraction

The following data was extracted from each study and included in the qualitative analysis:

1. The neoplastic conditions studied,
2. Number of patients enrolled in the study,
3. Baseline characteristics of the included patients,
4. The salivary biomarkers assessed in the study and their respective detection method,
5. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of each biomarker calculated in the study.

Analysis of the literature

The database search on the Internet retrieved a total of 3387 citations (321 from PubMed, 1052 from Scopus and 2014 from EMBASE) and an addition of 3 other citations were retrieved manually through alternative sources. After removal of duplicates a total of 2701 citations remained, amongst which a total of 2597 citations were excluded after screening, since irrelevance to the research question was obvious from their titles or abstracts. Among the remaining 104 studies which were assessed based on their full texts, a total of 47 studies did not contain relevant data for our research, 7 were review studies,, 2 were animal studies and two had cohorts of less than 20 participants, leaving a total of 46 studies included in the qualitative synthesis. The selection process is illustrated in Figure 1.

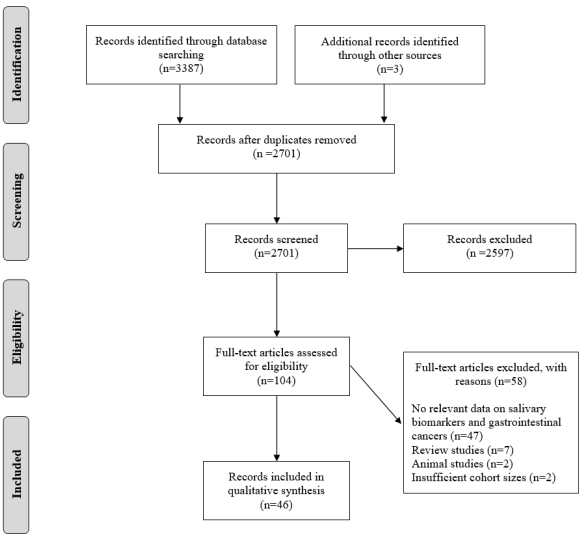

Fig. 1. PRISMA diagram of the inclusion process

The characteristics of all included studies are recorded in Tables 1–6.

Table 1. Characteristics of studies on detecting esophageal cancer

Study (Author, year, country)	Participants (n)	Histological subtype	Age of participants	Biomarker type	Biomarkers assessed	Method of assessment	Association in cancer patients /p	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Aznab et. al, 2023, Iran[10]	Cases: 6 Controls: 57 Total: 63	Not mentioned	Cases - 56 Controls - 42	Protein	E-cadherin	ELISA	0.59				
Graham et. al., 2016, United Kingdom[11]	Cases: 20 Controls: 20 Benign: 40 Total: 80	Adenocarcinoma	Unknown	RNA	TP53, CDKN2a, SMAD7, EGFR, AMY2, TLR6, KIT9, KRAS, PTEN and PIK3CA	RNA extraction -QIAzol and chloroform based process RNA expression analysis – RT-qPCR with 18s rRNA	<0.05	93%	73%		
Li et. al., 2022, China[12]	Cases: 340 Controls: 180 Total: 520 ESCC	Squamous cell carcinoma	Cases: 52–70.11 Controls: 51.93 – 70.33	RNA (sesncRNA)	1. tRNA-GlyGCC-5 2. sRESE (small RNA identified in Exosome from Saliva of ESCC patients) 3. Bi-sesncRNA signature (1+2 combined)	RT-qPCR	<0.001 for all	1. 79% 2. 77% 3. 90.50%	1. 91.67% 2. 88.33% 3. 94.20%	1. 94.05% 2. 91.67% 3. 96.28%	1. 72.37% 2. 69.73% 3. 85.61%
Li et. al., 2023, China[13]	Cases: 512 Controls: 254 Total: 766 ESCC	Squamous cell carcinoma	Training cohort: Cases - 61.09+7.77 and Controls -60.98+8.02 Internal validation cohort: Cases - 62.44+6.97 and Controls - 61.64+4.90 External validation cohort: Cases - 63.17+5.36 and Controls - 63.21+5.57	EVP miRNA	Increased in ESCC – EVP miR-1268a, miR-4505 Decreased in ESCC – miR1972, miR-4274, miR-4701-3p and miR-6126	RT-qPCR	<0.001 for all	Training Cohort – 87.39% Internal Validation Cohort – 86.43% External Validation Cohort – 88.05%	Training Cohort – 91.67% Internal Validation Cohort – 91.05% External Validation Cohort – 88.06%	Training Cohort – 95.10% Internal Validation Cohort – 95.28% External Validation Cohort – 94.59%	Training Cohort – 79.71% Internal Validation Cohort – 76.25% External Validation Cohort – 75.64%
Shu et. al, 2021, China[14]	Cases: 16 Controls: 25 Total: 41 ESCC	Squamous cell carcinoma	Cases: ≤ 60 = 43.75% >60 = 56.25 % Controls: ≤60 = 44% >60 = 56%	Glycoprotein	GalNAc, Gal, sialic acid and GlcNAc	MALDI-TOF/TOF-MS					
Stone et. al., 2024, United Kingdom[15]	Cases: 88 Controls: 168 Total: 256 Adenocarcinoma	Adenocarcinoma	Cases (mean) – 70.3 Controls (mean) – 62.2	Ca biomarkers based on DNA methylation (detection of epigenetic changes)	Used probes not specified	Illumina EPIC methylation arrays	NA	88%	31%		
Wu et. al, 2013, China[16]	Cases: - Controls: 50 Benign: - Total: -	Not mentioned		RNA	miRNA-144	RT-PCR	<0.05	Whole saliva - 74.6% Saliva supernatant – 53.7%	Whole saliva - 92% Saliva supernatant – 94%		
Xie et. al., 2013, China[17]	Cases: - 39 Controls: 19 Total: - 58	Not mentioned	Cases (mean ± SD): 59.7±9 Controls (mean ± SD): 55.9±8.8	RNA	Whole saliva markers: 1. miR-10b 2. miR-144 3. miR-451 Supernatant markers: 1. miR-10b 2. miR-144 3. miR-21 4. miR-451	RT-qPCR	Whole saliva markers: 1. 0.001 2. 0.012 3. 0.002 Supernatant markers: 1. 0.013 2. 0.036 3. 0.015 4. 0.006	Whole saliva markers: 1. 89.7% 2. 92.3% 3. 84.6% Supernatant markers: 1. 79.5% 2. 43.6% 3. 89.7% 4. 51.3%	Whole saliva markers: 1. 57.9% 2. 47.4% 3. 57.9% Supernatant markers: 1. 57.9% 2. 89.5% 3. 47.4% 4. 84.2%		
Xie et. al., 2012, China[18]	Cases: - 32 Controls: 16 Total: - 48	Not mentioned	Cases (mean ± SD): 61.6±9.3 Controls (mean ±SD): 57.5±7.1	RNA	Saliva supernatant miR-21	RT-qPCR	0.003	84.4%	62.5%		
Ye et. al., 2014, China[19]	Cases: - 100 (50 in stage I, 50 in stage II) Controls: 50 Total: - 150	Not mentioned	NA	RNA	miR-21	qPCR		Stage I – 90% Stage II – 88% Stage I+II combined – 89%	64% for all		

Table 2. Characteristics of studies on detecting gastric cancer

Study (Author, year, country)	Participants (n)	Histological subtype	Age	Biomarker type	Biomarkers assessed	Method of assessment	Association in cancer patients /p	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Aznab et.al., 2023, Iran [10]	Cases: 32 Controls: 57 Benign: 0 Total: 89	Not mentioned	Cases (mean): 56 Controls (mean): 42	Protein	E-cadherin	ELISA	Higher				
Bel'skaya et.al., 2020, Russia [20]	Cases: 11 Controls: 16 Benign: 0 Total: 27	Adenocarcinoma	Cases: 56.8 ± 5.5 Controls: 57.1 ± 6.4	Volatile organic compounds	Acetaldehyde, acetone, propanol-2, and ethanol	Capillary gas chromatography	Higher (p<0.05)	80.0%	100%		
Chen et.al., 2022, China [21]	Cases: 120 Controls: 20 Benign: 31 AG , 35 NAG, 26 GP Total: 232	Adenocarcinoma	Cases: Age<61: 56 Age≥61: 64 Controls: 56.90 ± 16.02 Benign: 62.13 ± 10.56 AG, 60.80 ± 14.54 NAG , 58.17± 15.26 GP	Microbe	Fusobacterium nucleatum	Digital droplet polymerase chain reaction (ddPCR)	Higher (p=0.780)	73.33%	82.14%	81.48%	74.19%
Huang et al., 2021, China [22]	Cases: 99 Controls: Benign: 101 SG, 93 AG (21 without IM and 72 with IM) Total: 293	Adenocarcinoma	Cases: 49.6 ± 8.8 Benign: 48.2 ± 10.2 SG, 49.9 ± 12.5 AG without IM and 48.5 ± 11.7 AG with IM	Microbes	Salivary Microbiota	Sequence analysis of the 16S ribosomal RNA gene	0.3582				
Kaczor-Urbanowicz et.al., 2022, USA[23]	Cases: 51 Controls: 49 Benign: Total: 100	Adenocarcinoma	Cases: 61.0 Controls: 60.0	RNA	Salivary exRNA	qPCR	0.0809				
Koopaie et.al., 2022, Iran [24]	Cases: 31 Controls: 31 Benign: Total: 62	Adenocarcinoma	Unknown	Protein	CSTB and DMBT1	ELISA and Machine Learning	CSTB p-value: 0.001 DMBT1 p-value: 0.002	CSTB sensitivity: 83.87% DMBT1 sensitivity: 80.65 %	CSTB specificity: 70.97 % DMBT1 specificity: 64.52 %		
Li et.al., 2018, Korea [25]	Cases: 163 Controls: 131 Benign: 0 Total: 294	Not mentioned	Cases: 51.9 ± 9.2 Controls: 55.7 ± 8.1	RNA	Extracellular RNA (exRNA)	RT-qPCR	Lower ANXA1: 0.008 CD24: 0.001 CSTB: 0.004 EIF3G: 0.945 ERO1A: 0.002 KRT4: 0.035 KRT6A:0.001 PPL: 0.007 RANBP9: 0.157 S100A10: 0.006 SEMA4B: 0.149 SPINK7:0.001 MIR140-5p: <0.001 MIR37a: <0.001 MIR454: 0.003 MIR15b: <0.001 MIR28-5p: 0.024 MIR301a: 0.01	82%	77%		
Shu et.al., 2018, China [26]	Cases: 51 Controls: 51 Benign: 0 Total: 102	Not mentioned	Cases: 54.1±7.4 Controls: 53.7 ± 5.5	Glycans	N- and O-linked glycans	MALDI-TOF	Six fucosylated N-linked glycans (?				

Shu et.al., 2017, China [27]	Cases: 64 Controls: 30 Benign: 0 Total: 94	Not mentioned	Unknown	Protein	Protein glycosylation patterns (15 lectins)	Lectin microarray	Higher and altered glycosylation patterns in GC	96%	80%
Swarup et.al., 2023, USA [28]	Cases: 10 Controls (gastritis): 10 Benign: 0 Total: 20	Not mentioned	Cases (mean): 59.4 Controls (mean): 40.5	DNA	Cell-free DNA	PCR	Higher (p= 0.0361)		
Xiao et.al., 2016, China [29]	Cases: 20 Controls: 20 Benign: 0 Total: 40	Not mentioned	Cases: 54.45 ± 11.14 Controls: 44.45 ± 12.54	Protein	Cystatin B (CSTB), triosephosphate isomerase (TPI1), and deleted in malignant brain tumors 1 protein (DMBT1)	ELISA	Lower (p<0.04)	85%	80%
Xu et.al.,2020, China [30]	Cases: 60 Controls: 60 Benign: 0 Total: 120	Adenocarcinoma	Cases: 60.17 ± 11.78 Controls: 58.80 ± 14.38	Protein and RNA	Carcinoembryonic antigen (CEA) and mRNAs (SPINK7, PPL, SEMA4B, SMAD4)	Immunoassay system and RT-qPCR	Carcinoembryonic antigen: Higher (p<0.001) SPINK7, PPL, SEMA4B and SMAD4: Lower (p<0.001)	Combined: 92%	Combined: 87%

Table 3. Characteristics of studies on detecting colorectal cancer

Study (Author, year, country)	Participants (n)	Age	Biomarker type	Biomarkers assessed	Method of assessment	Association in cancer patients /p	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Aznab et.al., 2022, Iran [10]	Cases:68 Controls:57 Benign:0 Total:125	49.66 ± 15.71 years	Soluble E-cadherin (sE-cadherin)	Levels of soluble E-cadherin in saliva and serum	Enzyme-linked immunosorbent assay (ELISA)	Elevated levels of soluble E-cadherin in cancer patients compared to healthy controls/p=0.031				
Bel'skaya et.al., 2020, Russia [20]	Cases:11(stomach Ca)+18(colorectal ca) Controls:16 Benign:0 Total:45	Age Range:30–70 Stomach Cancer Patients: Average age 56.8 ± 5.5 Colorectal Cancer Patients: Average age 58.2 ± 3.8	Volatile Organic Compounds (VOCs)	Acetaldehyde, Acetone, Propanol-2, Ethanol, Methanol, Lipid Peroxidation Products: triene conjugate and Schiff base	VOCs Analysis: Capillary gas chromatography	Significant differences in the levels of VOCs in saliva between cancer patients and healthy controls.	For Cancer/Control Classification: 95.7% For Stomach Cancer: 80.0% For Colorectal Cancer: 92.3%	For Cancer/Control Classification: 90.9% For Stomach and Colorectal Cancer: 100%		
Holten-Andersen et.al., 2012, Denmark [31]	Cases:56 Controls:105 Benign: Total:161	61 (range 25–87) Cancer individuals:68,2 Non-cancer individuals:56,6	TIMP-1 (Tissue Inhibitor of Metalloproteinases-1)	TIMP-1 concentration in saliva and plasma	ROC curves	Plasma TIMP-1 concentration significantly associated with the diagnosis of CRC (p = 0.0003)				
Kiseleva et.al, 2023, Russia[32]	Cases:100 Controls:66 Benign: Total:166	CRC: >61, LC: ≥54	miRNA-21	CMR-21(miRNA-21 in saliva) and PMR-21(miRNA-21 in blood plasma)	Reverse transcription polymerase chain reaction(RT-PCR)	CMR-21 and PMR-21 levels were statistically significantly different among CRC, LC, GCT, and healthy controls (p < 0.001) Higher CMR-21 in CRC with a small depth of tumor invasion (p = 0.004; p = 0.042)	CRC: SMR-21= 52%, PMR-21=61% LC: SMR-21=100%, PMR-21=78% GCT: CMR-21=88%, PMR-21=57%	CRC: SMR-21=89%, PMR-21=83% LC: SMR-21=86%, PMR-21=64% GCT: CMR-21=77%, PMR-21=71%		
Koopaei et.al., 2024, Iran[33]	Cases:42 Controls:33 Benign: Total:75	CRC patients: 54.12±13.98 years, healthy controls: 42.33±12.50 years	Salivary microRNAs	miR-29a miR-92a	Real-time quantitative reverse transcription PCR (RT-qPCR)	miR-29a and miR-92a expression levels: Significantly higher in CRC patients compared to controls.	miR-29a: 88% miR-92a: 85.3%	miR-29a: 93% miR-92a: 93%	miR-29a PPV:94%	
Kuwabara, et.al.,2019, Japan[34]	Cases:231 Controls:2272 Benign:99 Total:2602		Metabolites	Numerous hydrophilic metabolites in saliva	Capillary electrophoresis-mass spectrometry-based metabolomics	High area under the receiver operating characteristic curve (AUC ¼ 0.876; P < 0.0001) in the training data-set. This combination also showed high AUC values using the validation dataset (AUC ¼ 0.861; P < 0.0001).				
Kuwabara et.al., 2022, Japan [35]	Cases:235 Controls:2317 Benign:50 Total:2602		Salivary metabolites	122 quantified metabolites	Metabolite concentrations were calculated using CE-MS based on the ratio of peak area divided by the area of internal standards. Polyamine LC-MS was used redundantly to detect peaks. Data analysis included partial least squares discriminant analysis (PLS-DA) and machine learning models (ADTree and MLR).	63 metabolites showed a p-value < 0.05 between HC and CRC.				
Lazaro-Sanchez et.al., 2019, Spain [36]	Cases:15 Controls:10 Benign: Total:25		Soluble HLA-G (sHLA-G)	sHLA-G levels in saliva and serum	Measurement of sHLA-G levels in saliva and serum	Higher sHLA-G levels in advanced stages (III-IV) compared to early stages (I-II) in both saliva and serum. Significant association between higher sHLA-G levels and advanced tumor stages. No significant association with tumor location, DNA repair protein instability, or sex of patients. Higher sHLA-G levels in patients over 70 years old compared to those under 70 years. Difference between CRC patients and controls: p = 0.036 (saliva)				

Rapado-Gonzalez et.al., 2019, Spain [37]	Cases:51 Controls:37 Benign:19 Total:107		Salivary microRNAs (miRNAs)	miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p, miR-491-5p.	ROC curve analysis to discriminate CRC patients from controls based on salivary miRNA levels. Screening of 754 miRNAs in saliva samples using RT-qPCR.	No significant association between the five salivary miRNAs and age, lymph node metastasis, distant metastasis, tumor grading, serum CEA levels, TNM staging, tumor location, body mass index, alcohol intake, and smoking status. Levels of miR-186-5p, miR-29a-3p, and miR-29c-3p were significantly increased in males ($p < 0.05$)	miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-5p and miR-491-5p 72% (Combined with CEA levels: 89.36%)	miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-5p and miR-491-5p 66.67%
Rapado-Gonzalez et.al., 2018, Spain [38]	Cases:14 Controls:10 Benign:4 Total:28		MicroRNA (miRNA)	754 miRNAs analyzed, with a panel of specifically dysregulated miRNAs identified	Sample collection: Non-stimulated saliva RNA isolation: miRNeasy extraction Micro Kit (QIAGEN) miRNA expression analysis: TaqMan Array Low-Density Human MicroRNA Arrays (TLDA; Applied Biosystems, Thermo Fisher Scientific) Validation: qRT-PCR in an independent cohort			
Sazanov et.al., 2017, Poland [39]	Cases: 31 Controls: 34 Benign: 0 Total: 65	≤60	microRNA (miRNA)	miRNA-21	Real-time quantitative PCR (qPCR)	miRNA-21 expression levels were significantly higher in CRC patients compared to healthy controls/ P=5e-12	97%	91%
Waniczek, et.al.,2022, Poland [40]	Cases:39 Controls:40 Benign: Total:79	>60y.o	Proteins (chemerin, α-defensin 1, TNF-α)	Serum chemerin, Salivary chemerin, Serum α-defensin 1, Salivary α-defensin 1, Serum TNF-α, Salivary TNF-α	ROC Curve Analysis	The salivary and serum concentrations of chemerin, α-defensin 1, and TNF-α were significantly higher in cancer patients compared to the control group/p-values < 0.001 for all biomarkers tested	Salivary chemerin, a-defensin 1, TNF-a 100% Serum chemerin, a-defensin 1, TNF-a 100%	Salivary chemerin, a-defensin 1, TNF-a 100% Serum chemerin, a-defensin 1, TNF-a >90%
Zhang et.al.,2022, China [41]	Cases:207 Controls:117 Benign:76 Total:324	Median age:63y.o	Salivary DNA and Serum Levels of CEA and CA19-9	Fn DNA (Fusobacterium nucleatum DNA), CEA (Carcinoembryonic Antigen), CA19-9 (Carbohydrate Antigen 19-9)	Fn DNA levels were measured using qPCR and calculated as the ratio of the NusG gene level to the geometric mean of GAPDH and TERT levels	Fn DNA: Higher in CRC patients compared to control groups. CEA: Significantly higher in CRC patients compared to controls. CA19-9: Significantly higher in CRC patients compared to controls	Salivary Fn DNA: 71.5% CEA 22.2% and CA19-9 10.1%	Salivary Fn DNA: 82.1%

Table 4. Characteristics of studies on detecting pancreatic cancer

Study (Author, year, country)	Participants (n)	Age	Biomarker type	Biomarkers assessed	Method of assessment	Association in cancer patients /p	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Asai et.al., 2018, Japan [42]	Cases: 45 Controls: 10 Benign: 13 Total: 68	Unknown	Metabolomic	300 metabolites	CE-TOMS (capillary electrophoresis mass spectrometer)	Values of AUC (cases vs controls): spermine (0.8978) spermidine (0.7929) N1-Acetylspermidine (0.8526) N1,N8-Acetylspermidine (0.8526) N1,N12-Acetylspermidine (0.8526) Three metabolite-based MLR model's value of AUC (cases vs controls + benign): 0.8725	Unknown	Unknown	Unknown	Unknown
Asai et.al., 2018, Japan [43]	Cases: 39 Controls: 26 Benign: 14 Total: 79	Cases : 66.1 ± 9.8 Controls: 50.8 ± 16.4 Benign: 51.1 ± 12.4	Metabolomic	292 metabolites	CE-TOMS (capillary electrophoresis mass spectrometer)	(1) MLR model included four metabolites (cases vs controls + benign): AUC= 0.887; p = 0.002 Cases vs controls + benign (p<0.05): alanine; N1-acetylspermidine; 2-oxobutyrate; 2-hydroxybutyrat Cases vs controls (p<0,001): spermine; N1-acetylspermine; 2-aminobutanoat	(1) 0.71 (0.55-0.85)	(1) 0.95 (0.83-0.99)	(1) 0.93 (0.78-0.99)	(1) 0.77 (0.63-0.88)
Farrell et.al., 2009, USA [44]	Cases: 30 Controls: 30 Benign: 30 Total: 90	Unknown	Microbiome transcriptomic metabolomic	The combination of four biomarkers (mRNA biomarker MBD3L2, GLTSCR2; microbial biomarker Prevotella nigrescens, Neisseria elongata)	The Affymetrix U133 Plus 2.0 array; RT-PCR; CE-TOMS	AUC value (four biomarkers): (1) cases vs controls 0.940 (p < 0.001) (2) cases vs benign 0,900 6 microbial, 11 mRNA and 8 metabolites p<0.05 (cases vs controls + benign)	(1) 0.89 (0.73-0.98)	(1) 0.89 (0.73-0.98)	(1) 0.90 (0.73-0.98)	(1) 0.90 (0.73-0.98)
Gao et.al, 2014, China [45]	Cases: 30 Controls: 32 Total: 62	Cases: 33-78	Epigenomic	384 miRNAs	miScript miRNA PCR array human miRNome (384-well plate) from Qiagen; SYBR Green-based qPCR; qRT-PCR	Positive association p<0.05 (amount of 19 miRNAs)	Unknown	Unknown	Unknown	Unknown
Liu et al., 2017, China [46]	(1) Cases: 12 Controls: 12 Total: 24 (2) Cases 8 Benign: 4 Total: 12	Unknown	Transcriptomic	516 mRNAs (1,2) set of 29 of them	microarrays and qRT-PCR	Positive association p<0.01 (23 up-regulated and 12 down-regulated transcripts)	(1) 0.92 (0.62-1.00) (2) 1.00 (0.63-1.00)	(1) 1.00 (0.74-1.00) (2) 1.00 (0.40-1.00)	(1) 1.00 (0.72-1.00) (2) 1.00 (0.64-1.00)	(1) 0.92 (0.64-1.00) (2) 1.00 (0.40-1.00)
Machida et al., 2016, Japan [47]	Cases: 12 Controls: 13 Total: 25	Cases: 65 (45-84) Controls: 66 (53-83)	Epigenomic	miR-1246, miR-3976, miR-4306, miR-4644	high-performance liquid chromatography; latex agglutination; bromocresol green albumin; electrochemiluminescence immunoassays; Total Exosome Isolation Reagent RT-qPCR Mx3000P Real-Time QPCR System	(1) miR-1246: AUC 0.814; p = 0.008 (2) miR-4644: AUC= 0.763; p=0.026 (3) combination of miR-1246 and miR-4644: AUC=0.833; p= 0.005	(1) 0.667 (0.35-0.90) (2) 0.75 (0.43-0.95) (3) 0.83 (0.52-0.98)	(1) 1.00 (0.75-1.00) (2) 0.77 (0.46-0.95) (3) 0.92 (0.64-1.00)	(1) 1.00 (0.63-1.00) (2) 0.75 (0.51-0.90) (3) 0.91 (0.59-1.00)	(1) 0.76 (0.59-0.88) (2) 0.77 (0.54-0.90) (3) 0.86 (0.62-0.96)

Xie et al., 2016, China [48]	Cases: 55 Controls: 55 Benign tumors: 20 Total: 130	Cases: median: 61 (13–81) Controls: median: 61 (13–81) Benign: median: 49 (34–79)	Epigenomic	lncRNA: H19, HOTAIR, HOTTIP, MALAT1 PVT1	qPCR	Cases vs controls: (1) HOTAIR: p< 0.001; AUC: 0.880 (2) PVT1 p< 0.001; AUC: 0.870 (3) HOTAIR + PVT: p< 0.001; AUC: 0.909 Cases vs benign: (4) HOTAIR: p< 0.001; AUC: 0.863 (5) PVT1 p< 0.001; AUC: 0.846 (6) HOTAIR + PVT: p< 0.001; AUC: 0.909	(1) 0.78 (0.65-0.88) (2) 0.96 (0.87-1.00) (3) 0.78 (0.65-0.88) (4) 0.80 (0.67-0.90) (5) 0.69 (0.55-0.81) (6) 0.82 (0.70-0.91)	(1) 0.85 (0.73-0.93) (2) 0.64 (0.50-0.76) (3) 0.91 (0.80-0.97) (4) 0.90 (0.68-0.99) (5) 0.95 (0.75-1.00) (6) 0.95 (0.75-1.00)	(1) 0.84 (0.71-0.93) (2) 0.73 (0.61-0.82) (3) 0.90 (0.77-0.97) (4) 0.96 (0.85-0.99) (5) 0.97 (0.87-1.00) (6) 0.98 (0.88-1.00)	(1) 0.80 (0.67-0.89) (2) 0.95 (0.82-0.99) (3) 0.81 (0.69-0.90) (4) 0.62 (0.42-0.79) (5) 0.53 (0.35-0.70) (6) 0.66 (0.46-0.82)
Xie et al., 2015, China [49]	Cases: 40 Controls: 40 Benign: 20 Total: 100	Cases: median: 59,5 (13-81) Controls: median: 60,5 (23-71) Benign: median: 49 (34-79)	Epigenomic	miR-4433-5p miR-4665-3p miR-940 miR-1273g-3p miR-3676-5p miR-3679-5p miR-3940-5p miR-4327 miR-4442 miR-5100	qPCR ABI 7500 Real-Time PCR System (Life Technologies); SYBR Premix ExTaq (TaKaRa)	Cases vs (controls + benign): (1) miR-3679-5: p=0.002; AUC=0,688 (2) miR-940 p=0.001; AUC= 0,696 (3) MLR model (miR-3679-5p + miR-940): p<0.001; AUC= 0.763 Cases vs controls (4) miR-3679-5 p = 0.008; AUC: 0.673 (5) miR-940 p = 0.006; AUC: 0.680 (6) MLR model (miR-3679-5p + miR-940): p < 0,001; AUC: 0,750 Cases vs benign: (7) miR-3679-5 p = 0.007; AUC: 0.716 (8) miR-940 p = 0.004; AUC: 0.729, (9) MLR model (miR-3679-5p + miR-940): p < 0.001; AUC: 0,821	(1) 0.85 (0.70-0.94) (2) 0.90 (0.76-0.97) (3) 0.70 (0.53-0.83) (4) 0.83 (0.67-0.93) (5) 0.90 (0.76-0.97) (6) 0.73 (0.56-0.85) (7) 0.90 (0.76-0.97) (8) 0.63 (0.46-0.77) (9) 0.63 (0.46-0.77)	(1) 0.45 (0.32-0.58) (2) 0.42 (0.29-0.55) (3) 0.70 (0.57-0.81) (4) 0.43 (0.27-0.59) (5) 0.40 (0.25-0.57) (6) 0.70 (0.53-0.83) (7) 0.45 (0.23-0.68) (8) 0.75 (0.51-0.91) (9) 0.80 (0.56-0.94)	(1) 0.51 (0.38-0.63) (2) 0.51 (0.39-0.63) (3) 0.61 (0.45-0.75) (4) 0.59 (0.45-0.72) (5) 0.59 (0.46-0.72) (6) 0.71 (0.54-0.84) (7) 0.77 (0.62-0.88) (8) 0.83 (0.65-0.94) (9) 0.86 (0.68-96)	(1) 0.82 (0.65-0.93) (2) 0.86 (0.68-0.96) (3) 0.78 (0.64-0.88) (4) 0.71 (0.49-0.87) (5) 0.80 (0.56-0.94) (6) 0.72 (0.55-0.85) (7) 0.69 (0.39-0.91) (8) 0.50 (0.31-0.69) (9) 0.52 (0.33-0.70)
Wong et al., 2009, USA [50]	Cases: 30 Controls: 30 Benign: 30 Total: 90	Unknown	Transcriptomic and microbiome	ACRV1 DMXL2 DPM1 Neisseria longate Streptococcus mitis	The Affymetrix Human Genome U133+2.0 array, The Human Oral Microbe Identification Microarray (HOMIM)	MLR models: Cases vs controls: (1) ACRV1, DMXL2, DPM1 AUC: 0.974; p < 0.0001 (2) Neisseria elongata i Streptococcus mitis AUC: 0,895; p < 0,0001 Cases vs (controls + benign): (3) (ACRV1, DMXL2, DPM1, S. mitis) AUC: 0.949; p < 0.0001	(1) 0.93 (0.78-0.99) (2) 0.97 (0.83-1.00) (3) 0.93 (0.78-0.99)	(1) 0.90 (0.73-0.98) (2) 0.83 (0.65-0.94) (3) 0.85 (0.73-0.93)	(1) 0.90 (0.74-0.98) (2) 0.85 (0.69-0.95) (3) 0.76 (0.59-0.88)	(1) 0.93 (0.77-0.99) (2) 0.96 (0.80-1.00) (3) 0.96 (0.87-0.99)

Table 5. Characteristics of studies on detecting hepatocellular cancer

Study (Author, year, country)	Participants (n)	Age	Biomarker type	Biomarkers assessed	Method of assessment	Association in cancer patients /p	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Ding et. al., 2019 China [51]	Cases: 14 Controls: 14 Total: 28	Cases (mean): 61.64 +- 9.40 Controls (mean): 53.8 +-11.25	Protein	1. SOD-2 2. HP	iTRAQ, ELISA and LC-MS	1. 1.83×10^{-7} 2. 1.71×10^{-9}				
He et. al., 2022 China [52]	Cases: 90 Controls: 50 Benign: 100 Total: 240	34.6 – 63.96	Protein	1. ORM-1 2. AFP 3. ORM-1 + AFP	iTRAQ and ELISA	1. <0.001	1. 77.5% 2. 78.75% 3. 95%	1. 81.67% 2. 80% 3. 74.17%		
Hershberger et. al., 2021, USA [53]	Cases: 37 Controls: 43 Benign: 30 Total: 110	Cases (mean): 67.3 Controls (mean): 57.6 Benign (mean): 58	Metabolites (lipids, amino acids, peptides and sugars)	1.Acetophenone 2. Octadecanol 3. Lauric acid 4. 3-hydroxy-butyric acid	GC-TOF MS	1.Acetophenone – <0.001 2. Octadecanol – <0.001 3. Lauric acid – <0.001 4. 3-hydroxy-butyric acid – 0.002	87.9%	95.5%	29.3%	65.5%
Mariam et. al., 2022, USA [54]	Cases: 20 Controls: NA Benign: 19 Total: 39	Cases (mean): 67.9 Benign (mean): 57.2	RNA	miRNA: 1. hsa-mir-3198-2 2. hsa-miR-3198 3. hsa-mir-1246 4. hsa-miR-1246 5. hsa-mir-3648-2	qPCR	1. 6.56×10^{-8} 2. 5.96×10^{-9} 3. 1.54×10^{-8} 4. 1.18×10^{-8} 5. 3.59×10^{-8}				
Sun et. al., 2017, China [55]	Cases: 10 Controls: 10 Total: 20	Unknown	RNA	mRNA (5 ?	RT-qPCR	<0.01				

Table 6. Characteristics of studies on detecting bile duct cancer

Study (Author, year, country)	Participants (n)	Age	Biomarker type	Biomarkers assessed	Method of assessment	Association in cancer patients /p	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Machida et.al., 2016, Japan [47]	Cases: 12 (2 with bile duct cancer) Controls: 13 Benign: Total: 25	Range Case: 65 (45-84) Control: 66 (53-83)	RNA	miRNA	RT-qPCR	miR-1246/U6: p= 0.007 miR-4306/U6: p=1.000 miR-4644/U6: p=0.026	miR-1246: 66.7% miR-4644: 75.0 % miR-1246 + miR-4644: 83.3%	miR-1246: 100% miR-4644: 76.9% miR-1246 + miR-4644: 92.3%	miR-1246: 1.000 miR-4644: 0.750 miR-1246 + miR-4644: 0.909	miR-1246: 0.765 miR-4644: 0.769 miR-1246 + miR-4644: 0.857

Esophageal cancer

As Table 1 suggests, the data on salivary E-cadherin indicate that with a p-value of 0.59, further research is required to consider it a potential biomarker for detecting esophageal cancer. Likewise, the use of detecting epigenetic changes based on DNA methylation also require further studies, but provide promising opportunities as illustrated by Stone et al.[15] The glycoproteins GalNAc, Gal, sialic acid and GlcNAc, were significantly higher in patients with esophageal squamous cell carcinoma (ESCC) compared to the control group. According to results obtained by Graham et al. and Li et al., salivary RNA proved to be far higher in case groups than the control. This is particularly true for TP53, CDKN2a, SMAD7, EGFR, AMY2, TLR6, KIT9, KRAS, PTEN, and PIK3CA as well as tRNA-GlyGCC-5, sRESE and Bi-sesnucRNA.[11,12] The same results demonstrate a promising future for RNA as a salivary marker for esophageal cancer. Similarly, and with few exceptions, some miRNA biomarkers have been consistently elevated in the case groups compared to the control ones. These include miR-144, miR-451 and miR-10b at an overall p-value of <0.05.[16,18] Moreover, with a collective sensitivity of >84.4% and specificity of >47.4, miR-21 has also shown optimistic results.[17,19] Li et al. also identify two additional up-regulated miRNA salivary biomarkers in early stage ESCC: miR-1268a and miR-4505 with p-values of <0.001, but miR1972, miR-4274, miR-4701-3p and miR-6126 were also isolated but contrastingly, down-regulated in the case groups compared to the control ones.[12,13] It is also worth mentioning that RNA markers have been more studied in squamous cell cancers whereas protein and other markers more in adenocarcinomas.

Gastric cancer

As shown in the Table 2, there are several saliva biomarkers that can be used to detect gastric cancer. Research found that when assessed with ELISA, E-cadherin, which is a protein has a high association in cancer patients.[10] Acetaldehyde, acetone, propanol-2, and ethanol have also a high predictive value with sensitivity to be 80.0% and specificity 100%.[20] Some microbes can be used as a biomarker. In more details, Fusobacterium nucleatum has high association with gastric cancer (p=0.780) with sensitivity to be 73.33% and specificity 82.14%, but the Salivary microbiota shows lower association (p=0.3582).[21, 22] Studies shown that exRNA can be used as a biomarker. Salivary exRNA, when assessed with qPCR has low association (p= 0.0809) and when exRNA is assessed with RT-qPCR has sensitivity 82% and 77% specificity.[23] Additionally, research used CSTB (p=0.001) and DMBT1 (p=0.002). CSTB has 83.87% sensitivity and 70.97% specificity and DMBT1 has 80.65% sensitivity and 64.52% specificity. However, when assessed together CSTB, DMBT1 and TPI1 have lower association in cancer patients and the sensitivity is 85% and specificity 80%.[24] N- and O-linked glucans when assessed by MALDI-TOF may be used as a biomarker with association varying on the number and type of glycans.[26] Furthermore, protein glycosylation patterns when assessed with lectin microarray show higher and altered glycosylation patterns in GC with sensitivity 96% and specificity 80%. The cell-free DNA when evaluated with PCR has higher association (p=0.0361).[28] Finally, CEA and mRNAs (SPINK7, PPL, SEMA4B, SMAD4) after evaluation with the immunoassay system and RT-qPCR have shown 92% sensitivity and 87% specifiicty.[30]

Colorectal cancer

Numerous saliva biomarkers are available for the detection of colorectal cancer, as the Table 3 illustrates. According to research, there is a strong correlation between cancer patients' ELISA results for E-cadherin (substantially higher levels in cancer patients).[10] Also, significant differences were observed in the levels of acetaldehyde, acetone, propanol-2, ethanol, methanol, lipid peroxidation products: triene conjugate, and Schiff base among diseased and healthy patients, with sensitivity reaching 92.3% and specificity 100%.[20] Furthermore, measurements of TIMP-1 in saliva do not indicate the existence of colorectal cancer (CRC), so plasma TIMP-1 testing is still the standard for CRC identification (saliva TIMP-1 levels in cancer patients and non-patients with CRC are comparable).[31] Studies shown that miRNA-21 can be used as a cancer biomarker. Salivary miRNA, when assessed with qPCR has a sensitivity of 97% and specificity of 91%, but when assessed with RT-PCR, CMR-21(miRNA-21 in saliva) has a sensitivity of 52% and specificity 89%.[32, 56] On the other hand, miR-186-5p, miR-29a-3p, miR-29c-3p, miR-766-3p, and miR-491-5p presented a sensitivity of 72% (Combined with CEA levels: 89.36%) and specificity of 66.67%. Additionally, research has shown that miR-29a and miR-92a levels are significantly higher in cancer patients, with a sensitivity of 88% and 85.3% and specificity of 93% respectively.[33, 39] It is also worth noting that salivary metabolites show high potential as a screening tool for CRC. Additionally, according to a study, there was a significant association between higher sHLA-G levels and advanced tumor stages.[36] Proteins like chemerin, α-defensin 1, TNF-α showed a strong association in cancer patients with a sensitivity 100% in all three proteins and specificity of 100% in salivary chemerin/a-defensin 1 and over 90% in TNF-a.[40] Finally, Fn DNA (Fusobacterium nucleatum DNA), CEA(Carcinoembryonic Antigen), CA19-9 (Carbohydrate Antigen 19-9) have a sensitivity of 71.5%, 22.2%, 10.1% respectively , and the salivary specificity of Fn DNA is 82.1%.[41]

Pancreatic cancer

Biomarkers of pancreatic cancer, including metabolomic, microbiomic, epigenomic transcriptomic markers have been discovered in saliva (Table 4).[44,50] Differences have been found in the levels of various individual metabolites in pancreatic cancer patients ($p<0.05$).[42] Significant differences in the microbiome have been found in the saliva pancreatic cancer patients, including variations in the quantitative and qualitative flora *Neisseria elongata*, *Prevotella nigrescens* and *Streptococcus mitis* as well as the Leptotrichia/Porphyromonas ratio.[44] Statistically significant differences in the expression of some genes have also been observed in patients with pancreatic cancer. These differences have been described using MLRs based on several gene transcripts, reaching in all cases $p<0.01$: models included transcripts such as ACRV1, DMXL2, DPM1, and these three transcripts in combination with *Streptococcus mitis* and transcripts MBD3L2 GLTSCR2 in combination with *Prevotella nigrescens* and *Neisseria elongata*, as well as set of 29 different mRNAs.[44,50] Simultaneously, in pancreatic cancer patients, significant differences ($p<0.05$) in the amount of different salivary miRNAs have been described compared to the control groups, as well as to patients with benign changes.[47,49] In addition, differences in IncRNA (HOTAIR, PVT1 and MLR based on both) have observed between in the saliva of cancer patients and other patients such as controls and those with benign conditions.[48]

Hepatocellular cancer

SOD-2, HP, ORM-1, AFP and ORM-1 + AFP are proteins that have shown remarkable results as salivary biomarkers for hepatocellular cancer, all with p-values <0.001 and were considerably elevated in the case groups compared to the control groups.[51,52] Furthermore, different metabolites were analyzed by Hershberger et al., including acetophenone, octadecanol, lauric acid and 3-hydroxybutyric acid.[53] All four had p-values <0.05 with a sensitivity of 87.9% and specificity of 95.5% - all promising results for potential biomarkers. The 5 miRNA biomarkers distinguished by Mariam et al. have all been shown to be increased in apparently healthy individuals with a p-value of <0.001.[54] Moreover, Sun et al. identified 5 mRNA biomarkers that were isolated and upregulated in the case groups as well as 8 mRNA biomarkers that were down-regulated in the case groups.[55] All 13 had p-values of <0.01, demonstrating the use of the transcriptomic profile in early ECC is a useful asset for detecting hepatocellular cancer (Table 5).

Bile duct cancer

The salivary miRNA markers miR-1246 and miR-4644, also correlated with pancreatic cancer have been shown to detect bile duct cancer as well with adequate sensitivity and specificity (Table 6).

Quality assessment

The quality of the studies included in the meta-analysis was assessed according to the QUADAS-2 scale and the results are recorded in Table 7. No serious concerns were found regarding the applicability of the studies.

Discussion

Gastrointestinal (GI) cancers, which include esophageal, gastric (stomach), colorectal, pancreatic, liver, and biliary tract cancers, contribute significantly to global cancer burden. By examining a range of biomarkers across various gastrointestinal cancer types, this study contributes valuable new insights to the critical field of cancer detection and diagnosis. It is known that early detection and treatment improve prognosis, but many GI cancers are diagnosed late, contributing to poor survival outcomes.[57] So, the introduction of biomarkers that promote early cancer identification is of primary importance. Nevertheless, it is worth mentioning that the salivary profile can be affected by the viscosity of saliva based on patient's hydration status, the intensity of stimulation of salivary flow and time of day saliva is collected. Viscous saliva in dehydrated patients may affect concentration of molecules in saliva leading to inaccurate results in ELISA.[58] Moreover, stimulated saliva may contain higher concentrations of certain biomarkers compared to unstimulated saliva.[59,60] It is also worth noting that salivary biomarkers may show circadian variation and differ after consumption of meals as well as after fasting.[61]

In esophageal cancer, certain glycoproteins, such as GalNAc, Gal, and sialic acid, along with specific RNAs and miRNAs like miR-21 and miR-144, have demonstrated potential for cancer detection and indeed show better diagnostic values compared to plasma biomarkers.[12,14,19] However, additional studies are needed to confirm their reliability. For gastric cancer, protein markers like E-cadherin, volatile organic compounds (e.g., acetaldehyde and acetone), and microbial markers such as *Fusobacterium nucleatum* have shown high sensitivity and specificity in identifying the disease.[21,25,27] These findings suggest that salivary biomarkers could serve as effective diagnostic tools in gastric cancer cases. In colorectal cancer, salivary miRNAs such as miRNA-21 and miR-29a have exhibited significant diagnostic potential, with higher accuracy compared to plasma miRNAs.[39] Nevertheless, some limitations were noted in certain biomarkers like TIMP-1 in saliva, which did not correlate well with cancer detection.[31] Pancreatic cancer research has identified promising biomarkers based on metabolomic, microbiome, and gene transcript analyses. Variations in metabolites, bacterial profiles, and specific gene transcripts have shown strong predictive power for distinguishing between cancer and non-cancer patients.[42,44,49] For hepatocellular can-

Table 7. Quality assessment of studies in the meta-analysis

Study (Author, year)	Risk of bias				Applicability concerns		
	Patient selection	*Index test*	*Reference standard*	*Flow and timing*	*Patient selection*	*Index test*	*Reference standard*
Asai et.al., 2018 [42]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Asai et.al., 2018 [43]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Aznab et. al, 2023 [10]	Low risk	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk
Bel'skaya et.al., 2020 [20]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Chen et.al., 2022 [21]	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk	Low risk
Ding et. al., 2019 [51]	Low risk	Low risk	Low risk	High risk	Low risk	Low risk	Low risk
Farrell et.al., 2009 [44]	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk	Low risk
Gao et.al, 2014 [45]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Graham et. al.,2016 [11]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
He et. al., 2022 [52]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Hershberger et. al., 2021 [53]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Holten-Andersen et.al., 2012 [31]	Low risk	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk
Huang et al., 2021 [22]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Kaczor-Urbanowicz et.al., 2022 [23]	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk	Low risk
Kiseleva et.al, 2023 [56]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Koopaie et.al., 2022 [24]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Koopaei et.al., 2024 [33]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Kuwabara, et.al.,2019 [34]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Kuwabara et.al., 2022 [35]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Lazaro-Sanchez et.al., 2019 [36]	Low risk	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk
Li et.al., 2022 [12]	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk	Low risk
Li et.al., 2023 [13]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Liu et.al., 2017 [46]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Machida et.al., 2016 [47]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Mariam et.al., 2022 [54]	Low risk	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk
Rapado-Gonzalez et.al., 2019 [37]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Rapado-Gonzalez et.al., 2018 [38]	Low risk	Low risk	High risk	Low risk	Low risk	Low risk	Low risk
Sazanov et.al., 2017 [39]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Shu et. al., 2021 [14]	Unclear	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Shu et.al., 2018 [26]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Shu et.al., 2017 [27]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Stone et. al.,2024 [15]	Low risk	Low risk	Low risk	High risk	Low risk	Low risk	Low risk
Sun et. al., 2017 [55]	Unclear	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Swarup et.al., 2023 [28]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Waniczek, et.al.,2022 [40]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Wong et al., 2009 [50]	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk	Unclear
Wu et. al, 2013 [16]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Xiao et.al., 2016 [29]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Xie et. al., 2013 [17]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Xie et. al., 2012 [18]	Unclear	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Xie et al., 2016 [48]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Xie et al., 2015 [49]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Xu et.al.,2020 [30]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Ye et.al., 2014 [19]	Low risk	Low risk	Unclear	Low risk	Low risk	Low risk	Low risk
Zhang et.al.,2022 [41]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk

cer, salivary proteins like AFP and certain metabolites, including acetophenone and lauric acid, have demonstrated strong sensitivity and specificity.[54,55] Additionally, miRNA and mRNA profiles have been highlighted as valuable tools for early detection.

The future of salivary biomarkers in cancer detection is highly promising, with exciting potential for non-invasive, accessible, and accurate screening across various cancer types. While significant progress has been made in understanding the role of these biomarkers, further advancements are needed to ensure they become reliable tools for routine cancer diagnosis.

In esophageal cancer, future research will likely focus on improving the specificity and reliability of the markers, which could lead to non-invasive screening options for early detection. Given the poor prognosis of advanced esophageal cancer, early diagnostic tools could drastically improve patient outcomes.

For gastric cancer, protein markers and microbial markers have already demonstrated high sensitivity and specificity. Future prospects include the development of personalized medicine approaches, specially involving RNA markers that tailor treatments based on a patient's unique biomarker profile.[62] Additionally, advances in microbiome research could provide a more comprehensive understanding of gastric cancer development. As technology evolves, rapid, at-home testing devices may allow patients to monitor these biomarkers in real-time, making diagnosis more convenient and accessible, a method used today for detecting respiratory infections.[63,64]

In colorectal cancer, the future could see the creation of combined biomarker panels that improve diagnostic accuracy by integrating miRNAs, proteins, and volatile compounds. Early and routine screening for colorectal cancer using saliva could reduce mortality rates by identifying the disease at more treatable stages.[65]

For pancreatic cancer, salivary biomarkers identified through metabolomic, microbiome, and gene transcript analyses have shown strong predictive power. Given that pancreatic cancer is often diagnosed in late stages, the future holds great potential for developing saliva-based tools for early detection, which could significantly increase survival rates.[66] With ongoing research, new biomarkers are likely to be discovered, leading to more comprehensive diagnostic panels. Non-invasive saliva tests could replace more invasive methods and provide a simpler way to monitor high-risk individuals.

In hepatocellular cancer, salivary proteins have already demonstrated strong sensitivity and specificity, and miRNA and mRNA profiling have shown value in early detection. The future may see further advancements in metabolomic profiling, offering more precise diagnostic tools that differentiate between various liver diseases. Additionally, combining biomarker data with genetic and lifestyle factors could help identify high-risk patients earlier, allowing for preventive interventions before cancer develops.[67] Saliva-based tests could also provide affordable and scalable screening tools in regions with high liver cancer incidence, such as Asia and Africa, where access to traditional diagnostic resources, such as imaging, is limited.[68]

Looking more broadly, salivary biomarkers hold the potential to revolutionize cancer screening across the board. As research progresses, these tests could be widely adopted as part of routine medical care, replacing invasive procedures like biopsies and endoscopies. Future diagnostic tools may integrate salivary biomarkers with other "omics" technologies (e.g., genomics, proteomics, metabolomics) to create more accurate, personalized cancer diagnostics.[69,70] With the development of portable, cost-effective devices, cancer detection could become more accessible to underserved populations, potentially transforming global cancer care. Artificial intelligence and data analytics will likely play a key role in the future of salivary biomarker research.[71] AI could enhance the interpretation of complex biomarker data, leading to more accurate predictions and personalized treatment plans. As a result, the integration of salivary biomarkers into cancer diagnosis could not only improve early detection but also provide tailored therapeutic approaches based on individual risk profiles.[72]

In spite of the fact that the present study was performed according to PRISMA guidelines, three different electronic data bases were searched and quality assessment showed no serious risk of bias, it had some notable limitations. Firstly, different laboratory methods used in each study may cause biased results. At the same time, some studies had very small cohorts making the results unreliable, and such studies must by investigated further by researchers in the future. Moreover, most studies were performed in Caucasian and Asian populations, possibly leading to a small degree of bias. It is also worth mentioning that the literature was limited to articles written in the English language and some notable articles in other languages may have been missed.

Conclusion

In summary, the future of salivary biomarkers in cancer detection is bright, with potential applications in early diagnosis, personalized treatment, and global accessibility. Ongoing advancements in technology, bioinformatics, and large-scale clinical studies will be crucial in ensuring these biomarkers become reliable tools in the fight against cancer.

Declarations

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Author contributions

Conceptualization, D.K.; Methodology, A.A., D.K. and Chara. C.; Software, D.K.; Validation, A.A., D.K. and Chara. C.; Formal Analysis, A.A. and D.K.; Investigation, A.A., D.K., Chara. C, M.G., L.F., S.K., J.K. and Z.C.; Resources, D.K.; Data Curation, A.A., D.K., Chara. C, M.G., L.F., S.K. and J.K.; Writing – Original Draft Preparation, A.A., D.K., Chara. C, M.G., L.F., S.K. and J.K.; Writing – Review & Editing, A.A., D.K., Chara. C, M.G., L.F., S.K. and J.K..; Visualization, D.K..; Supervision, Charal. C. and D.A.; Project Administration, D.K.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

Not applicable.

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Not applicable.

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REVIEW PAPER

Characterization of anti-steatogenic long noncoding RNAs and their epigenetic influence on the development of metabolic fatty liver disease – a systematic review

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ABSTRACT

Introduction and aim. Numerous transcriptomic studies have demonstrated that the development of metabolically associated fatty liver disease is accompanied by changes in the expression level of long noncoding RNAs (lncRs). The aim: to present a brief description of the role of anti-steatogenic lncRs in the epigenetic influence on the development of metabolically associated fatty liver disease, analyzing the data of modern scientific literature.

Material and methods. An analysis of 64 reports over the past 10 years was conducted from the databases PubMed; MEDLINE; EMBASE; Cochrane Systematic Reviews Database; BIOSIS which were selected using the indicated keywords. Quality aspects were assessed using the adapted Newcastle–Ottawa Scale, PROSPERO CRD420250652980.

Analysis of the literature. Hypoexpression of AC012668 – increased representation of miR-380-5p, activation of *LRP2*; B4GALT1-AS1/lncSHGL, MEG3 – activation of lipogenesis, gluconeogenesis in hepatocytes; FLRL2 – inhibition of *BMAL1* and *SIRT1*; Gm16551 – increased expression of *ACC1, SCD1*; HR1 – activation of *SREBP1c*. LncLSTR – activation of cytochrome Cyp8b1 transcription; MRAK052686 reduction of *FABP7* expression.

Conclusion. The formation of hepatosteatosis is supported by a decrease in the expression level of anti-steatogenic lncRs, such as AC012668, B4GALT1-AS1/lncSHGL, MEG3, FLRL2, Gm16551, lncHR1, lncLSTR, MRAK052686. LncRs overexpression is compensatory in escalating inflammation, hyperglycemia.

Keywords. anti-steatogenic long noncoding RNAs, epigenetic regulation, metabolically associated fatty liver disease, obesity

Introduction

Long noncoding RNAs (lncRs) actively participate in the regulation of the functioning of various biological processes. Aberrant lncR expression may be a key molecular driver of the development of metabolic diseases, in particular, obesity, type 2 diabetes mellitus (T2DM), arterial hypertension, dyslipidemia, and metabolic-associated fatty liver disease (MAFLD).[1-5] Numerous transcriptomic studies have demonstrated that the development of MAFLD is accompanied by changes in lncR expression levels.[6–8] A genome-wide screening performed in key metabolic organs of mice identified 359 metabolically responsive lncRs. Unlike previous studies that have demonstrated the role of lncR-mediated epigenetic regulation in the development of MAFLD, our review contains a systematic analysis of only statistically verified reports on this issue. It is believed that lncRs, which act as regulators of subtle metabolic reprogramming of cells, and their transcription mechanisms in the near future may become a target for drugs in the implementation of drug treatment of metabolic, inflammatory, autoimmune, neurodegenerative, oncological and other diseases.[9-12]

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Brief description of long noncoding RNAs

Currently, at least 28,000 lncRs have been identified in humans, with transcript lengths exceeding 200 nucleotides, hence the name long lncRs. lncR genes are transcribed by RNA polymerases I, II, and III.[13-17] Due to the different location in the genome of lncR coding sequences relative to protein-coding genes, lncRs are grouped into five different classes: long intergenic noncoding RNAs, antisense RNAs, sense RNAs, sense intronic RNAs, and enhancer lncRs. Depending on the functions they perform, there are: 1) signaling lncRs, which are specifically associated with intracellular molecular signaling pathways; 2) lncR traps, which interact with transcription factors and remove them from chromatin; 3) targeting lncRs, which bind to protein complexes and direct them to gene promoters or specific sites in the genome; 4) scaffolding lncRs, which connect various protein complexes, control gene expression. Notably, antisense transcription, which occurs through interaction with complementary sense transcripts encoded by the opposite gene, prevents transcription altogether.[18] Currently, there are several databases that provide updated information on lncRs (Table 1).

Table 1. lncR databases

Databases	Website	Website characteristics
CLS FL	–	Contains annotations for 807 lncRs
Lnc2Cancer database v3.0	http://www.bio-bigdata.net/lnc2cancer or http://bio-bigdata.hrbmu.edu.cn/lnc2cancer	Provides information on 2659 lncRs associated with 216 human cancer subtypes
lncATLAS	https://lncatlas.crg.eu/	LncATLAS displays subcellular localization for user-selected lncRs. Only GENCODE annotated lncR genes are present, which can be accessed using their identifier or official gene name
LncExpDB	https://ngdc.cncb.ac.cn/lncexpdb/	Covers expression profiles of 101,293 high-quality human lncR genes, predicts potential functional lncRs and their interacting partners
LncRNADisease v3.0	http://www.rnanut.net/lncrnadisease	Provides information on 2297 lncR interactions
Mammalian ncRNA-Disease Repository v3.1	http://rna-society.org/mndr/	Presents 40,000 human lncR and disease associations
MiTranscriptome (v2)	https://www.mitranscriptome.org	The full catalog includes over 91,000 genes. Contains annotations for 63,615 ncRs
NONCODE (v5)	http://v5.noncode.org/	Contains annotations for 96,308 human ncRs. The most comprehensive resource

lncR genes, compared to protein-coding genes, are characterized by a high level of tissue specificity. Thus, it has been shown that tissue specificity is characteristic of 78% of lncRs and only 19% of mRNAs. The lncR pool contains a larger number of organ-specific and clone-specific RNAs. The activity of lncR expression depends on the state of tissues and the presence of pathological processes. The biogenesis of most lncRs is similar to mRNA synthesis. LncRs are transcribed by RNA polymerase II, after which the lncR transcript undergoes capping, polyadenylation, and splicing, resulting in mRNA-like transcripts. Unlike mRNAs, which are localized in the cytoplasm, lncRs are predominantly located in the cell nucleus. A certain portion of lncRs is exported to the cell cytoplasm, where they are distributed to various intracellular organelles.[19-22]

LncRs have the ability to bind to DNA, RNA, and proteins, and therefore they participate in the regulation of: 1) gene expression at the level of transcription and posttranscriptional processes; 2) alternative splicing, and 3) the formation of protein complexes. Some lncRs bind to target mRNA transcripts and can enhance or repress their subsequent translation, provided that these lncRs are expressed from a gene silencer or enhancer. LncRs can regulate the expression of genes distant from the location of the lncR, they promote the formation of DNA loops, bringing the enhancer and the target site of the gene promoter closer to each other.[23]

It has been demonstrated that lncRs function as "sponges" for microRNAs (miRs) in the cytoplasm of the cell or as host genes for miR transcription in the cell nucleus. In addition, lncRs mediate DNA methylation and act as modular scaffolds for chromatin-modifying complexes.[24,25] lncRs are also involved in chromosome inactivation, induction of pluripotency, and cell differentiation.[26,27]

Recently, data have been obtained that some lncRs with open reading frames can be translated into small proteins or micropeptides that play a significant role in the body's immune response and carcinogenesis.[28,29]

It has been demonstrated that in liver biopsies of patients with MAFLD, 1735 lncRs and 1485 mRNAs are differentially expressed. Increased expression is characteristic of 535 lncRs and decreased expression is characteristic of 1200 lncRs.[30]

The role of anti-steatogenic lncRs in the development of hepatic steatosis in MAFLD

The development of hepatic steatosis in MAFLD is supported by a decrease in the expression level of anti-steatosis lncRs, such as AC012668, FLRL2, Gm16551, LncHR1, B4GALT1-AS1/lncSHGL, MEG3, and others (Table 2).

Aim

To present a brief description of the role of anti-steatogenic long noncoding RNAs in the epigenetic influence on the development of metabolically associated fatty liver disease, analyzing the data of modern scientific literature.

Table 2. Hypoexpression of long noncoding RNAs associated with the development of hepatic steatosis in MAFLD[31,32]

lncR	Effect	Molecular target	Signaling cascade
AC012668	Promotes lipid accumulation	miR-380-5p	miR-380-5p/LRP2
B4GALT1-AS1/ lncSHGL	Activates lipogenesis and gluconeogenesis		PI3K/AKT; hnRNPA1/ CaM
FLRL2	Activates lipogenesis, inflammatory response		Arnt1/Sirt1
Gm16551	Activates lipogenesis de novo		ACC1 i SCD1
LncHR1	Promotes lipid accumulation		SREBP1c, PDK1/AKT/ FoxO1
lncLSTR	Promotes lipid accumulation		Cyp8b1/FXR/apoC2
MEG3	Stimulation of lipogenesis	miR-21	LRP6/AKT/ mTORC1
MRAK052686	Promotes lipid accumulation		ZBTB20

Material and methods

An analysis of 64 literature sources over the past 10 years was conducted from the databases PubMed, MEDLINE; EMBASE; PreMedline In-Process & Other Non-Indexed Citations; The Cochrane Systematic Reviews Database, DARE, NHS EED and HTA databases; Web of Knowledge Science Citation Index; Web of Knowledge ISI Proceedings; CRD databases; BIOSIS, which were selected using the following keywords: long non-coding anti-steatogenic RNAs, epigenetic regulation, metabolically associated fatty liver disease, obesity. Inclusion criteria were based on the PECOS approach, depending on the sample types:[33]

Type 1 (n=2) – MAFLD patients (P (Population): people over 18 years of age; E (Exposure): diagnostic criteria for MAFLD;[34] C (Comparison): MAFLD patients vs. healthy individuals; O (Outcome): decreased expression of lncRs, assessed by RT-qPCR with escalation of MAFLD factors; S (Study design): Cross-sectional study (CSS)/Dynamic prospective study of the "case-control" type (DPCC)).

Type 2 (n=12) – MAFLD modeling *in vivo* (P: 8-week-old mouse line with an average body weight of 18-20 g; E: High-fat diet (HFD) according to 12 weeks, assessed by analysis of biological samples from participants; C: MAFLD mice vs. healthy mice; O: decreased expression of lncR, assessed by RT-qPCR with escalation of MAFLD factors; S: Dynamic prospective experimental studies (DPES).

Type 3 (n=10) – MAFLD modeling *in vitro* (P: human hepatocytes of cell lines or mice; E: FFA treating followed by transfection of lncRs activators/inhibitors, cell culture analysis, and assessment of human homologs using the Basic Local Alignment Search (BLAST) tool of the National Center for Biotechnology Information; C: FFA-treating of cells versus intact cells; O: decreased expression of lncRs, assessed by RT-qPCR with escalation of MAFLD factors; S: DPES.

Studies were excluded if the determination of lnsR expression was carried out by methods other than the analysis of biological samples of participants, if the effect in MAFLD modeling was assessed over a shorter period of time, and the criteria for diagnosing MAFLD were not considered the main outcome. Quality aspects were assessed using the adapted Newcastle–Ottawa Scale. Studies that received a score of 7 were considered to have a low risk of bias; 6 - medium risk of bias; 5 or less - high risk of bias.[35]

Analysis of the literature

Of the 64 reports highlighting the contribution of lncRs to MAFLD development, we focused on 35 reports associated with lncRs expression as a biomarker for MAFLD development, namely: 2 (AC012668); 3 (B4GALT1-AS1/lncSHGL); 7 (FLRL2); 1 (Gm16551); 2 (LncHR1); 2 (LncLSTR); 15 (MEG3); 4 (MRAK052686). Most of them had a moderate risk of bias. Ultimately, only 16 (24 non-randomized clinical trials (nRCT)) out of 35 reports contained reliable information and met the standardized criteria for inclusion in the study. The bias for low risk was 54.2%, for intermediate risk 29.2%, and for high risk 16.6%. The quality of the nRCTs on the contribution of lncRs to the development of MAFLD using NOS is presented in Table 3.

At the same time, a compensatory overexpression of MEG3 was noted under the additional influence of metabolically unfavorable factors associated with persistent meta-inflammation (the presence of concomitant diabetes type 2, obesity) or the cultivation of hepatocytes in a medium with TNF-α.[46;50]

AC012668. It was found that in MAFLD, the expression activity of lncR AC012668 (1.642 nt URS-00004FA763_9606) is suppressed, while overexpression of lncR AC012668 is accompanied by suppression of the expression of genes associated with lipogenesis (*SREBP1c, FASN, SCD1*) and inhibition of triglyceride accumulation processes in hepatocytes. AC012668 sequesters miR-380-5p, whose molecular target is low density lipoprotein receptor-related protein 2 (LRP2). Decreased expression of lncR AC012668 leads to increased expression of miR-380-5p, which suppresses expression of the *LRP2* gene, which promotes lipid accumulation in liver tissue. Administration of exogenous AC012668 inhibits the progression of hepatic steatosis.[36]

B4GALT1-AS1/lncSHGL. In liver biopsies of people with MAFLD, a decrease in the expression level of homo sapiens (human) beta-1,4-galactosyltransferase 1 antisense RNA 1 (B4GALT1-AS1; 1,185 nt URS000075AF86_9606; in mice, designated as lncSHGL) is noted. B4GALT1-AS1/lncSHGL is a non-secretory lncR, which under physiological conditions is highly expressed in liver tissue and has the ability to inhibit lipogenesis and gluconeogenesis.[37,52] Overexpression of lncR lncSHGL is accompanied by phosphorylation of AKT, which leads to inhibition of gluconeogenesis and

Table 3. Analysis of the quality of nRCTs associated with the contribution of AC012668, B4GALT1-AS1/lncSHGL, FLRL2, Gm16551, LncHR1, LncLSTR, MEG3, MRAK052686 to the development of MAFLD using NOS*

Author, year	Individual participant data, as a model of the MAFLD	Lnc Rs expression levelin hepatocytes (h) or serum (s), RE[a] (M±m)		S[b]	C[c]	E[d]/O[e]	Risk of study bias
		MAFLD$^+$	MAFLD$^-$				
	AC012668						
Chen et al., 2021[36]	MAFLD modeling in C57BL/6J mice (n=10/10) [DPES]	0.3 ±0.04 (h)	1 ±0.07 (h)	3	2	3	8
	MAFLD modeling in human hepatocyte cell culture LO_2 [f, DPES]	0.4 ±0.02	1.1 ±0.03	3	2	3	8
	B4GALT1-AS1/lncSHGL						
Wang et al., 2018[37]	MAFLD modeling in C57BL/6J mice (n=6/8) [DPES]	0.6±0.01 (h)	1 ±0.1 (h)	3	2	3	8
	MAFLD patients [CSS] (n=6/6)	0.7 ±0.13 (h)	1.1 ±0.2 (h)	3	2	2	7
	FLRL2						
Chen et al., 2017[38]	MAFLD modeling in C57BL/6J mice (n=4/4) [DPES]	0.2 ±0.01 (h)	0.96 ±0.02(h)	2	2	2	6
Chen et al., 2019[39]	MAFLD modeling in C57BL/6J mice (n=6/6) [DPES]	0.46 ±0.05(h)	0.9 ±0.03 (h)	3	2	2	7
	MAFLD modeling in human hepatocyte cell culture [DPES]	0.25 ±0.02	0.82 ±0.01	3	2	2	7
	Gm16551						
Di Mauro et al., 2021[40]	MAFLD modeling in C57BL/6J mice (n=8/8) [DPES]	↓(h)	N (h)	3	1	2	6
	LncHR1						
Li et al., 2017[41]	MAFLD (HCV) modeling in C57BL/6J mice (n=6/6) [DPES]	1.12 ±0.07(h)	2.93 ±0.09(h)	3	1	2	6
Li et al., 2018[42]	MAFLD modeling in human hepatocyte cell culture Huh7 [g, DPES]	↓	↓/N (p>0.05)	2	2	2	6
	LncLSTR						
Li et al., 2015[43]	MAFLD modeling in C57BL/6J mice (n=7/8) [DPES]	0.08 ±0.001	0.93 ±0.003	3	2	3	8
	MEG3						
Zhu et al., 2016[44]	MAFLD modeling in C57BL/6J mice (n=?) [DPES]	↓ (h)	N (h)	1	1	3	5
	MAFLD modeling in mouse hepatocyte cell culture [DPES]	↓	N	1	1	3	5
Wang et al., 2018[45]	MAFLD modeling in C57BL/6J mice C57BL/6J (n=?) [DPES]	↓(h)	N (h)	1	2	3	6
	MAFLD modeling in mouse hepatocyte cell culture [DPES]	↓	N	1	2	3	6
Zhu et al., 2019[46]	MAFLD (T2DM/ob/ob[i]) modeling in C57BL/6J mice (n=10/10[i] [DPES]S	2.8 ±0.08 (h)	1.1 ±0.03 (h)	3	1	3	7
	MAFLD (T2DM/ob/ob) modeling in cell culture of primary mouse hepatocytes [DPES]	2.3 ±0.1	1.1 ±0.08	3	1	3	7
Huang et al., 2019[47]	MAFLD modeling in C57BL/6J mice C57BL/6J (n=?) [DPES]	↓(h)	N (h)	1	1	3	5
	MAFLD modeling in human hepatocyte cell culture $HepG_2$ [j, DPES]	↓	N	1	1	3	5
Zou et al., 2022[48]	MAFLD (MASH[k]) modeling in C57BL/6J mice (n=10/15/15) [DPES]	0.6±0.06 (0.3±) h	1.12 ±0.09 (h)	3	2	3	8
	MAFLD modeling in mouse hepatocyte cell culture [DPES]	0.4 ±0.01	0.9 ±0.05	3	2	3	8
Erdem et al., 2023[49]	MAFLD patients (n=180/60) [DPCC]	0.41 ±0.02(s)	1.68 ±0.03(s)	3	2	3	8
Meng et al., 2024[50]	MAFLD (TNF-α[l]) modeling in human hepatocyte cell culture HepG[2DPES]S	4.1 ±0.1	0.9 ±0.07	3	1	2	6
	MRAK052686						
Yuan et al., 2015[51]	MAFLD modeling in human hepatocyte cell culture Huh7[DPES]	0.64±0.04	1.7±0.08	2	2	3	7

* RE[a] – relative expression by real time PCR (qPCR) of differentially expressed genes in the microarray analysis; S[b] – selection; C[c] – comparability; E[d] – exposure; O[e] – outcome; LO_2[f] – the human normal hepatocyte; Huh7[g] – an immortal cell line composed of epithelial-like, tumorigenic cells; ob/ob[i] – mice are hyperphagic, obese, hyperinsulinemic, and hyperglycemic, so they are used as a model for diabetes and obesity; $HepG_2$[j] – a hepatoblastoma cell line; MASH[k] – metabolic dysfunction-associated steatohepatitis; TNF-α[l]– Tumor necrosis factor α

lipogenesis gene expression (*PEPCK, G6Pase, FAS*). It is believed that lncR lncSHGL recruits heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), which increases the translation efficiency of calmodulin (*CALM*) gene mRNA. In turn, calmodulin independently of insulin and calcium stimulates the PI3K/AKT-associated signaling pathway in hepatocytes. It is believed that drug activation of the lncR B4GALT1-AS1/hnRNPA1 signaling pathway is a potential new direction for the treatment of both hepatic steatosis and type 2 diabetes.[37] It is possible that lncR B4GALT1-AS1 is involved in the development of liver fibrosis.[53]

FLRL2. Fatty liver-related lncR (FLRL): FLRL1, FLRL2, FLRL6 – are involved in the regulation of circadian rhythm.[36,38] It has been established that in experimental animals with a model of MAFLD, the expression activity of lncR FLRL2, which is highly associated with the development of hepatic steatosis, is significantly reduced.[38] Overexpression of FLRL2 inhibits lipogenesis activity, contributes to the reduction of steatosis and liver inflammation in MAFLD. Given that the sequence encoding FLRL2 is located in the intronic region of the AHR nuclear translocator-like protein gene (basic helix-loop-helix ARNT like 1 – BMAL1), its expression

level corresponds to the expression of both the *BMAL1* gene and the closest sirtuin 1 (*SIRT1*) gene. FLRL2 promotes Bmal1 transcription and accumulation of Bmal1 protein in the cytoplasm, which translocates into the cell nucleus and binds to the *Sirt1* gene promoter, enhancing its transcription. Decreased FLRL2 expression in MAFLD leads to inhibition of *BMAL1* and *SIRT1*. Thus, the lack of expression of lncR FLRL2 is associated with a low level of *SIRT1* gene expression, which leads to: 1) increased activity of NR1H3/LXRα and SREBP1c with increased lipogenesis and patatin-like phospholipase domain-containing protein 3 (PNPLA3);[5] 2) inhibition of peroxisome proliferator activated receptor alpha (PPARα), reducing the efficiency of lipid β-oxidation; 3) disruption of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB), which induces inflammation.[36,37,52-55] It is known that overexpression of the *SIRT1* gene is accompanied by recovery from MAFLD, while deficiency of SIRT1 production contributes to the progression of the manifestations of this disease. Cheng Tian and colleagues believe that insufficient SIRT1 activity contributes to the development of MAFLD by increasing lipid accumulation in hepatocytes, reducing the efficiency of autophagy, developing oxidative stress in hepatocytes, and inflaming the liver tissue.[56]

Gm16551. Decreased expression of the anti-steatogenic lncR Gm16551 (mus musculus (mouse) predicted gene 16551; 3.162 nt URS0000AAF6F6_10090), which is observed in MAFLD, promotes increased expression of the *ACC1* and *SCD1* genes, and induces the development of hepatic steatosis. Interestingly, administration of coffee to mice fed a HFD restores Gm16551 expression levels to values observed in lean mice.[40]

LncHR1. Decreased expression of the transcript 1 regulated by the hepatitis C virus (homo sapiens (human) lncR induced by HCV, regulator of SREBF1 – lncHR1; 420 nt URS000038DCB1_9606), which causes the development of hepatic steatosis, is pathognomonic for MAFLD. Anti-steatogenic lncHR1 is a potent negative regulator of SREBP1c. LncHR1 inhibits SREBP1c activity by phosphorylating components of the PDK1/AKT/FoxO1 signaling cascade.[42] Overexpression of lncHR1 is accompanied by a decrease in the expression level of the SREBP1c factor, which is induced by oleic acid, and inhibition of the process of triglyceride accumulation in lipid droplets of hepatocytes.[41] Decreased lncHR1 expression promotes activation of the SREBP1c factor, which induces lipogenesis and the development of hepatic steatosis.

LncLSTR. With the development of MAFLD, the level of expression of long intergenic RNA liver-specific triglyceride regulator (LSTR) in blood serum significantly decreases. Mice with liver-specific depletion of lncLSTR show a marked reduction in serum triglyceride levels. lncLSTR has been shown to directly bind to TAR DNA binding protein (TARDBP), which represses the transcription of cytochrome p450 family 8 subfamily b member 1 (Cyp8b1). Inhibition of Cyp8b1 activity leads to significant changes in the composition of bile acids. The altered composition of bile activates the expression of farnesoid X receptor (FXR). Farnesoid X receptor is a bile acid-activated receptor that is mainly expressed in liver and intestinal tissues and has 2 forms in mammals: FXRα and FXRβ. FXR is known to modulate several metabolic pathways, including bile acid synthesis and lipid metabolism. For example, FXR activation leads to the induction of apolipoprotein C2 (apoC2). Patients with homozygous apoC2 deficiency have elevated serum triglyceride levels and significantly reduced LPL activity, suggesting that apoC2 is an activator of LPL. An increase in the activity of apoC2 leads to a powerful activation of LPL and leads to an increase in the clearance of triglycerides from the peripheral blood stream. Suppression of the increased expression of apoC2 in lncLSTR KD mice induces restoration of the reduced serum triglyceride levels caused by lncLSTR knockout. Decreased lncLSTR expression leads to increased lipid content in hepatocytes.[43,57]

MEG3. Homo sapiens (human) maternally expressed gene 3 (MEG3; 1,595 nt URS0000759EA9_9606), which is located in the imprinted locus DLK1-MEG3 in the 14q32.3 region of human chromosome, is highly generated in the physiological state and is reduced in the development of MAFLD. It has been demonstrated that activation of MEG3 expression inhibits lipogenesis. It is worth noting that the level of reduction in MEG3 expression is associated with the severity of MAFLD. The decrease in MEG3 expression is likely due to the increase in miR-136 expression.[29,45,47] Long non-coding RNA MEG3 is a competing endogenous RNA (ceRNA) for some miRNAs, among which miR-466i-5p, miR-574-5p, miR-770-5p are represented. The target genes of miR-466i-5p are integrin alpha M (*ITGAM*), prolyl 3-hydroxylase 2 (*P3H2*), serine/threonine kinase 10 (*STK10*); miR-574-5p – FAM20C Golgi associated secretory pathway kinase (*FAM20c*) gene; miR-770-5p – collagen type VI α chain 2 (*COL6A2*) gene. They are expressed in adipose tissue and regulate meta-inflammation, fibrosis and insulin resistance.[58,59] The MEG3 transcript has the ability to directly interact with miR-10b-5p, miR-21-5p, miR-27a-3p, miR-29a-3p, miR-33a-5p, miR-99a-3p, miR-99b-3p, miR-122-5p, miR-139-5p, miR-146b-3p, miR-214-3p, miR-222-5p, miR-296-3p, miR-302a-3p, miR-378a-5p.[49,60] Decreased MEG3 expression induces increased serum triglyceride, cholesterol, and ALT and AST concentrations, while MEG3 deficiency promotes increased expression of nuclear factor 2 of erythroid origin, similar to basic leucine zipper (bZIP) transcription factor 2 (NFE2L2). In hepatocytes, increased activity of the transcription factor

NFE2L2 enhances the expression of PPARγ and associated lipogenic genes, and, conversely, knockout of the *NFE2L2* gene in hepatocytes attenuates the expression of the *PPARG* gene. NFE2L2 activity induces increased VLDL levels and causes hepatic steatosis. NRF2-dependent expression of the PPARγ receptor gene in hepatocytes is believed to be a critical factor that initiates the development of MAFLD, and pharmacological inhibition of NFE2L2 in hepatocytes may be a key approach to prevent the development of hepatic steatosis.[45,50] It has been demonstrated that overexpression of MEG3 significantly reduces the expression of FoxO1, ACC1 and FASN and, as a result, inhibits lipid accumulation in HepG2 cells. It has also been found that MEG3 inhibits the translocation of the FoxO1 factor into the cell nucleus, inhibiting lipogenesis *de novo*. Whereas inhibition of MEG3 expression is accompanied by an increase in the levels of mRNA and proteins of FoxO1, ACC1, FASN, as well as the intensity of lipid accumulation in hepatocytes.[44] At the same time, it was shown that MEG3 sequesters miR-214-3p, promoting the activation of the expression of activating transcription factor-4 (ATF4), which has the ability to potentiate glucose synthesis *de novo* by stimulating FoxO1 transcriptional activity.[61] A decrease in the activity of the transcription factor ATF4 leads to a decrease in gene expression of the protein, CCAAT-enhancer-binding protein homologous protein (CHOP), and inhibition of cell apoptosis.[62] MEG3 sequesters miR-21, which targets low-density lipoprotein receptor-related protein 6 (LRP6), PPARα, and SMAD7. It has been found that miR-21 causes effects similar to those of the expression of genes involved in lipogenesis, including ACC1, SCD1, the SREBP1c factor, and the NR1H3/LXRα receptor. Overexpression of MEG3 is accompanied by an increase in the level of LRP6, which leads to a decrease in lipid accumulation in hepatocytes, an increase in the activity of PPARα receptors, which enhance lipid uptake and β-oxidation, and the inhibitory factor SMAD7, which suppresses the activity of the synthesis of profibrotic molecules.[60,63,64] While MEG3 deficiency is accompanied by overexpression of miR-21, which suppresses the expression of *LRP6, PPARA, SMAD7* genes, contributing to the development of both steatosis and liver fibrosis.[48] Experimental studies have shown that MEG3 activates the genes *SIRT6* and enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*), which are involved in the regulation of triglyceride accumulation and liver inflammation. Insufficient expression of MEG3 also causes the formation of hepatic fibrosis.[51]

LncR MRAK052686. The lncR gene MRAK052686 is located near the zinc finger and BTB domain containing 20 (ZBTB20) protein gene. The function of ZBTB20 is closely related to the antioxidant NFE2L2.[65] Decreased expression of lncR MRAK052686 is associated with suppression of NFE2L2 gene expression and increased expression of fatty acid binding protein 7 (*FABP7*) gene.[57] Decreased NFE2L2 activity is accompanied by increased endoplasmic reticulum stress and induction of inflammation. Fatty acid binding proteins are involved in intracellular lipid metabolism, such as fatty acid uptake, transport, oxidation, synthesis, and storage, and also play a role in regulating the activity of nuclear receptors.[66-68] An increase in the activity of FABP7 leads to the accumulation of lipids in the lipid droplets of the cytoplasm of hepatocytes.[64]

Study limitations and strengths

This systematic review was performed in accordance with the Preferred Reporting Items for Systematic Reviews (PRISMA) guidelines using a validated method for quality analysis of stratified 64 reports.[69] All studies that examined lncRs expression in both MAFLD patients and in MAFLD animal models (*in vivo*) and hepatocyte cell cultures (*in vitro*) were included, primarily focusing on identifying anti-steatogenic lncRs. In MAFLD modeling, we used only representative results from studies. In vivo: experimental subject – 8-week-old male C57BL/6J mice, weighing 18-20 g (negative exposome factor: HFD 12 weeks); in vitro: the object of the experiment is a cell culture of human or mouse hepatocytes (negative exposome factor: cultivation with higher fatty acids for 24 hours, followed by transfection with small interfering RNA, miR mimic/inhibitor and small interfering target gene involved in the corresponding signaling pathway). This systematic review presented the relationship of a wide range of steatogenic signaling pathways associated with the development of MAFLD and suppressed by the physiological expression of anti-steatogenic lncRs. This review includes the rationale for target points of regulation of lncR physiological expression for future precision therapy of MAFLD. and data demonstrate the efficacy of MEG3 in protecting hepatocytes from FA-induced lipogenesis and inflammation. The systematic review was not based on the analysis of randomized clinical trials in patients with MAFLD due to their limitations and the high invasiveness of obtaining biopsy material in "blinded" conditions, as well as the ethical feasibility of collecting it in a control group.

Conclusion

Thus, in MAFLD, hypoexpression of lncRs is observed, which pathogenically affects the main mechanisms of hepatic steatosis development, enhancing lipogenesis *de novo*, inhibiting β-oxidation of fatty acids and lipid export from hepatocytes (Fig. 1).

The development of hepatic steatosis is supported by a decrease in the expression level of anti-steatogenic lncRs, such as AC012668, B4GALT1-AS1/lncSH-

GL, MEG3, FLRL2, Gm16551, lncHR1, lncLSTR, MRAK052686. LncRs overexpression is compensatory in escalating inflammation and hyperglycemia. Future research should aim to achieve consensus on the systematic presentation of results and facilitate the presentation of a core data set on the contribution of lncRs in MAFLD. The research group also aims to expand the current policy to encourage study authors to make RCT data available for meta-analysis purposes.

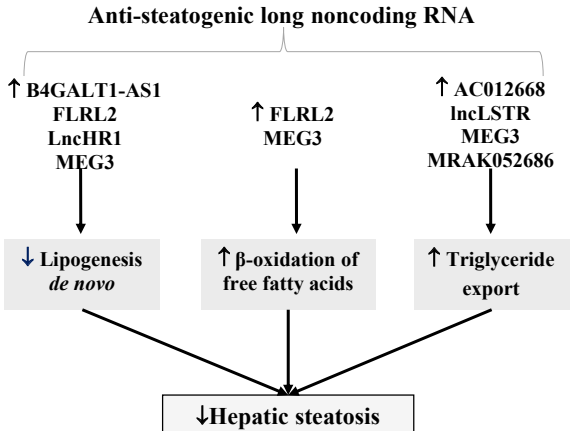

Fig. 1. Effect of anti-steatogenic lncR on the development of hepatic steatosis in MAFLD

Declarations

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Author contributions

Conceptualization, O.A. and A.N.; Methodology, O.A.; Validation, O.A. and A.N.; Formal Analysis, O.A. and A.N.; Investigation, O.A. and A.N.; Resources, O.A. and A.N.; Writing – Original Draft Preparation, O.A.; Writing – Review & Editing, O.A. and A.N.; Visualization, O.A. and A.N.; Supervision, O.A. and A.N.; Project Administration, O.A.

Conflicts of interest

All authors declare that they have no conflicts of interest.

Data availability

No datasets were generated or analyzed during the current study.

Ethics approval

Not applicable.

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REVIEW PAPER

Genetic determinant of metabolic syndrome – a review

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ABSTRACT

Introduction and aim. Metabolic syndrome is a pathological condition that is a combination of insulin resistance, hypertension, dyslipidemia, and abdominal obesity. Every metabolic syndrome trait has an estimated hereditary factor greater than fifty percent. Many mutations related to distinct traits have been successfully identified through genetic studies. With respect to the advancements in our knowledge of the genetics of obesity, it is a major contributor to metabolic syndrome.

Metabolic syndrome is associated with many diseases and is closely related to overweight / obesity and lack of activity. Genetic predisposition also plays an important role in it. Therefore, lifestyle modification is the initial and main intervention that can be implemented for such a population. This review pinpoints the literature on the definition of metabolic syndrome, epidemiology, pathogenesis, and its gene polymorphism and treatment approach comprising metabolic syndrome.

Material and methods. The literature review was carried out using databases such as PubMed, Scopus, Google Scholar and the Web of Science for studies looking into metabolic syndrome and its complications. This review provides a comprehensive analysis of the role of genetic and lifestyle changes in the development of metabolic syndrome.

Analysis of the literature. Metabolic syndrome is a collection of diseases that include lower levels of high-density lipoprotein (HDL), increased triglyceride levels (TG), high blood pressure, more waist circumference, and elevated fasting blood glucose. It is closely related to overweight / obesity and lack of activity. Genetic predisposition also plays an important role in it. So lifestyle modification is the initial and main intervention that can be implemented for this population.

Conclusion. Genetic, lifestyle changes, and other environmental changes contribute to the development of metabolic syndrome. Metabolic syndrome leads to many health issues; for a better healthy outcome, lifestyle modifications such as healthy dietary habits, following regular physical activity, quitting smoking and alcohol, balanced weight, stress management are required.

Keywords. genetics, lifestyle modification, metabolic syndrome, obesity

Introduction

Metabolic syndrome is a collection of elevated risk factors for cardiovascular disease (CVD) and diabetes. These include decreased levels of high-density lipoprotein (HDL) cholesterol, obesity, increased triglyceride (TG), high blood pressure, and glucose level.[1]

Metabolic syndrome is one of the greatest threats to type 2 diabetic mellitus (T2DM) and CVD in this twenty-first century. In addition, a sedentary lifestyle, increased food intake, and the ensuing abdominal obesity may be the basic cause of metabolic syndrome. However, it is now understood that metabolic syndrome is a frequent, complicated reality rather than an illness.[2]

Obesity is associated with an increased risk of comorbidities and difficulties caused by being overweight. Hypertension and metabolic abnormalities, such as elevated TG, fasting blood sugar (FBS) levels, and decreased HDL levels, are linked to the deposition of fat.[3,4] Individuals with abdominal obesity, particularly those who have more of visceral or intra-abdominal adipose tissue, ex-

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hibit metabolic abnormalities associated with insulin resistance.[5] The prevalence of obesity and type 2 diabetes often correlates with the prevalence of metabolic syndrome.[6]

The components of the metabolic syndrome produce a cardiovascular illness. But there is still a risk associated with overweight that includes various types of heart disease, such as microvascular dysfunction, coronary atherosclerosis, myocardial infarction, cardiac dysfunction, and heart failure.[7]

The complete etiology of metabolic syndrome has yet to be discovered. Studies on genetic associations could shed insight into the precursors of metabolic syndrome. According to research, the risk of metabolic syndrome is primarily hereditary. The epidemiological study states that only a minor portion of the genetic variance in metabolic syndrome is explained by the single nucleotide polymorphism (SNP) for which a genetic association with metabolic syndrome has been demonstrated. Consequently, there are still many genetic variations to be found. SNPs linked to lipid levels, insulin resistance, and abdominal obesity in the genome-wide association study (GWAS) are probably candidates for a relationship with the metabolic syndrome itself.[8]

Now, metabolic syndrome has become a problem for public health and it is concerned of its high prevalence. The estimated prevalence with 95% uncertainty interval of metabolic syndrome is 1.4% to 6.7% among children and 4.8% among teenagers. This corresponds to approximately 25.8 million children (around 12.6 to 61 million) and 35.4 million teenagers (around, 21.3 to 63 million) are affected by metabolic syndrome. This prevalence of metabolic syndrome has been estimated worldwide in 2020 at 2.8%. This prevalence has differed among countries based on the income of that country.[9] Research has indicated that the adjusted prevalence of metabolic syndrome in urban Indian populations was found to be around 25% (roughly 31% in women and 18.5% in men).[10] It clearly shows that metabolic syndrome is quite common nowadays and is in the position of rising prevalence worldwide, this emphasizes the increase in obesity and sedentary lifestyle.[1]

Modifications in lifestyle habits, such as increased exercise and decreased calorie consumption, are essential for both preventing and treating metabolic syndrome. Due to the fact that its pathophysiology involves a positive energy balance, it is stressed to achieve optimal body weight, so that excess fat does not deposit in the body.[11]

It has come to know that obesity as a primary cause of metabolic syndrome, linking it to metabolic complications such as insulin resistance and lipid abnormalities. It also clearly shows that obesity is a key determinant of insulin resistance and CVD risk. Here it is mentioned that the genetic basis of metabolic syndrome suggested that SNP associated with insulin resistance, lipid metabolism, and obesity may contribute to disease susceptibility. Although genetic predisposition plays a role, lifestyle factors such as poor dietary habits and physical inactivity are also predominant contributors to the development of metabolic syndrome.

The discussion on the epidemiology reinforces metabolic syndrome among children, adolescents, and adults, and highlights its widespread impact across age groups in different demographic groups. This emphasizes the increasing prevalence of metabolic syndrome due to increasing obesity rates, sedentary lifestyle, and genetic predisposition. This sets the stage for further exploration of its pathophysiology, risk factor, and guess that are associated with metabolic syndrome.

Aim

This review pinpoints the literature on the definition of metabolic syndrome, epidemiology, pathogenesis, and its gene polymorphism and treatment approach comprising metabolic syndrome.

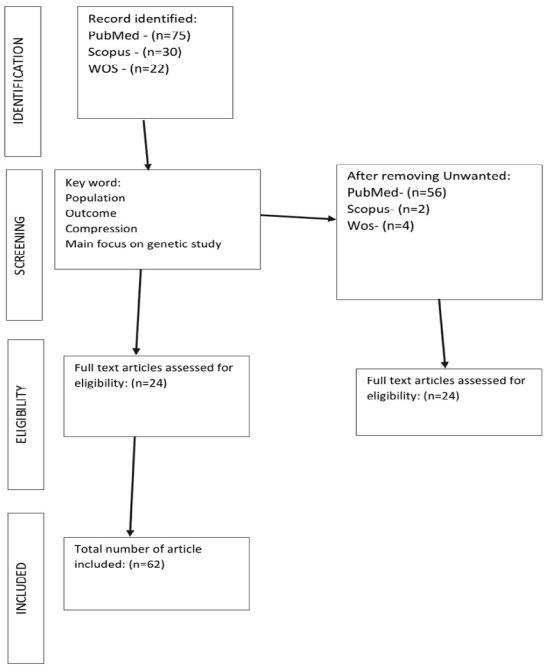

Fig. 1. PRISMA flow chart

Material and methods

The literature review was carried out using databases such as PubMed, Scopus, Google Scholar and the web of science for studies looking into metabolic syndrome and its complications. This review provides a comprehensive analysis of the role of genetic and lifestyle changes in the development of metabolic syndrome. Inclusion criteria: peer-reviewed review paper,, research paper and meta-analysis papers relevant to metabolic syndrome. Studies with various study designs. Studies

involving various age group related with metabolic syndromes. Studies published in the last 15 years, with exception of older highly relevant studies and studies that suggest interventions and preventive measures for metabolic syndrome. Exclusion criteria: Studies that are irrelevant to this review article. Poor methodology and high risk of bias. The selection and inclusion of papers have been mentioned in Figure 1.

Table 1. Criteria proposed for the clinical finding of metabolic syndrome*

Clinical tests	WHO (1998)	EGIR (1999)	NCEP:ATPIII (2001)	AACE (2003)	IDF (2005)	IDF, AHA, NHLBI definition
Insulin resistance	IGT, IFG (>100 mg/dL), T2DM, and with any 2 of the following criteria	Insulin resistance >75th percentile of non-diabetic patients, with two of the following	Nothing, but any 3 of the following criteria	IGT or IFG with any of the following criteria	None	None, but any 3 of the following criteria
Abdominal obesity	Waist-to-hip ratio: >0.9 in men and >0.85 in women, BMI: >30 kg/m^2	WC 94 cm in men and ≥80 cm in women	WC > 100 cm and above in men, and >88 cm and above in women	BMI ≥25 kg/m^2	BMI >30 kg/ m^2. And following 2 criteria	WC 102 cm or more for men, and for women 88 cm or more
Blood pressure	140/90 mmHg or above that	BP 140/90 mmHg or higher or on hypertensive drugs	130/85 mm Hg and more	BP 130/85 mmHg or higher.	BP- 130/85 mmHg or more	BP 130/85 mmHg or more
Lipid profile	TG 150 mg/dL and more than that and High-density lipoprotein (HDL) cholesterol <40 mg/dL in men and <50 mg/dL in women	TG 150 mg/dL or greater and / or high density lipoprotein-cholesterol <39 mg/dL	TG 150 mg/dL or higher. HDL cholesterol <40 mg/dL in men and <50 mg/dL in women	TG 150 mg/dL and higher and HDL cholesterol <40 mg/dL in men and <50 mg/dL in women	TG 150 mg/ dL or more and HDL <40 mg/dL or more	TG 150 mg/ dL and higher, and HDL cholesterol <40 mg/dL in men and <50 mg/dL in women
Glucose	IGT, IFG or T2DM	IGT or IFG (but non-diabetes)	FBS 110 mg/ dL and more	IGT and IFG	FBS 100 mg/dL or more	FBS 100 mg/ dL or more
Others	Microalbuminuria: Urinary excretion of >20 mg/min or albumin: creatinine ratio of >30 mg/g	–	–	Insulin resistance	–	–

* WHO World Health Organization, EGIR – European Group for the Study of Insulin Resistance, NCEP: ATPIII – The National Cholesterol Education Program: adult Treatment Panel III, IDF – International Diabetes Foundation, NHLBI – National Heart, Lung, and Blood Institute, AHA – American Heart Association), T2DM type 2 diabetes Miletus, TG triglyceride, HDL – high density lipoprotein, WC waist

Analysis of the literature

Pathophysiology

The primary pathophysiological feature of metabolic syndrome involves excessive abdominal fat accumulation that results in obesity and insulin resistance, fasting glucose is affected and decreased glucose tolerance, which may alter the gene leading to single nucleotide polymorphism.[12] This shows the dietary changes such as high calorie intake, high fat diet, and life style changes like lack of physical activity, these two are the main contributor to the development of metabolic syndrome (Fig. 2). This determines that visceral adiposity is the main cause of most of the pathways linked to metabolic syndrome.[13] The patient's metabolic disarray becomes a syndrome if they exhibit any three of these conditions showed in the Table 1.

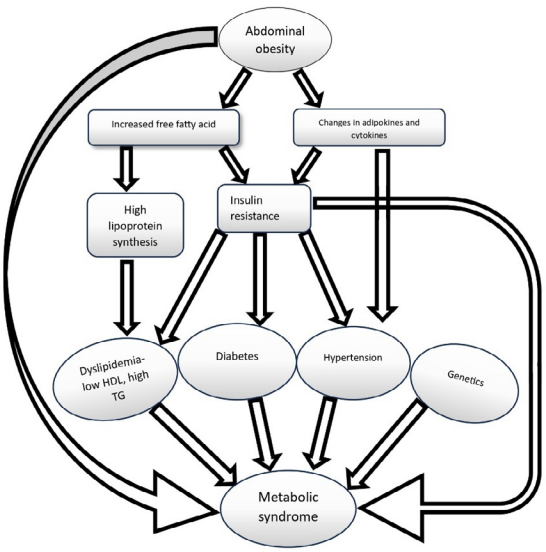

Fig. 2. Characteristics and cause of metabolic syndrome

Obesity

Obesity is a non-communicable disease, which has become a major health issue around the world.[14] According to the World Health Organization (WHO), health is at risk when obesity or overweight is due to unusual or extra fat deposition in the body.[15] When body mass index (BMI) falls under 25 to <29 is considered overweight, and if the BMI is more than 30, is considered as obesity. A person with obesity is at high risk of central obesity, high blood pressure, dyslipidemia, cardiovascular disease (CVD), diabetes, gastrointestinal disorder, muscle and joint problems, respiratory problems, sleeping disorder, and ultimately leads to metabolic syndrome,[16] as obesity is one of the co-occurrence of metabolic syndrome. The overview of obesity is that, it is caused due to the body stores surplus energy than its energy expenditure,[17] lack of physical activity, overconsumption of high calorie and high fat foods, and genetics also plays a major role in developing obesity. Among the components of metabolic syndrome, abdominal obesity is found to be more prevalent than the central obesity.

Anthropometric measurements such as waist hip ratio and BMI help in the partial prediction of metabolic syndrome, as this will show whether the person in having obesity and abdominal obesity proved that the best

anthropometric measure for identifying metabolic syndrome differed depending on the subsets of age and sex subsets.[18]

Insulin resistance

Insulin resistance, characterized by reduced biological activities of the insulin stimulant of target tissue, especially adipose tissue, muscle, and liver. In this phase, it decreases glucose disposal leading to an increase in beta cells and subsequently causes hyperinsulinemia.[19] Insulin resistance is one of the root causes of more pathophysiology problem in the process and development of many non-communicable diseases, including dyslipidemia, T2DM, obesity, hypertension, CVD, sleep problem, cancer, nonalcoholic fatty liver disease (NAFLD), poly cystic ovary disease (PCOD), commonly it contributes to metabolic syndrome.[20,21] Mostly Insulin resistance will be present in the person having metabolic syndrome. The body produces insulin to transport glucose into cells for its process of producing energy, so people with metabolic syndrome are mainly obese, which makes it more difficult for cells to response to the insulin. says that the researcher has observed that most patients with high level of plasma insulin and insulin resistance also had hypertension.[22] Insulin resistance and diabetes also cause many kinds of heart disease.

In the diagnosis of metabolic syndrome by gender, the insulin resistance area under the curve for the homeostatic model assessment of insulin resistance (HOMA-IR) in men with metabolic syndrome are (0.7000) and for women with metabolic syndrome (0.6779). for the quantitative insulin sensitivity check index (QUICKI) (0.7016) and (0.65779) in men and women with metabolic syndrome. The prediction accuracy is similar for men, indicating effective performance in both HOMA-IR and QUICKI. For women both show lower area under the curve value suggesting predictive capability. Sensitivity and specificity differ between sexes. [23,24]

Dyslipidemia

Dyslipidemia is a fundamental part of metabolic syndrome. It refers to an unusual level of lipid in the bloodstream. Increased triglyceride, low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and reduced level HDL. The criteria for metabolic syndrome are that increased triglycerides 150 mg/dL or more per dL of blood and reduced HDL cholesterol, below 50 mg/dL and 40 mg/dL in women and men, both define hypertriglyceridemia.[25]

Recently, many findings have shown that people with metabolic syndrome are more prone to heart-related diseases such as cardiovascular disease, myocardial infraction, stroke, coronary heart disease. The highly atherogenic lipid profile is often the result of having an unhealthy eating habit and unhealthy lifestyle changes such as smoking, alcohol, lack of physical activity, leading sedentary life or genetic predisposition.[26,27]

Genetic predisposition to dyslipidemia and type 2 diabetes share more phenotypic feature of metabolic syndrome such as (insulin resistance, hypertension, abdominal obesity, and abnormal cholesterol levels). Mostly, it seems to present a higher risk of cardiovascular disease. Therefore, according to the guidelines, diabetic patents are also treated for dyslipidemia.[28]

Hypertension

Hypertension is one of the other components of metabolic syndrome. Hypertension is highly correlated with dyslipidemia, insulin resistance, obesity, which are considered as metabolic syndrome.[29] Hypertension is one of the basic reasons for the complication of diabetes, atherosclerosis, CVD and other heart-related problems, and even depression.[30] Nowadays, it is more common among adults due to their frequent stress, unhealthy eating habits, obesity, sedentary life style, high salty foods and processed food are the main cause for the cause of hypertension.[31] Many studies have found that genetic predisposition causing hypertension, where pathways and other molecular mechanism has impact on blood pressure which influences metabolic syndrome.[32]

Role of genetics in the development of metabolic syndrome

Genetics

In particular, lifestyle changes, environmental factors, diet, and exercise are the primary source for the onset of metabolic syndrome. However, genetic factors contribute a crucial role in the onset of metabolic syndrome. Researchers have found that many genes are associated with obesity, dyslipidemia, insulin resistance.[33] However the genetic research to examine metabolic syndrome is much less. Family-based studies have been concerned with identifying the genetic factors that contribute to various diseases such as metabolic syndrome.[34] As the sedentary lifestyle is more common these days. The increasing incidence of metabolic syndromes among young adults and children is more prevalent. Deeper understanding of the genetics that are related to metabolic syndrome will give proper insight into the inception of the disease.[35] Different countries and various clinical findings have different criteria to find metabolic syndrome, in addition to genetic, will also help in finding the metabolic syndrome and prevent future complications. This genetic study may be more consistent. Recent GWAS have allowed the identification of many polymorphisms associated with metabolic syndrome. Genetic markers can be used for early detection of metabolic syndrome as a biomarker for screening for the development of its related diseases. Here are some genes associated with various diseases interlinked with metabolic syndrome that are mentioned below.

Adiponectin (ADIPOQ)

It controls glucose metabolism and lipid metabolism, enhances insulin sensitivity, manage food intake, and body weight. The ADIPOQ gene is located on the 3q27 chromosome and this locus is susceptible for it to be associated with cardiovascular risks such as hypertension, diabetes, LDL-C and TG.[36,37] SNP in adiponectin gene negatively correlated with HDL, body weight, waist circumference, fasting insulin, TG, insulin resistance and HOMA IR. show the association with adiposity, insulin secretion and T2DM. The study conducted with the Caucasian population found that high triglycerides, low adiponectin concentration, and insulin resistance are associated with the GG rs17300539 genotype, which will lead to a higher risk of metabolic syndrome.[38] Punjabi population concludes that, ADIPOQ -3971A>G (rs22396) and +276G>T (rs1501299) SNP could be clinically helpful in assessing metabolic syndrome and its related problem among that population.[39] Investigation done with the Crimean individuals in the population of Crimean has found that GT genotype is more prevalent variant of ADIPOQ (rs2241766) among patients with metabolic syndrome. It is also associated with high systolic blood pressure and high BMI. Whether high diastolic blood pressure, high HBA1c glucose value, high value are associated with the ADIPOQ individuals carrying the genotype (rs2241766). Their finds show that this genetic variant is very closely linked to metabolic syndrome in this population (Fig. 3).[40]

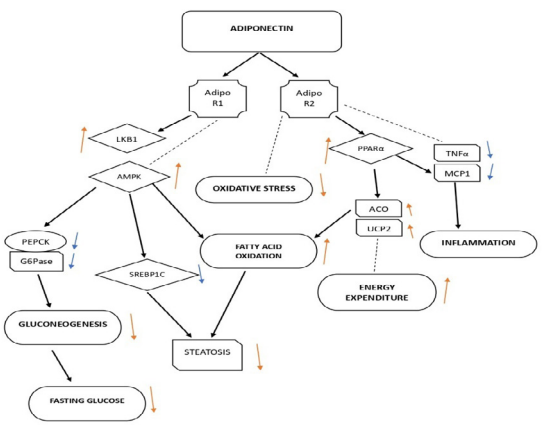

Fig. 3. Mechanism of adiponectin (AdipoR1 – adiponectin receptor 1, AdipoR2 – adiponectin receptor, LKB1 – liver kinase B, AMPK – AMP-activated protein kinase, PEPCK – phosphoenolpyruvate carboxy kinase, G6Pase – glucose-6-phosphatase, SREBP1c – sterol regulatory element binding protein 1c, PPARα – peroxisome proliferator-activated receptor alpha, ACO – acyl-CoA oxidase, UCP2 – uncoupling protein 2, TNFα – tumor necrosis factor alpha, MCP1 – monocyte chemoattractant protein 1)

Peroxisome proliferator – activated receptor gamma co-activator 1 – alpha (PPARGC1A)

Shows the association with adiposity, insulin secretion indexes, and type 2 diabetes.[41]SNP in PPARGC1A, became more inflexible and may have altered the protein's catalytic activity and structural structure.[42] It may also have caused coronary artery disease (CAD), nonalcoholic fatty liver disease (NAFLD), and T2DM. According to the genotype, the parameters among the 2-study group do not show any significant variation. Therefore, statistical significance does not show the risk of metabolic syndrome.[43] The Gly482Ser polymorphism (rs8192678) may facilitate in the onset of metabolic syndrome, but the effect might be less. A meta-analysis performed using data collected from Asian, African and European populations concludes that the PPARGC1A gene locus might be one of the risk factors for type 2 diabetes (Fig. 4).[44]

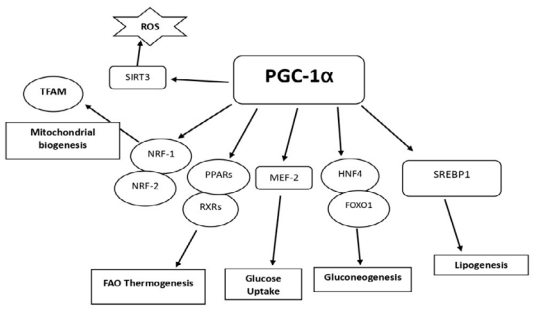

Fig. 4. Mechanism of PPARGC1A (SIRT3 sirtuin 3, ROS – reactive oxygen species, nuclear respiratory factors NRF-1 and NRF-2, mitochondrial transcription factor A, TFAM mitochondrial transcription factor A, PPAR peroxisome proliferator activated receptors, RXRs – retinoid X receptors, FAO fatty acid oxidation, MEF-2 – myocyte enhancer factor 2, HNF4 nuclear factor 4, FOXO1 – fork head box protein O1 SREBP1 sterol regulatory element-binding protein 1)

Fat mass obesity associated (FTO)

Studies have shown that the FTO gene is very much associated with obesity. FTO is present in the hypothalamus, so it is associated with energy expenditure and in regulation of food intake.[45] FTO is located on the chromosome at 16q12.2. The first gene that is associated with obesity is FTO, which was found by GWAS. As diabetes and obesity is characteristics of metabolic syndrome, it is believed that FTO gene polymorphism might be linked to the risk of metabolic syndrome. A study found that the SNP of FTOrs9939609 gene was correlated with the risk among the Temiar subtribe.[46] A study done with the FTO rs9939609 gene, their results show that, the subjects with AA allele have significantly low HDL cholesterol levels than subjects with TT allele. Therefore,

they concluded that the FTO rs9939603 gene is one of the causes of genetically risk factor for metabolic syndrome among the Egyptian population. And they also highlight that the HDL component may be the reason for this association.[47] FTO gene (rs9939609) is used to study the southern Italian polymorphism in the population among the subjects with Metabolic Syndrome, their results show that, TA allele heterozygous the SNP of FTO gene (rs9939609) is significantly correlated among the individuals with metabolic syndrome.[48]

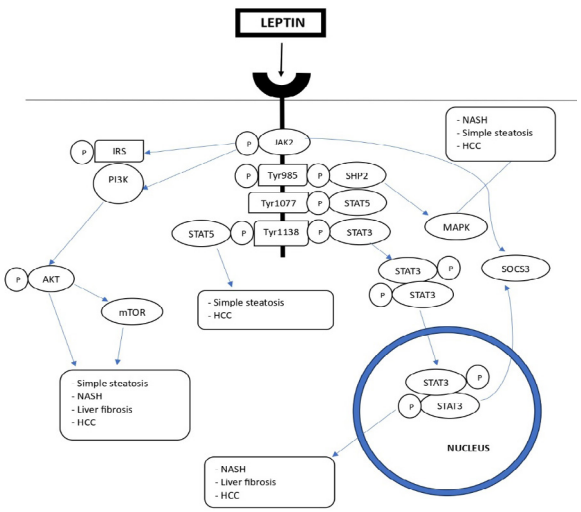

Fig. 5. Mechanism of leptin (JAK2 phosphorylates Tyr985, Tyr1077, and Tyr1138 residues on Ob-Rb, STAT signal transducers and activators of transcription, SOCS3 suppressor of cytokine signaling 3, PI3K – phosphoinositide 3-kinase. IRS – insulin receptor substrate. AKT – protein kinase B, mTOR – mammalian target of rapamycin, P – phosphorylation, NASH - nonalcoholic steatohepatitis, HCC – hepatocellular carcinoma)

Leptin (LEP)

When obesity develops, plasma LEP levels increase and when weight is lost, they drop. The hypothalamus and brain stem contain the majority of leptin receptors, and signals from these receptors regulate neuroendocrine function, energy expenditure, and satiety. Therefore, many overweight people will have leptin resistance.[49] Adiposity and plasma leptin concentrations are associated, and hyperleptinemia is in fact believed to be a separate risk factor for CVD and also nonalcoholic steatohepatitis (NASH).[50] Research carried out in Kyrgyz population, the leptin receptor gene shows the association with a higher level of insulinemia and glycemia, therefore, this gene may be the marker of insulin resistance.[51] Their research shows that LEP AA genotype carriers has the higher incidence of Metabolic Syndrome and the G2548A gene of leptin polymorphism BMI and FBS is correlated with the G2548A gene of leptin polymorphism. So, the research confirms that Leptin G2548A loci will alone be the risk factor for Metabolic Syndrome. Their findings show that the homozygous genotype LEP 2548AA is a risk factor for developing high cholesterol levels (Fig. 5).[52]

Lipoprotein lipase (LPL)

The LPL gene is located on the human chromosome 8p22 and helps in the transport and metabolism of lipoprotein. The SNPs for each gene were associated with an increased risk of obesity, and the LPL protein interacts functionally to regulate lipid metabolism. The study shows that the C allele and the CC allele of the rs13702 C/T SNP of LPL gene is significantly correlating with type 2 diabetes. Their research states that this may be due to increased level of LPL that will be leading to insulin resistance.[53] An interventional study was conducted in Taiwan among metabolic syndrome, who are subjected to do aerobic exercise. Their result shows that the polymorphism of the LPL gene (rs3779788) expressed in TT carrying individuals who did aerobic exercise shows the improvement in metabolic syndrome compared with CC/CT carrying individuals.[54]

Apolipoprotein A1 (APOA-1)

Apoa1 gene is located in chromosome 11q23. the main protein found in HDL particles, which provides protection against CVD risk. The SNP in the APOA-I gene affects the HDL level, leading to many CVD risks.[55] A study conducted with 1000 adult obese, states that, Apoa1 rs570 gene has an influence on glucose, HOMA-IR, insulin and anthropometric measurement. Central obesity, low HDL levels, diabetes are shown in males without A alleles.[56] Studies conducted in the Malaysian population states that patients with DM2, Apoa1 -75G>A genetic polymorphism can be considered as susceptibility to myocardial infraction. The variation of the Apoa1 gene is also associated with arterial stiffness, since the Apoa1 gene is involved in the synthesis, transport, and HDL process.[57] Research conducted in Tehran shows that, individual who are regularly eat the western diet pattern and fat-sweet dietary pattern are associated with Apoa1 gene polymorphism linked with Metabolic Syndrome risk.[58]

Apolipoprotein A2 (APOA-2)

Apoa2 is the second most abundant protein in HDL. Apoa2 appears to affect the process of reverse cholesterol transport and the antioxidant role of HDL cholesterol, which is consistent with the observation that increased Apoa2 levels and SNP in this gene promote the development of atherosclerosis and other CVD risks. Research done in Egypt states that CC genotype SNP is associated with insulin resistance.[59] Study conducted among Egyptian adolescents, results show that, after adjusting for multivariate, individuals with homozygous CC genotype exhibit higher levels of body fat percentage,

more WC, visceral adipose tissue, and HDL cholesterol when compared to the TT or TC genotype. In common, these individuals were consuming more food, so they conclude that individuals with homozygous C allele have more risk of obesity than the individuals with T allele. And they mentioned the link between Apoa2 c.-492T>C SNP and food intake.[60] Apoa2 rs5082 polymorphism can serve as marker to forecast the effectiveness of digital healthcare and lifestyle interventions, where genotype-based personalized medicine is required.[61]

Genetics causes various changes in the body, especially by increasing certain diseases or due to inherited genetic factors. Genetic predisposition is the probability of having a high risk of metabolic syndrome. Therefore, genetic screening is also highly important to predict metabolic syndrome and its related disease. A limitation of these studies is that they are often population-specific.

The limitations of these studies are often population-specific, focusing on particular ethnic groups. The findings may not be generalizable to other populations with different genetic backgrounds, lifestyle factors, or environmental exposures. Results from these populations may not apply to larger and more diverse populations. Depending on the study, sample sizes may be relatively small, limiting the ability to detect the effects of genetic variants on metabolic syndrome. Smaller sample sizes also increase the risk of type I or type II errors, leading to negative results. Genetic variants may not have uniform effects among individuals within the same population due to genetic heterogeneity. The influence of specific SNPs may vary based on other genetic factors, making it difficult to predict individual responses to these variants. Environmental factors, including lifestyle choices (eg, diet, exercise), environmental exposures, and other external factors, can interact with genetic factors in influencing metabolic outcomes. If these factors are not properly considered, they could confound the observed genetic associations. Genotyping technologies and the choice of SNPs may vary between studies, potentially leading to inconsistencies in the results. Additionally, the relevance of certain SNPs might be influenced by gene-environment interactions or epigenetic modifications that are not captured in these studies.

Lifestyle modification

As sedentary lifestyle is more common these days, the rising incidence of metabolic syndrome among young adults and children is more prevalent. Lifestyle changes in daily life will help in preventing various diseases. Metabolic syndrome is one of the diseases, where it can be treated by altering individual lifestyle habits like, regular physical activity, quitting smoking and alcohol, following healthy diet patterns, reducing or managing stress, maintaining healthy weight, having good quality of sleep, regular ingestion of medication, in case of hypertension, diabetes, dyslipidemia. Lifestyle modification plays a key role in the metabolic syndrome.[62]

Conclusion

Metabolic syndrome is the characterization of a combination of physiological, biochemical, clinical, metabolic factors, genetic factors, diet and lifestyle modification, and environmental changes. This will increase the risk of hypertension, obesity, cardiac related problems, insulin resistance, T2DM, dyslipidemia, genetic predisposition and stress. Metabolic syndrome can be treated by lifestyle modifications such as healthy eating habits, following regular physical activity, quitting smoking and alcohol, balanced weight, and stress management. Lifestyle modification is required to overcome metabolic syndrome. medications will be required if the metabolic syndrome is not treated. Genetic predisposition is the major factor in developing metabolic syndrome. Many studies are undergoing to find various genetic polymorphism markers to detect the risk of metabolic syndrome risk. In general, it provides a strong foundation for discussing metabolic syndrome by demonstrating its significance in both clinical and public health contexts.

Declarations

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Author contributions

Conceptualization, H.N. and S.G.; Methodology, H.N.; Software, H.N.; Validation, S.G. and H.N.; Formal Analysis, S.G.; Investigation, S.G.; Resources, H.N. and S.G.; Data Curation, H.N.; Writing – Original Draft Preparation, H.N.; Writing – Review & Editing, H.N.; Visualization, H.N.; Supervision, H.N.; Project Administration, H.N.; Funding Acquisition, H.N. and S.G.

Conflicts of interest

The authors declared no conflicts of interest.

Data availability

The dataset supporting the finding of this study are derived from publicly available database and articles, which are cited in the reference list.

Ethics approval

Ethical approval was not required for this study as it is a review of previously published literature and does not involve human participants or animal subjects.

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REVIEW PAPER

Effects of *Ganoderma lucidum* ("lingzhi") supplementation on blood lipids – a systematic review of clinical trials

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ABSTRACT

Introduction and aim. Impaired blood lipid profile is a major risk factor for cardiovascular disease, the leading cause of mortality worldwide. Supplementation of *Ganoderma lucidum* ("lingzhi") has been postulated to have a positive impact on blood lipids. This review aims to evaluate the evidence on this topic.

Material and methods. A systematic search of Google Scholar, PubMed and clinicaltrials.gov databases was performed to identify randomized clinical trials and cross-over trials which assessed the effects of lingzhi supplementation on blood lipids. The gathered data were reviewed and analyzed in accordance with PRISMA guidelines.

Analysis of the literature. Of the 805 records identified in the initial search, a total of 7 studies met all the inclusion criteria and were qualified for the review. None of them reported a statistically significant change in triglycerides, high-density lipoprotein, or low-density lipoprotein during lingzhi supplementation. Only 2 out of 7 trials showed a moderate decrease in total cholesterol level.

Conclusion. There is no evidence to support any effect of *G. lucidum* supplementation on triglycerides, high-density lipoprotein or low-density lipoprotein. Its influence on total cholesterol also remains highly doubtful based on the present systematic review.

Keywords. cardiovascular disease, cholesterol, *Ganoderma lucidum*, lingzhi, lipids

Introduction

Cardiovascular disease is the leading cause of death worldwide, accounting for 27% of global mortality.[1] Impaired blood lipid profile, especially elevated level of low-density lipoprotein, presents a major, yet potentially modifiable risk factor for its development.[2,3] For this reason, statins – the most common lipid-lowering drugs – play a key role in the primary prevention of cardiovascular disease.[4] However, the use of statins is associated with several side effects, which has stimulated extensive research into alternative therapies.[5]

Ganodrema lucidum (commonly known as "lingzhi" or "reishi") is a mushroom widely used in traditional medicine of many Asian countries, notably in traditional Chinese medicine. It is believed to possess immunomodulating, bacteriostatic, hepatoprotective, and life-prolonging properties.[6] Some research also suggests that *G. lucidum* has a beneficial impact on impaired blood lipid profile and could be used in the cardiovascular disease prevention.[7–9]

Aim

Despite considerable clinical and research interest in this topic, a methodologically rigorous summary of the existing literature is lacking. No up-to-date review has systematically assessed the potential effects of *G. lucidum* supplementation on blood lipids in the general population. This article aims to fill this gap by critically

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analyzing existing clinical trials, including the latest research.

Material and methods

The study was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses 2020 (PRISMA 2020) statement,[10] but was not prospectively registered. The focus was on the effect of lingzhi supplementation on blood lipids, namely: triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL,) and low-density lipoprotein (LDL).

Search strategy

PubMed and Google Scholar databases were searched using the following phrases: ("Ganoderma lucidum" OR "Reishi" OR "Lingzhi") AND ("Lipids" OR "Cholesterol" OR "Triglycerides" OR "Cardiovascular"). No restrictions regarding language, publication period or publication type were applied. Moreover, ClinicalTrials.gov repository was screened for all studies, which used "Ganoderma" as an intervention/treatment. In addition, reference lists of included articles were manually checked to identify more potentially relevant publications. The final search took place on September 9, 2024.

Study selection

All identified studies were entered into Rayyan software[11] and duplicates manually removed. A single author analyzed each unique study based on title and abstract, and then evaluated the full texts of publications that passed the initial assessment. Studies were considered to be eligible for the review if they fulfilled all of the following criteria: randomized controlled trials or controlled crossover trials; *G. lucidum* in a single-component preparation; changes in TG, TC, HDL, and LDL as an outcome; full text in English language. Conversely, the exclusion criteria were: in vitro studies, animal studies, no-intervention studies, case reports, reviews or meta-analyses; studies lacking randomization or a control group; studies that only administrated *G. lucidum* in combination with other herbal medicine; publications in languages other than English.

Data extraction

Data from all included publications were extracted and analyzed by a single researcher. Extracted information included: first author's last name and publication date; study design; sample size; participants health status; administered dose of *G. lucidum*; duration of the intervention; statistically significant outcomes regarding blood lipids.

Risk of bias assessment

Risk of bias was judged by a single author in accordance with the recommendations of the Cochrane Handbook of Systematic Reviews of Interventions.[12] The applied criteria were: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other sources of bias. Each criterion was rated as "low risk", "unclear risk" or "high risk."

Analysis of the literature

Literature selection

The electronic search yielded 805 records. Of these, 95 were excluded as duplicates, and 695 because they were not relevant to the purpose of the review. In the latter case, the main reasons behind exclusion were: unrelated topic (studies discussing biotechnology, biochemistry or ecology of *G. lucidum*), wrong study type (reviews, laboratory studies or animal studies), non-English language studies. The remaining 15 publications were retrieved and subjected to rigorous evaluation, of which 8 did not meet the inclusion criteria due to wrong outcome (n=3) and flawed study design, i.e. lack of randomization or appropriate control group, (n=5). Ultimately, 7 studies were included in the review (Fig. 1).

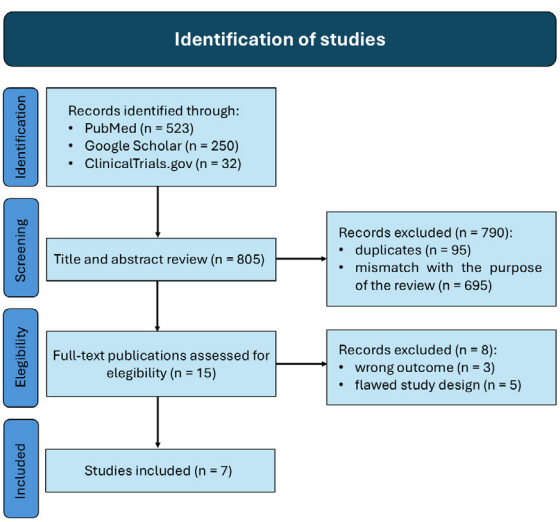

Fig. 1. PRISMA flowchart of this systematic review

Characteristics of the included studies

As the articles included in this study vary greatly in terms of research methodology and data reporting practices, it seems appropriate to discuss them in detail. The Wachtel-Galor 2004a[13] study was a double-blind placebo-controlled crossover trial which enrolled 10 healthy patients. Half of the subjects were non-selectively assigned to begin daily supplementation with 720 mg *G. lucidum*, and another half with suitable placebo. Supplementation lasted 10 days, and after at least two-week-long washout period, patients received the alternative treatment. The obtained data suggested a negligible decline in blood lipids (TG, TC, HDL, LDL) levels after *G. lucidum* supplementation, but the changes were not statistically significant.

Wachtel-Galor 2004b[14] was a follow-up to the above-mentioned study. Its protocol was very similar to Wachter-Galor 2004a, the major differences being the number of participants (n=18), the daily dose of *G. lucidum* (1440mg per day), and the duration of the trial (4 weeks). The authors again reported a slight, statistically insignificant trend towards a decrease in blood lipids levels after *G. lucidum* supplementation.

Gao 2004a[15] was a randomized controlled clinical trial that evaluated the effect of supplementation with 5400 mg *G. lucidum* in patients suffering from type II diabetes mellitus. After 12 weeks, no statistically significant changes were observed in TG, TC, HDL, and LDL levels in either the treated or control groups.

The Gao 2004b[16] study was a double-blind randomized controlled trial conducted among 170 patients with coronary heart disease. Participants received 5400 mg *G. lucidum* daily or placebo for 12 weeks. The authors noted a significant decrease in TC level in the *G. lucidum*-treated group, with no significant change in the control group. Nevertheless, the authors did not present between-groups comparison of these changes. Additionally, no data have been published regarding TG, HDL, and LDL levels.

Chu 2011[17] was another double-blind crossover study conducted to assess whether *G. lucidum* supplementation has any effect on blood lipids levels. It recruited 26 hypertensive and/or dyslipidemic patients, who were randomly allocated to start with 1440 mg *G. lucidum* per day or a placebo. After 12 weeks of supplementation and 4 weeks of a crossover period, participants switched to the corresponding groups and received alternative treatments. The results of Chu 2011 were hard to interpret. A significant crossover effect prevented the authors from conducting a proper analysis of changes in TG and HDL levels. The data obtained from the first treatment period showed a mild decline in TG and a moderate increase in HDL in the *G. lucidum*-treated group, however, comparison of changes between groups did not show statistical significance. No statistically significant differences were also observed between groups in terms of changes in TC and LDL levels based on data from both treatment periods.

During the Klupp 2016 trial, 84 patients with type II diabetes mellitus and metabolic syndrome were randomized into one of three groups to receive either 3000 mg lingzhi, 3000 mg *G. lucidum* plus 1000 mg *Cordyceps sinensis,* or placebo.[18] The supplementation period lasted 16 weeks. Due to the small sample sizes, the authors decided to combine both treatment groups for further analysis. This approach did not reveal any significant changes in any of the blood lipids in the combined-treatment group compared to the control group.

The potential effects of *G. lucidum* supplementation in 72 overweight patients were investigated in the Babamiri 2022 trial.[19] The study was double-blind, randomized, and placebo-controlled, with each participant receiving 750 mg of *G. lucidum* per day. After 6 weeks, a slight reduction in TC levels in the *G. lucidum* group was the only parameter that changed significantly compared to the placebo group. Improvements in TG, HDL and LDL levels have been proven to be negligible.

Overall, of the seven analyzed studies, only Gao 2004b and Babamiri 2022 reported a significant decrease in TC levels after *G. lucidum* supplementation, and the first one did not compare this change with the control group.[16,19] Other, smaller but more methodologically rigorous, studies did not confirm these positive results. In addition, *G. lucidum* supplementation caused significant changes in TG, HDL and LDL levels in none of the reviewed studies, suggesting a lack of a noteworthy effect in this regard. A concise description of analyzed studies is provided in Table 1. A more quantitative synthesis could not be performed due to insufficient quality and availability of the data.

Table 1. Characteristics of the included studies

Author	Study design	Sample	Health status	Dose	Duration	Significant outcomes
Wachtel-Galor 2004a[13]	double-blind crossover study	n=10	healthy	720 mg	10 days	no significant changes in blood lipids
Wachtel-Galor 2004b[14]	double-blind crossover study	n=18	healthy	1440 mg	4 weeks	no significant changes in blood lipids
Gao 2004a[15]	RCT	n=71	type II diabetes mellitus	5400 mg	12 weeks	no significant changes in blood lipids
Gao 2004b[16]	double-blind RCT	n=170	coronary heart disease	5400 mg	12 weeks	decrease in TC (not compared to the control group)
Chu 2011[17]	double-blind crossover study	n=26	hypertension and/or dyslipidemia	1440 mg	12 weeks	partial data suggest decrease in TG and increase in HDL, which proven to be insignificant when compared to the control group; no significant between-groups changes in TC and LDL
Klupp 2016[18]	double-blind RCT	n=84	type II diabetes mellitus ant metabolic syndrome	3000 mg	16 weeks	no significant changes in blood lipids
Babamiri 2022[19]	double-blind RCT	n=72	overweight	750 mg	6 weeks	decrease in TC compared to the control group

Risk of bias

With the exception of Klupp 2016, no study reviewed provided sufficient information on randomization and allocation concealment of participants. Most studies also lacked a detailed description of blinding practices, but the objective nature of biochemical assessment of blood lipid levels allowed most studies to be designated as "low risk" with respect to blinding criteria. Deficiencies in reporting missing data and lack of available study protocol were another common flaws reflected in

"unclear risk" or "high risk" ratings. The author did not find other sources of bias in any of the reviewed studies. A complete summary of the risk of bias assessment is depicted in Figure 2 and Figure 3.

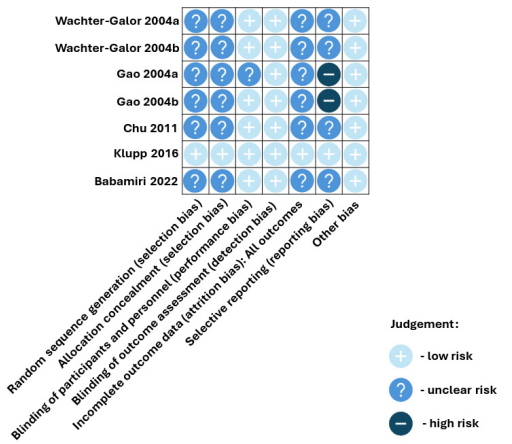

Fig. 2. Domain of risk of bias assessment

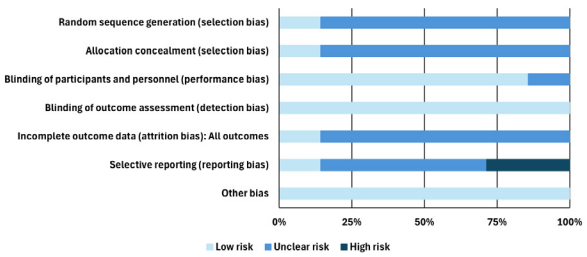

Fig. 3. The overall results of risk of bias assessment

Discussion

The literature evaluating the metabolic effects of *G. lucidum* supplementation remains relatively sparse. It consists mainly of animal studies, the vast majority of which have yielded positive results regarding blood lipid levels. A recent meta-analysis summarizing these trials concluded that *G. lucidum* effectively improves impaired lipid profile in a dose-dependent manner.[20] However, such findings cannot be directly applied to medical practice, as all animal trials are performed in strictly controlled laboratory conditions and use models which may not be representative for human physiology.

A more clinically relevant evidence comes from 2015 meta-analysis that evaluated the use of *G. lucidum* among patients at risk for cardiovascular disease.[21] Nonetheless, even this study suffered from some limitations. It excluded the two trials discussed in this review that were conducted in healthy patients (Wachtel-Galor 2004a and Wachtel-Galor 2004b studies) and did not include the newest Babamiri 2022 study.[13,14,19] As a result, it only qualified three studies to the final meta-analysis, all of which were conducted in diabetic patients. The authors confirmed the lack of statistically significant effect of *G. lucidum* on concentrations of TC, TG, HDL and LDL in this subpopulation.

Such results are in good agreement with the findings of the present study, which is, to the author's knowledge, the first one to collectively assess all good-quality human trials, regardless of participants health status. The data gathered show that the TC-lowering effect of *G. lucidum* is highly questionable. If it exists, it is probably small in size and requires long-term supplementation. The accumulated evidence also suggest that *G. lucidum* does not affect TG, HDL, and LDL levels. Still, further research is needed to definitively confirm these findings.

This study has several limitations. It included only publications in English, which may have led to the omission of potentially relevant research reported in other languages. In addition, *G. lucidum* formulation and standardization methods varied between included studies, a factor that could have affected the observed outcomes. The qualitative synthesis of gathered evidence was performed by a single author and a quantitative analysis was not conducted due to insufficient quality of the data. Finally, the risk of bias assessment was also conducted by a single author, which could have influenced the obtained results.

Conclusion

The effect of *G. lucidum* supplementation on blood lipid levels remains highly doubtful. Most studies do not confirm its positive impact on TC. No study reports any significant influence on TG, HDL, and LDL. Although a beneficial effect of *G. lucidum* supplementation on blood lipid levels seems unlikely, it cannot be definitely ruled out based on the current limited data. A bigger, longer-term, and more methodologically rigorous studies are needed to draw any stronger conclusions.

Declarations

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Author contributions

Conceptualization, J.Z.; Methodology, J.Z.; Software, J.Z.; Validation, J.Z.; Formal Analysis, J.Z.; Investigation, J.Z.; Resources, J.Z.; Data Curation, J.Z.; Writing – Original Draft Preparation, J.Z.; Writing – Review & Editing, J.Z.; Visualization, J.Z.; Supervision, J.Z.; Project Administration, J.Z.; Funding Acquisition, J.Z.

Conflicts of interest

Author states no conflict of interest.

Data availability

All data generated or analyzed during this study are included in this published article.

Ethics approval
Not applicable.

Use of AI and AI-assisted technologies in the writing process
During the preparation of this work the author used Rayyan software in order to screen the literature and detect duplicates. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the published article.

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REVIEW PAPER

The effect of exercises on the quality of life in individuals undergoing hip arthroplasty following hip fractures – a systematic review and meta-analysis

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ABSTRACT

Introduction and aim. The increasing burden of hip fractures and hip arthroplasties (HA) requires a deeper understanding of physiotherapy rehabilitation, facilitating the development of evidence-based strategies to enhance patient recovery. The aim of this study is to evaluate the effectiveness of exercises in improving quality of life after HA.

Material and methods. A systematic search of PubMed, Scopus, Web of Science, CINAHL, Pedro, Embase, and the Cochrane Central Register of Controlled Studies (CENTRAL) was conducted from inception until April 2023. Randomized controlled trials (RCTs) were recovered that compare exercises with any comparator to improve quality of life were retrieved. The quality was evaluated using PeDro. Data were pooled using random-effects models.

Analysis of the literature. Analysis of eight RCTs, comprising 1,560 individuals, revealed that comprehensive exercise programs produced superior outcomes in quality of life compared to standard treatment, with a notable effect size (SMD, 0.68; 95% CI, -0.01 to 1.37; I^2=89%) and progressive resistance exercises have a statistically significant positive impact on quality of life.

Conclusion. This review underscores the importance of comprehensive exercise programs, including progressive resistance training to improve quality of life in patients undergoing hip arthroplasty, and recommends further investigation to determine the most effective exercise parameters for the development of personalized rehabilitation programs.

Keywords. exercises, hip arthroplasty, quality of life

Introduction

Hip fractures are the most common fractures that require hospitalization, associated with significant deterioration in quality of life and an increased risk of mortality.[1] Hip arthroplasty (HA) is the preferred surgical intervention for people with displaced femoral neck fractures, total hip arthroplasty suitable for younger and more active patients, and bipolar hemiarthroplasty more suitable for older patients with significant comorbidities.[2,3] While the hip arthroplasty procedure is analogous for both hip fractures and osteoarthritis, distinct variations are evident in the surgical findings of the capsule, ligaments, and sur-

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rounding muscles in osteoarthritic hips, differing significantly from those in healthy hips. The physiological stress associated with hip fractures, as opposed to elective hip replacements, may be a key factor underlying the inferior outcomes and increased mortality seen in patients with hip fractures after surgery.[4-7]

The increasing burden of hip fractures and hip arthroplasties requires a deeper understanding of the rehabilitation process, which facilitates the development of evidence-based strategies to improve patient recovery and outcomes. The American Physical Therapy Association (APTA) clinical practice guidelines recommend that physical therapists implement structured exercise protocols and provide post-discharge follow-up care in various settings, including homes and outpatient facilities, to target existing impairments and functional limitations.[8]

Exercises should be implemented methodically with the primary objective of improving the patient's quality of life. Recent systematic reviews on HA have evaluated the effects of physical therapy on functional outcomes, gait and balance, pain management, and the effectiveness of aquatic exercises in improving clinical outcomes.[9-12] The scope of these reviews was restricted to trials featuring exclusively osteoarthritic individuals, with no consideration of patients who underwent HA for hip fracture treatment.

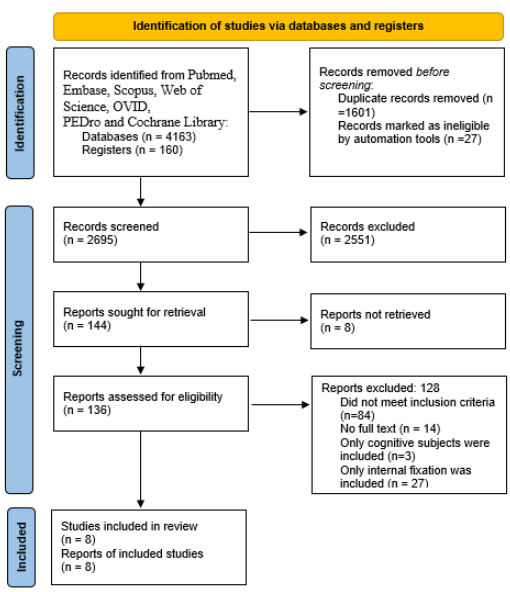

Fig. 1. PRISMA flow chart

The 2016 JOSPT clinical practice recommendations for hip fractures affirm that structured exercise improves mobility and that home or community-based workout programs are effective in promoting functional independence and improving daily living activities.[13,14] Some studies have concluded that exercise has a positive impact on functional outcomes after hip fracture.[15,16] In the published systematic reviews on hip fracture, the patient population is likely a mix of internal fixation and arthroplasty cases, which may limit generalizability due to differing postoperative protocols, including precautions and weight-bearing restrictions. The primary objective of this study was to systematically review and analyze research articles focused on arthroplasty outcomes in a large population of patients with hip fractures.

Inconsistency in research findings highlights the challenges posed by methodological heterogeneity, underscoring the importance of careful consideration and critical evaluation of study designs. Some investigations confirm the value of structured exercise regimens in improving mobility and quality of life, but other studies yield limited or ambiguous results, highlighting the need for more research.

Aim

The purpose of this study was to systematically review and meta-analyze randomized controlled trials to evaluate the impact of exercise on quality of life in patients undergoing hip arthroplasty following hip fractures.

Material and methods

The review protocol was registered with PROSPERO (CRD42023415893). This systematic review and meta-analysis were conducted in accordance with the Cochrane Collaboration guidelines and reported following the 2020 PRISMA statement (Fig. 1).

Data source and search strategy

We used the (PICOTS) framework, i.e., population, intervention, comparison, results, time frame, and study type, to develop the inclusion criteria. PubMed / Medline, Physiotherapy Evidence Database (PEDro), Web of Science, Scopus, Cumulated Index to Nursing and Allied Health Literature (CINAHL), Embase, Cochrane Systematic Reviews and additional sources Google Scholar, ProQuest were searched electronically from their inception to January 2023 with appropriate search terms (see Table 1 in supplementary file). Grey literature was also considered.

Study selection

Studies were included if they were randomized controlled trials using any type of exercise, physical therapy, or training related to HA for hip fractures. Comparators included placebo, no intervention, sham treatment, different exercise therapies, or other physiotherapeutic approaches. The primary outcome is quality of life, assessed using any of the following tools: Short Form 36 or Short Form 12, EuroQOL, Barthel Index, Patient-Specific Functional Scale, Instrumental Activities of Daily Living (IADL), or any comparable outcome measures indirectly measuring quality of life. Other physical function outcomes like physical activity, gait, and balance

were also included. Trials were omitted if the treatment group did not contain exercise or physiotherapy.

Primary outcome

The primary outcome of this study was self - report measures of health-related quality of life, defined by the World Health Organization as "an individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards, and concerns".[17]

Secondary outcomes

The effectiveness outcomes covered in the studies were evaluations of functional activities of daily living (ADL), muscular strength of the lower limb, range of motion of the hip joint and performance-based outcomes.

Data extraction

Data from included studies were retrieved by two independent reviewers and recorded in an Excel spreadsheet in compliance with the PRISMA guidelines.[18] Duplicate studies were detected and eliminated through the Mendeley desktop application. Initially, two independent reviewers extracted data from the chosen studies according to specific research attributes: author names, publication year, country of origin, study characteristics (eg design, setting), participant characteristics (e.g., sample size, age, gender), type of surgery, type of training, exercise dosage, results, exposures, and timelines. If consensus was not possible, the disparities or conflicts were addressed through discussion or by employing a third reviewer. The analysis was performed using RevMan 5.4.1 software.

The second stage involved a thorough assessment and the extraction of additional articles. Both descriptive and analytical methods were employed to synthesize the collected data... The results data were quantified as mean, standard deviations, mean differences and effect sizes, with effect sizes interpreted as small (0.2), moderate (0.5) or large (0.8).[19]

Risk of bias, methodological quality, and level of evidence assessment

Two independent reviewers (CM/SK) assessed the methodological quality of the included studies using the Cochrane RoB 2 checklist and the Pedro scale to evaluate the methodological quality. The PEDro scores were interpreted as poor (<4), fair (4–5), good (6–8) and excellent (9–10), with scores >5 indicating high-quality studies and <5 indicating low-quality studies. Disagreements were addressed through discussion and a third reviewer was called if needed.[20,21]

Data synthesis and analysis

When at least two comparable trials evaluated the effects of exercise interventions versus control therapies on quality of life or activities of daily living, using standardized outcome measures, meta-analyses were conducted after descriptive analysis of all included studies. As the included studies used various tools to measure quality of life and activities of daily living, standardized mean differences were applied.

Data heterogeneity assessment

The pooled odds ratio (OR) in the random effect model summarized the results. The *I2* value identified heterogeneity, and the p values measured significance. The forest plots showed the dispersion of effect sizes between the studies and meta-analysis was conducted after allocating weights to the included studies based on their variance, considering the quality and characteristics.

Analysis of the literature

Studies selection

The study selection process is detailed in the PRISMA flow diagram (Fig. 1). Initially, 4163 records were identified through database searches and 160 through supplementary sources, including reference lists. After deduplication and selection of title/abstract/full text, 8 studies were deemed eligible and included in the review.

Characteristics of included studies

Table 1 presents an overview of the characteristics of the included studies. There were eight randomized controlled trials, with publication years spanning from 2002 to 2023. The number of participants ranged from 11 to 1,873, with follow-up periods spanning 15 days to 12 months, and patient ages varying from 65 to 95 years. Studies were conducted in numerous countries around the world, including two from Australia and others from the United States (Pennsylvania), Germany, Finland, South Korea, Taiwan, and China.

The studies evaluated different structured exercise interventions, including high intensity progressive resistance exercise (2 trials) and comprehensive multidisciplinary approaches (6 trials). All studies had directly supervised interventions, with one study supervision via tele-rehabilitation. Interventions in all studies were administered to participants after discharge from hospital inpatient settings to nursing care facilities or home settings. Among home therapies, one study involved rigorous geriatric rehabilitation, while two trials focused on home-based resistance exercises.

Studies evaluated several types of interventions, including interdisciplinary geriatric rehabilitation programs, supervised tele-rehabilitation initiatives, and leg strengthening exercises performed under direct supervision.[22-25] The timing of the interventions varied, from immediately post-operative to post-hospital discharge and extending up to six months after surgery.[24,26,27]

The intervention was delivered in various settings; including home programs with direct supervision and telerehabilitation,[24,25] a nursing facility,[23] and a study included the intervention beginning from an inpatient setting to home-based.[22] The intervention period ranged from 2 weeks to 12 months.[22,28]

Three of the studies had used SF 36[25,28,29] and one study used DEMQOL/EQ 5D[26] to measure quality of life and other studies did not have a direct quality of life measure, instead they had used Barthel Index and IADL which were an indirect method of measurement.[22-24,27]

Participants

The pooled analysis included 1560 participants from 8 studies, with women comprising the majority of the study population. The included studies covered a spectrum of hip fractures, including femoral neck and intertrochanteric fractures, and employed various surgical interventions. Although both internal fixation (using hip sliding screws and intramedullary nailing) and hip arthroplasty (hemi and total) were represented, the latter represented the majority of cases.

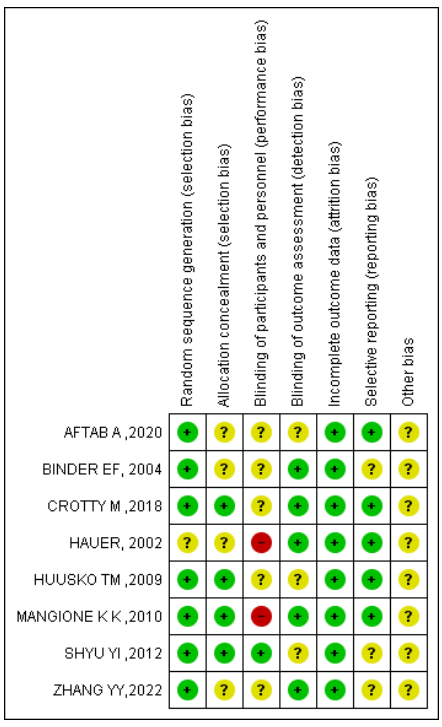

Fig. 2. Summary of risk of bias

Risk of bias

A detailed quality assessment for each included study is shown in Supplementary Appendix Table 2. Studies generally had a moderate risk of selection bias, with good reporting of randomization techniques. However, the absence of participant and researchers introduced a high risk of bias across all studies. Two RCTs were at high risk of bias due to additional threats to the randomization process. The outcome assessment in one study was performed by blinded evaluators.[25] The included studies scored 6-8 on the PEDro scale, categorizing them as good quality. The inter-rater reliability analysis showed a kappa value greater than 0.90, indicating almost perfect agreement between the raters. The results of the risk of bias assessment are given in Figs. 2 and 3.

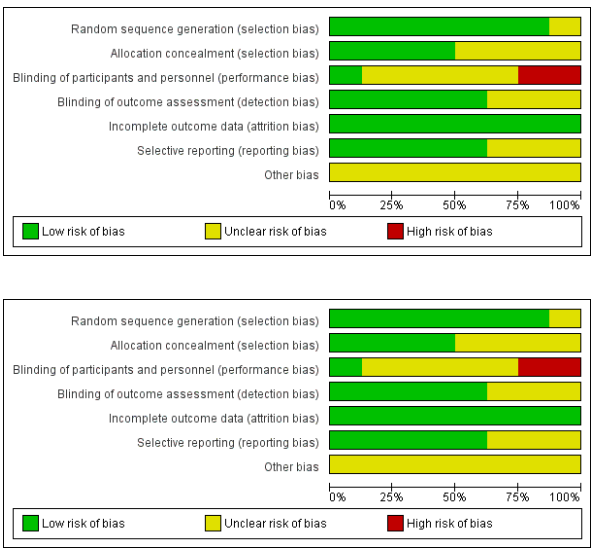

Fig. 3. Risk of bias graph

Data analysis

Overall effect size of exercise therapy on quality of life after total hip arthroplasty

All investigations affirmed that hip fractures adversely affect health-related quality of life (HRQOL). Physical functioning was significantly impaired in the initial weeks after hip fracture; however, with intervention, functional improvement steadily improved. The quality of life assessment was derived from four investigations employing Physical Function (SF 36) and DEMQOL approximately six months after surgery. Significant differences were observed in the comparison of exercise treatment with the control group in terms of quality of life (SMD, 0.68; 95% CI -0.01 to 1.37; I^2=89%). The overall effect suggests statistical significance, but the high heterogeneity and borderline significance indicate that the result should be interpreted with caution. The evidence is not strong and further investigation is needed to confirm the finding and understand the sources of heterogeneity (Fig. 4).

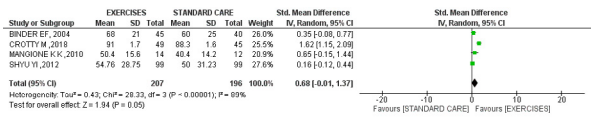

Fig. 4. Overall effect size of exercise on quality of life after approximately 6 months of hip surgery

Effect of exercise on activities of daily living

The impact of exercise on activities of daily living was examined through two trials with a three-month and a

Table 1. Study characteristics*

Author Name, year	No. of patients Con:Ex	HA: IF	Age group	Total sample	Female	Baseline	Intervention	Intervention duration	2nd measurement	Final measurement	Total follow up	Outcomes measured	ADL	QOL measurement	Central tendency measure
Aftab A, 2020[22]	19:20	not mentioned	65–95	39	32	2nd post op day	intensive rehabilitation	2 week	15th post op day	same	2 weeks	Barthel. KOVAL MMSE	Barthel	–	median values
Mangione KK, 2010[25]	12:14	8:18	81	26	21	6 mon after surgery	leg strengthening exercises	10 week	2 mon from baseline (8 mon)	12 mon	12 mon	strength, bal, gait, physical performance test	–	SF 36	mean
Binder E, 2004[29]	44:46	37:53	81	90	34 33	4 mon after surgery	supervised physical therapy	6 mon	3 mon from 4 mon baseline (8th month)	6 mon from 4 mon baseline (1 yr)	12 mon	strength, bal, gait, physical performance test	IADL Score	SF 36	mean
Shyu Y, 2012[28]	99:101:99	134:165	76.17–76.91	299	201	immediate after surgery	interdisciplinary comprehensive care	12 mon	1 mon, 3 mon, 6 mon	12 mon	12 mon	SF 36	–	SF 36	
Zhang YY, 2022[24]	24:27	31:17	77 (7.89). 75.17 (7.73)	51	33	immediate after surgery	home-oriented postoperative rehabilitation management	3 mon	1 mon	3 mon	3 mon	TUG, SPPB	HHS/FIM	–	mean
Crotty M, 2018[26]	121:119	97:240	88.6	240	87 91	immediate after surgery	comprehensive geriatric assessment and PT	4 week	4 week	3 mon/12 mon	12 mon	DEMQOL	M Barthel Index, Pain Depression	DEMQOL/EQ 5D	mean
Huusko TM, 2009[27]	125:125	not mentioned	80 (69–97)	250	174	immediate after surgery	intensive geriatric rehabilitation	2 week	3 mon	12 mon	12 mon	IADL/ADL	IADL/ADL	–	median
Hauer, 2002[23]	13:15	18:10	81.7./80.8	28	28	4 week	regime of high intensity PRT	3 mon	4 mon	7 mon	3 mon	Barthel/IADL	IADL	–	mean

* Con – control, Ex – experimental, HA – hip arthroplasty, IF – internal fixation, mon – month, yr – year, bal – balance, PRT– Progressive resistance training PRT, IADL Quality of Life – Instrumental Activities of Daily Living, IADL Quality of Life, EQ5D – EuroQol 5-Dimension 5-Level, HHS – Hip Harris Score, FIM – Functional Independence Measure

twelve-month follow-up. In the research conducted by Hauer et al.23 the mean Barthel ADL score for the intervention group (93±8.2) was lower than that of the control group (96.1±8.2), with 12 patients in each group evaluated 4 months after surgery. Crotty M et al.26 reported that the mean score for the Modified Barthel Index about five months after surgery was superior in the intervention group (27.4±2.5) compared to the control group (32.3±2.4), with 59 patients in the intervention group and 66 patients in the control group. The total mean difference of -1.21 (-2.81, 0.38) with p=0.13 indicates that the disparity in ADL scores is minimal and statistically not significant in favor of the exercise group. The chi-squared heterogeneity statistics is 12.8, I^2=92%, dof=1, and p=0.0005, indicating high variability between the two investigations. The overall effect test yields a Z value of 1.50 and a p value of 0.13, from the overall effect test suggesting that the observed effect may be attributed to chance, and the evidence is not strong enough to conclusively determine a significant difference between the intervention and control groups. (Fig. 5).

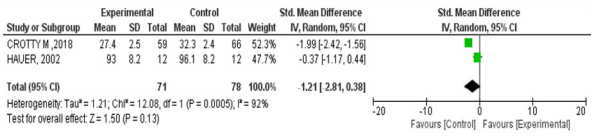

Fig. 5. Effect of exercise on activities of daily living scores

Effect of strengthening exercise on quality of life

Two studies used progressive resistance exercises, measuring quality of life by the Physical Function Score of the SF-36, and were therefore included in the meta-analysis.[25,29] Multidisciplinary therapy including bed exercises, mobility training and strengthening exercises was the most beneficial. Quality of life (QoL) was assessed with the DEM QOL, EQ-5D-5L, SF-36, and the Barthel index was used to evaluate functionality. In the telerehabilitation study conducted by Zhang et al. the FIM score was used, whereas Tina Huskko et al. employed the IADL score to elucidate changes in functional status.[24,27]

Although four studies did not report direct quality of life measures, they were still included in the review, as they provided indirect quality of life through IADL, FIM score and Barthel index. One study was excluded due to its data being presented in median and interquartile ranges.

Figure 6 provides a meta-analytic overview of the quality of life findings (SF-36) from two comparable studies. In the study by Binder et al., the mean score for physical function on SF-36 in the intervention group (68±21) at 7 months of follow-up was superior to that of the control group (60±25), which had a total of 88 participants (IG 45, CG 43).[29] Mangione KK et al. reported that the mean difference for the intervention group (50.4±15.6) exceeded that of the control group (40.4±14.2), with 14 participants in the intervention group and 12 in the control group, approximately 8 months after surgery.[25] The total mean difference of 8.83 (1.44-16.22) with p= 0.02 indicates that the change in quality of life is statistically significant in favor of strengthening exercises. The chi-squared heterogeneity statistics is 0.07, I^2=0, df=1, and p=0.79, indicating a lack of variability between the two studies. The general effect test yields a Z value of 2.34 and a p value of 0.02, indicating a significant benefit of resistance training in terms of quality of life when considering the entire impact.

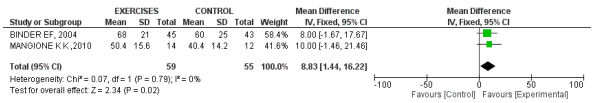

Fig. 6. Quality of life scores using physical function in short form 36

Discussion

This systematic review sought to consolidate information on the efficacy of exercise therapies in improving quality of life for patients undergoing hip arthroplasty, while also identifying deficiencies in the current literature. Taking into account the expected increase in hip fractures, it is crucial to identify the most effective exercise, the duration of rehabilitation, the dosage of exercise, the requirement for supervised versus unsupervised sessions, and the qualifications of the personnel who administer the exercise to improve the overall quality of life, reducing morbidity and mortality. According to the study's objective, comprehensive inclusion and exclusion criteria were established and carefully evaluated the impact of various aspects of rehabilitation on quality of life in patients with hip fractures receiving hip arthroplasty.

The review included trials utilizing several exercise modalities, including resistance, balance, and mobility exercises, along with comprehensive multidisciplinary programs that integrated physical therapy, fall prevention, nutritional, and psychological interventions. Trials demonstrate considerable variability in exercise interventions.

The results of this study demonstrate that progressive resistance training improves the quality of life for people with hip fractures in the medium to long term after surgery. Seven studies in this review used progressive resistance training; Of these, three studies specified parameters of progressive resistance exercise intensity and load progression,[23,25,29] while four studies mentioned resistance training as part of the exercise regimen without

detailing parameters.[24,26-28] Notwithstanding the favorable outcomes of resistance training in the previously stated studies, there were variations existed in the timing of resistance exercises, with some starting as early as 4 weeks after surgery and others at 6 months. Our results are supported by a recent narrative study by Avola et al., which also found resistance training to be particularly advantageous for people with hip fractures.[30]

Consistent with the systematic review by Lee et al., our findings demonstrate that prolonged intensive rehabilitation incorporating progressive resistance exercises yields improved clinical outcomes and quality of life, improving mobility, balance, strength, and task performance in hip fracture patients.[15] Resistance exercises implemented in the last stages of rehabilitation, especially after six weeks are effective in augmenting and boosting long-term quality of life.

The results of this study align with current clinical practice guidelines and highlight the findings of a 2020 systematic review, highlighting the need for more research to establish a definitive, evidence-based exercise protocol for optimal outcomes in elderly patients with hip fractures. Rehabilitation and exercise protocols varied between studies in terms of setting, methodology, supervision, duration, and follow-up and were consistent with the findings of a recent consensus study.[16,31]

Comprehensive geriatric rehabilitation, incorporating a geriatrician, physiotherapist, occupational therapist, and nutritionist optimizes recovery in this population. The research did not produce conclusive results on exercise metrics; nonetheless, it suggested that integrated geriatric care, in conjunction with structured physical therapy and prolonged follow-up, was more effective in improving the quality of life of this patient population. By identifying effective exercises for the prevention and rehabilitation, this review aims to promote healthy lives, reduce health inequalities, and contribute to creating more age-friendly communities.[32]

Study strengths

This study effectively addresses several limitations found in other published reviews by focusing on a specific population of hip arthroplasty, emphasizing key determinants of treatment effectiveness: timing of exercise initiation, the type of exercise, duration of rehabilitation, members of the rehabilitation team members, ensuring methodological rigor and identifying research gaps. These contributions enhance the impact on the existing body of literature and provide valuable insights for future research and clinical practice.

Study limitations

Although this study provides useful findings on exercise types, it is limited by the lack of data on exercise intensity and duration, which are likely crucial determinants of treatment effectiveness in improving quality of life outcomes. Although this review focused on hip arthroplasty (HA) patients, most of the studies included mixed populations, combining HA patients and internal fixation for hip fracture. The paucity of studies focusing solely on hip arthroplasty patients with hip fractures necessitated the inclusion of studies with broader cohorts, potentially compromising the generalizability of the results, to the specific population of patients. Most studies included in the review were found to have a risk of bias issues, primarily due to a lack of blinding, which may raise concerns about the reliability of the findings, as biases can significantly affect the results reported. We considered studies that assessed activities of daily living, even if they did not use specific quality of life measures, as patients' ability to perform these tasks comfortably implies improved quality of life and satisfaction with surgery, which is considered a limitation here.

Conclusion

In conclusion, this review underscores the importance of comprehensive geriatric care, incorporating structured exercises, including progressive resistance training, to improve quality of life in patients undergoing hip arthroplasty, and recommends further investigation to determine the most effective exercise parameters for the development of personalized rehabilitation programs.

Declarations

Funding

This study was not funded.

Author contributions

Conceptualization, C.M., A.L.A., A.J.J. and M.S.M.; Methodology, C.M., A.L.A., A.J.J. and M.S.M.; Software, C.M., A.L.A., A.J.J., M.S.M. and S.K.,; Validation, C.M., A.L.A., A.J.J. and M.S.M.; Formal Analysis, C.M. and A.L.A..; Investigation, C.M., S.K. and A.L.A.; Resources, C.M. and A.L.A.; Data Curation, C.M., S.K. and A.L.A..; Writing – C.M., S.K. and A.L.A.; .Original Draft Preparation, C.M., S.K. and A.L.A.; Writing – Review & Editing, C.M., S.K. and A.L.A.; Visualization, C.M., A.L.A., A.J.J. and M.S.M.; Supervision, A.L.A., A.J.J. and M.S.M.; Project Administration, C.M. and A.L.A.

Conflicts of interest

All authors declare that there exist no commercial or financial relationships that could, in any way, lead to a potential conflict of interest.

Data availability

Data supporting the results of this study is attached as supplementary and if any document needed shall, upon request, be available from the corresponding author.

Ethics approval

This systematic review and meta-analysis did not involve direct human participation or interaction; hence ethics approval and consent to participate were not required. However, research ethical guidelines and best practices were adhered to in the selection, review, and reporting of the studies included in this analysis.

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CASE REPORT

Pharmacogenetic aspects of therapy for autoimmune hepatitis against the background of degenerative-dystrophic joint lesions

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ABSTRACT

Introduction and aim. The pathogenesis of autoimmune diseases, including musculoskeletal, gastrointestinal, and endocrine manifestations, involves the interaction of genotype and environmental factors. Pathologies demonstrate comorbidity and clinical heterogeneity even within a single family. Genetic polymorphisms of one-carbon metabolism are key regulators of cellular processes that become therapeutic targets.

Description of the case. The study describes personalized therapy for a patient with an autoimmune comorbid disease, with an emphasis on genetic and metabolic characteristics. The treatment regimen is adapted to the features of the one-carbon metabolism profile of a patient with chronic autoimmune hepatitis and degenerative-dystrophic joint disease. Family history includes autoimmune thyroiditis, vitiligo, Parkinson's disease, cardiovascular diseases. The patient's genotype for single nucleotide polymorphisms rs1801133, rs1801131, rs1801394, rs1805087, and rs3733890 of the one-carbon metabolism genes is associated with elevated plasma homocysteine levels. After treatment, changes in biochemical parameters were observed: alanine aminotransferase (72→53 U/L), aspartate aminotransferase (53→44 U/L), gamma-glutamyltransferase (129→89 U/L), alkaline phosphatase (313→125 U/L) and homocysteine (15.1→17.0 μmol/L).

Conclusion. Positive dynamics after personalized therapy demonstrates the importance of an interdisciplinary approach to etiopathogenetic treatment, emphasizing the need to support hepatobiliary function along with muscular and skeletal therapy.

Keywords. autoimmune hepatitis, betaine-homocysteine methyltransferase, one-carbon metabolism genes, personalized therapy

Introduction

Personalizing pharmacotherapy for patients, especially for severe chronic noncommunicable diseases, is a global and, in particular, European trend. The main focus in practical activities is the application of the results of pharmacogenetic studies and the accumulated experience in this area to predict therapeutic and side effects, the consequences of the interaction of several simultaneously prescribed drugs, and improving treatment outcomes. The implementation of prediction is based primarily on

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knowledge of the patient's genetic profile. At the same time, it is necessary to take into account a large amount of patient data on other biological and environmental parameters such as life and family history.[1,2]

The development of molecular types of analysis has created opportunities for early assessment of pleiotropic effects of mutations and the development of individual treatment methods for patients, in particular, with autoimmune diseases, which at the same time may have clinical manifestations from the endocrine musculoskeletal, gastrointestinal tract, and other systems.[2-6] Common foundations and mechanisms of disease pathogenesis involve a complex interaction of genetic, embryological, immunological, and exogenous factors.[7] For example, autoimmune polyendocrine syndrome (APS) includes a diverse group of clinical conditions and is characterized by functional impairment of many endocrine organs due to loss of immune tolerance.[8-10] Autoimmune thyroiditis is associated with stomach disorders in 10–40% of patients.[7-10] Patients with type 1 diabetes often have concomitant multisystem autoimmune diseases, including thyroid, parathyroid gland pathologies, celiac disease, vitiligo, gastritis, dermatoses, and rheumatic diseases.[2]

Genetic studies of autoimmune diseases allow exploring pathogenesis and drug efficacy. It has been established that genetic factors of autoimmune hepatitis coincide with factors of other autoimmune diseases. Certain ethnic features regarding candidate genes and alleles predisposing to autoimmune hepatitis in Europeans, Latin Americans, and Japanese are presented.[11] Authors note that the results of genetic studies explain the phenotypic heterogeneity of autoimmune hepatitis and identify biological markers of drug response. The polymorphism of some genes associated with the pharmacokinetics and pharmacodynamics of drugs can modify the gene expression, causing changes in treatment in patients with autoimmune diseases.[12]

One-carbon metabolism potentially plays a role in the regulation of homeostasis and is also a target for drugs that affect the digestive system and metabolism processes of antineoplastic, antiepileptic, and other drugs.[13-19] Certain genotypes for one-carbon metabolism genes, particularly the *MTHFR* gene, may predispose to increased levels of circulating homocysteine, causing inflammation through oxidative stress. Resulting vascular dysfunction affects tissue function, leading to the development of cardiovascular, cerebrovascular, thromboembolic diseases, inflammatory syndromes, and neurodegenerative pathologies. An increase in homocysteine levels is observed in various liver diseases, including metabolically associated fatty liver disease. Hyperhomocysteinemia is noted in patients with rheumatoid arthritis. A link between hyperhomocysteinemia and complications of diabetes has been established since patients with diabetic angiopathy had high homocysteine levels.[20-25]

One pathway of homocysteine conversion to methionine is provided by betaine-homocysteine methyltransferase (BHMT). Certain genotypes for the *BHMT* gene may provide normal or reduced enzyme activity.[26] Under the appropriate genotype, maximum expression of BHMT is observed with a diet low in methionine but high in methyl donors such as betaine, choline, or betaine sulphonium analogs. Betaine functions as an osmolyte and methyl group donor, with its main physiological effects studied in animals and humans, especially against the background of complex therapy for hepatobiliary system pathology including hepatocellular carcinoma.[27-31]

Over the last decades, the literature widely discusses the experience of successful simultaneous use of drugs from different pharmacotherapeutic groups, in particular, corticosteroids, lipotropic substances used in liver diseases, drugs that affect the digestive system and metabolic processes, amino acids and their derivatives, including autoimmune pathologies of the hepatobiliary system.[32-35] At the same time, it is advisable to take into account the genetics of drug metabolism in patients with comorbid pathology against the background of the appointment of complex therapy, which can provide support for clinical decisions to improve medication management in medical care. Despite the universality of many aspects of genetic information, it is important to study the practical experience in each country or region, considering the ethnic, sociocultural and other characteristics of patients and the environment.[36]

For example, the diet may involve products that interact with different effects with the same enzymes as the prescribed drugs.[37] Therefore, personalization of complex therapy based on the analysis of the genotype and other features of the comorbid patient with autoimmune pathology is relevant.

Aim

The study aims to analyze the possibility of an individual approach to treatment, taking into account the genetic and metabolic characteristics of a patient with comorbid autoimmune pathology and degenerative-dystrophic joint lesions.

Description of the case

The patient D., Eastern European male, 53 years old, was observed and underwent treatment at the municipal non-commercial enterprise "City Clinical Hospital No. 13" of the Kharkiv City Council and the municipal non-commercial enterprise "City Multidisciplinary Clinical Hospital No. 17" of the Kharkiv City Council, Kharkiv, in 2021–2024.

Laboratory and instrumental investigations were carried out.[38,39] Laboratory studies, including general clinical, biochemical, immunological, and molecular genetics

were performed using samples of blood, urine, and feces. General clinical biochemical, immunofermentative, immunofluorescent, electrochemiluminescence, PCR qualitative types of analysis were conducted using analyzers Mindray BC-3000 Plus (Mindrey), Cobas 8000/Cobas Pro/Cobas6000/Cobas e411(Roche Diagnostics), Euroimmun Analyzer I Eurostar III Plus, and a fluorescent microscope (Euroimmun). Molecular-genetic analysis of polymorphic variants C677T (rs1801133) and A1298C (rs1801131) of the *MTHFR* gene (OMIM: 607093), A2756G (rs1805087) of the *MTR* gene (OMIM: 156570), A66G of the *MTRR* gene (rs1801394) (OMIM: 602568), G742A (rs3733890) of the *BHMT* gene (OMIM: 602888) was carried out by PCR-RFLP using Thermal Cycler Biometra T3000 (Biometra).[16,17,40]

Instrumental methods included ultrasound examination (Philips Clear Vue 550, Philips), multislice computed tomography (Philips Brilliance 64, Philips), electrocardiography (Heaco 300 G; Heako), radiography. A clinical-genealogical analysis allowed obtaining and studying data on relatives of the first to third degrees of kinship.

The presented work is done in accordance with the main positions of the "Rules of Ethical Principles for Conducting Scientific Medical Research Involving Human Subjects" adopted by the Helsinki Declaration (1964-2013), ICH GCP (1996), EEC Directive No. 609 (11/24/1986), Orders of the Ministry of Health of Ukraine No. 690, No. 944, No. 616. The patient in our project was informed about the purpose, design, methods of the study and provided written informed consent to be a participant in this completely anonymous project.

We provide an example of using genetic testing results for an individual approach to pharmacotherapy of a comorbid patient with autoimmune pathology.

Anamnesis

The patient has a main diagnosis: autoimmune hepatitis (ANA 1:320) with minimal activity cytolytic and cholestatic syndromes. Hemangioma of the right part of the liver (d up to 1cm). Comorbid diagnosis: Systemic involvement of connective tissue, unspecified. Bilateral deforming gonarthrosis II-III stages, arthrogenic contracture of both knee joints. Deforming arthrosis of the acromioclavicular joint 1–2 stages right side. Sclerosis of the large tubercle of the right shoulder bone with impairment of the right upper limb. Mixed contracture of the right shoulder joint. Osteoarthritis of the lumbar spine, spondyloarthrosis. Impaired joint function I stage. Pain syndrome. Chronic recurrent urticaria. Vitiligo. Alopecia. chronic atrophic autoimmune gastritis, *Helicobacter pylori* negative, in the acute stage. Post-arthroscopy state of the left knee joint, right knee joint, varicocele.

The family history of the patient is burdened with autoimmune thyroiditis, vitiligo, Parkinson's disease, and cardiovascular diseases, including varicose veins, hypertensive disease, myocardial infarction in relatives of 1–3 degrees of kinship. Does not smoke or drink alcohol.

The patient has been suffering from vitiligo since childhood, chronic urticaria for more than 18 years, arthrosis for more than 15 years. Due to arthralgia, he repeatedly turned to the trauma department, where diagnostic and therapeutic measures were carried out.

The general condition of the patient is of moderate severity, consciousness is clear, position is active. Skin and visible mucous membranes are pale pink, moist. Height 184 cm, weight 100 kg. Body mass index (BMI) – 29.5 kg/m^3. Peripheral lymph nodes are not palpable. In the lungs, breathing is vesicular, no rales. Heart tones are muffled, rhythmic. Blood pressure (BP) – 140/90 mm Hg Art., HR=Ps=70 bpm. Tongue moist, coated with a dense white-yellow plaque. The abdomen on inspection is symmetrical, no hernial protrusions, actively participates in the act of breathing, on superficial palpation painless, unstrained, peritoneal symptoms negative, Mendel's symptom negative, on methodical deep sliding palpation by Obraztsov-Strazhesko – the abdomen is soft, painless. The liver is not palpable; the edge of the liver is soft, painless, smooth. Dimensions of the liver by Kurlov 12×11×10 cm. Tenderness at the points of Mayo-Robson and Kacha is absent; tenderness in the zone of Schoffar is absent. The spleen is not palpable. Pasternatsky's symptom is negative on both sides. Stool daily formed without pathological admixtures, diuresis – urine of normal color, urination free, painless. Peripheral edema – no.

Joints: sizes and shapes are changed, mobility impaired. Pain on palpation and movement.

Laboratory study data

Analysis of laboratory test results in dynamics, before and after treatment, are within the average reference values for general blood test, electrolytes, proteinogram, lipidogram, with the exception of Low-density lipoproteins (LDL) (1.37 mmol/L, 1.68–4.53) and High-density lipoproteins (HDL) (0.82 mmol/L, 1.04–1.55). The results of laboratory studies in dynamics are presented in Table 1.

Indicators of general clinical analysis of urine, microscopic and physico–chemical properties are within the norm. Ketone bodies not found. No bacteria. Urine analysis for diastase – 166 U/L against the norm of 0–450 U/L. Coprogram without pathological deviations. Stool analysis for ova/helminths – not found. Pancreatic elastase in feces – 720 μg/g against a norm of more than 200 μg/g.

Table 1. Results of laboratory tests of the patient

Indicator	Result before treatment	Result after treatment	Reference values
Biochemical blood test			
Blood albumin, g/L	43	52	35–50
Thymol test, units	2.2		up to 4
Total bilirubin, μmol/L	15.9	10.4	5–21
Bilirubin direct, μmol/L	5.4	4.0	2.20–5.13
Bilirubin indirect, μmol/L	10.5	6.4	6.3–15.4
Alanine aminotransferase (ALT), U/L	72	53	0–41
Aspartate aminotransferase (AST), U/L	53	44	0–37
Gamma-glutamyl transferase (GGT), U/L	129	89	0–55
Alkaline phosphatase (ALP), U/L	313	125	53–128
Alpha-amylase, U/L	87	34	20–104
Creatinine, μmol/L	78.4	93.3	38.8–93.2
Calurea, μmol/L	5.3		0–8.3
Blood glucose, mmol/L	5.76	5.76	4.11–5.89
Homocysteine, μmol/L	15.1	17.0	up to 15.0
Serum iron, μmol/L	22.2		11.6–31.3
Transferrin, g/L	3.0		2–3.6
Ferritin (FER), μg/L	108.0		30–220
Ceruloplasmin, mg/dL	19		15–30
Rheumatic tests			
C-reactive protein, mg/l	up to 0.6		up to 6
Rheumatoid factor, IU/ml	negative		up to 12
Antistreptolysin-O, IU/ml	81		up to 200
Coagulogram (Coagulation tests)			
Prothrombin time, seconds	11.7		12–18
Prothrombin index, %	107		90–105
International normalized relationship (INR)	0.92		0.85–1.15
Prothrombin index according to Quick, %	129		70–130
Activated partial thromboplastin time (APTT), s	27.9		24–34
Blood test for hormones			
Thyroid stimulating hormone (TSH)	2.0		0.3–4
Immunological tests			
Autoimmune panel STD-X, serum (Ro/SS-A 52, La/SS-B, CENP-B, Scl-70, dsDNA, Jo-1, MPO, PR3, AMA M2, LC 1, LKM 1, PM/Scl 100, SRP 54, Sp 100, gp 210, Ku, Sm, U1-snRNP), kU/L	0.18–0.28		<0.3 – negatitve result
Antibodies IgG SS-A-52 (Ro-52), SS-A-60 (Ro- 60), SS-B (La), RNP/Sm, RNP-70, RNP-A, RNP-C, Sm-BB, Sm-D, Sm-E, Sm-F, Sm-G, Scl-70, Jo-1, dsDNA, ssDNA, polynucleosomes, mononucleosomes, complex histones, histone H1, H2A, H2B, H3, H4,Pm-Scl-100, centromeres B	3.0		<1.0 – negative result
Antinuclear antibodies (ANA)	1:320		<1:100 – negative result
Parietal cells of the stomach, antibodies IgG (PCA)	2.45		<1.00 – negative result
Analysis for HbS Ag and anti–HCV			
Hepatitis B	Not found		Not found
Hepatitis C	Not found		Not found
Genetic tests			
MTHFR			
C677T	CT		CC. CT. TT
A1298C	AC		AA. AC. CC
MTR			
A2756G	AG		AA. AG. GG
MTRR			
A66G	AG		AA. AG. GG
BHMT			
G742A	GG		GG. GA. AA

Data of instrumental studies

Electrocardiogram (ECG): HR=55 bpm. Sinus rhythm. Left ventricular myocardial hypertrophy.

Ultrasound of abdominal organs

The liver is not enlarged. Right lobe KBP 157 mm. Left lobe thickness 114 mm KKP 74 mm. Contours are even, clear, edge sharp. Parenchyma is homogeneous, finely granular, increased echogenicity. Hyperechoic formation in SgV up to 1 cm in d. The gallbladder is enlarged V=49 cm^3. Deformed due to the septum in the neck. Walls thickened to 28 mm. In the lumen, homogeneous bile. Hepatocholedochus is not dilated 0.4 cm in d, walls are not thickened. The pancreas is well visualized, not enlarged, head 30 cm, body 21 cm, tail 28 cm. Wirsung's duct is not dilated – 1 mm. Contours are clear, even. Echogenicity is normal. Structure is homogeneous. Portal vein 0.95 cm in d, splenic vein 0.8 cm in d, periportal fibrosis absent. Morrison's pouch does not contain fluid. The spleen is not enlarged 1.08x0.54 cm. Echogenicity is normal. Kidneys are typically located, shape unchanged, mobility sufficient. Right kidney 1.09x0.62 cm. Left kidney 1.27x0.59 cm. Contours are even, clear. Parenchyma is homogeneous, parenchyma height not reduced (right 21 mm, left 23 mm). Cavity system unchanged. Conclusion: Diffuse changes in liver parenchyma of the type of steatosis II stage. Hemangioma of the right lobe of the liver.

According to the Revised Original Score for Autoimmune Hepatitis (AIH), a pre–treatment score of 10 or greater and a post–treatment score of 12 or greater indicates "probable" AIH. A pre–treatment score of 10 has a "sensitivity" of 100%, a specificity of 73%, and a diagnostic accuracy of 67%. The patient's total pre–treatment score was 12.

Prescribed treatment

Therapy was prescribed in accordance with the Ukrainian clinical protocol for providing medical care to patients with autoimmune hepatitis, international recommendations Practice Guidance and Guidelines and manufacturers' instructions.[38,39] Basic therapy – budesonide, 9 mg per day, additional therapy – ursodeoxycholic acid, 1000 mg per day, ademetionine, 1000 mg per day, betaine 2 g per day. The patient is recommended to continue the prescribed therapy for up to two months. Against the background of the therapy, positive clinical and laboratory dynamics are observed.

Discussion

Interactions between drugs affect the effectiveness of therapy and the possible toxicity of active substances, after which the absence of the expected therapeutic result and unpredictable side effects may be observed. Taking into account the features of the simultaneous use of drugs and their interactions is important for pre-

dicting the results of treatment and preventing negative events.[41]

The metabolism of budesonide is mediated by the CYP3A4 enzyme, as well as CYP3A5 and CYP3A7.[42] Accordingly, the need for simultaneous use of inhibitors, inducers or substrates of CYP3A4 requires an assessment of the risk of systemic side effects and dose adjustment of the drugs. Ursodeoxycholic acid (UDCA) is an inducer of P450 3A. At the same time, according to the manufacturer's instructions, induction is not observed when interacting with budesonide.

According to the literature, it is known that combination therapy with ursodeoxycholic acid and budesonide was more effective than ursodeoxycholic acid monotherapy for primary biliary cirrhosis–autoimmune hepatitis overlap syndrome. In addition, budesonide has fewer side effects, for example, compared to prednisone.[33] Another potential long-term benefit of budesonide therapy is the preservation of bone mineral density.[39] Results from several studies suggest that combination therapy with ursodeoxycholic acid + [corticosteroids and/or antimetabolites] may be superior to ursodeoxycholic acid and corticosteroids ± azathioprine for the treatment of autoimmune hepatitis-primary biliary cirrhosis, but the authors clarify that further studies are needed to definitively demonstrate this.[32] Results of the simultaneous use of UDCA and budesonide are more effective than UDCA and placebo.[34]

S-adenosyl-L-methionine (SAMe) is known to be a hepatoprotector related with methylation. Also SAMe has been shown to suppress autoimmune reactions in PBC. The authors suggest potential therapeutic applications of SAMe.[43]

Data on the combination therapy of ursodeoxycholic acid and S-adenosyl-L-methionine in the treatment of gestational cholestasis are presented in the EASL Clinical Practice Guidelines on the management of liver diseases in pregnancy 2023.[35]

In vitro, SAM and betaine have been shown to enhance the antiviral effects of PegIFN-alpha-2a/2b and ribavirin. Both substances positively affect interferon-induced gene expression in individuals with viral hepatitis who do not respond to pegIFNα/ribavirin. SAM prevents CCl4-induced liver cirrhosis, inhibits cancer growth, and has a pro-apoptotic effect on hepatocellular carcinoma and MCF-7 breast cancer cells.[29] Combinations of SAMe with taurine and/or betaine have hepatoprotective effects against ethanol-induced liver damage and antioxidant functions in addition to anti–inflammatory effects against bacterial and/or viral inflammation.[30,31] Thus, the addition of ursodeoxycholic acid, ademetionine, and betaine to basic budesonide therapy potentiates positive dynamics.

When planning the treatment regimen, the patient's genetic and metabolic features were taken into account.

From the manufacturer's instructions for ademetionine, it is known that a contraindication to its prescription is genetic defects affecting the methionine cycle and/or causing homocystinuria and/or hyperhomocysteinemia during ademetionine therapy. Because the patient's genotype for five SNP genes of one–carbon metabolism – *MTHFR* 677CT/*MTHFR* 1298AC/*MTR* 2756AG/*MTRR* 66AG/*BHMT* 742GG, it may predispose to increased levels of homocysteine in the blood plasma.

It is known that heterozygous genotypes for the main genes of folate/methionine metabolism are associated with the highest indicators of homocysteine content in blood plasma.[22–24] Given such a genotype, it is advisable to conduct a study of the homocysteine level in the blood plasma. Before treatment, the indicator was 15.1 μmol/L against a reference value of 15 μmol/L (Table 1). Considering that the patient has a homozygous genotype G742G for the functional allele rs3733890 of the *BHMT* gene, the prescription of a dietary supplement with betaine and arginine will promote the remethylation of homocysteine into methionine and prevent an increase in homocysteine levels. Since hyperhomocysteinemia is an independent risk factor for cardiovascular and other pathologies for a patient with a burdened family history and own diseases, its prevention is particularly relevant. Therefore, the combined prescription of ademetionine with a dietary supplement with betaine and arginine is rational, with which changes in biochemical indicators in dynamics were noted: ALT (72 and 53 U/L), AST (53 and 44 U/L), GGT (129 and 89 U/L), ALP (313 and 125 U/L), homocysteine (15.1 and 17 μmol/L) (Table 1). The dynamics of homocysteine are not statistically significant.

The role of betaine as a dietary supplement in the prevention/mitigation of liver diseases associated with metabolic disorders and alcohol consumption is considered well–studied.[44] Betaine therapy alone has been shown to prevent vascular events in homocystinuria and also has benefits in other conditions associated with hyperhomocysteinemia as adjunctive therapy. Since betaine increases the level of methionine, reducing and maintaining the level of homocysteine is possible and effective with a diet limiting methionine and dietary supplements with betaine.[45,46] When planning a diet, it is also advisable to consider the needs for B vitamins, as well as other metabolic features associated with pathologies of the gastrointestinal tract and bone tissue according to the genotype.[47] Patients who have been taking dietary supplements with betaine for a long time should monitor the level of methionine and homocysteine in blood plasma.

Since recommendations regarding the diet must take into account the peculiarities of all drugs metabolism during treatment, and the peculiarities of drug–food interactions, it is important to note that budesonide

is a substrate of the cytochrome P450 3A4 isoenzyme CYP3A4. A drug or product that alters CYP3A4 activity may increase or decrease budesonide levels in the body. For example, grapefruit is a potent CYP3A4 inhibitor and may increase systemic exposure to budesonide.[48] Seville oranges, often used to make orange marmalade, pomelo, and tangelo may have the same effect as grapefruit juice. In addition, ursodeoxycholic acid is an inducer of P450 3A. Consumption of CYP3A4 inhibitors is not recommended during treatment with drugs that are metabolized by this enzyme. It is advisable that dietary advice to patients includes information on undesirable and preferred components and their combinations during treatment and, if possible, taking into account the patient's genotype as a metabolizer.[49]

In addition to understanding the interactions between drugs and products, it is advisable to consider pharmacogenetic and therapeutic recommendations for specific drug-gene pairs to predict treatment outcomes depending on the patient's genetic and metabolic status as a metabolizer. In the presented case, it would be useful to assess the patient's genotype for the CYP3A4 gene, since the two prescribed drugs are metabolized by this enzyme.[49] It would be possible to more accurately predict the therapeutic effect and dosages. At the same time, standard protocols do not involve assessing the patient's genotype before prescribing the mentioned drugs, so we consider such a project as promising.

We have not found data in the literature on the combination in patients of autoimmune hepatitis and arthrosis, although joint pain is a symptom of both autoimmune hepatitis and diseases of the musculoskeletal system. Provided that the patient's genotype for genes associated with autoimmune hepatitis and arthrosis is unknown, we can discuss the etiology of the patient's multifactorial pathologies using various hypotheses.

Comorbidity may be caused by the pleiotropic effect of one of the genes included in the genetic networks of both pathologies. It is known that genetic features of vitamin D reception are associated with both the development of autoimmune hepatitis and bone tissue disorders. Dysfunctional products of genetic variants or deficient levels of the gene product can disrupt homeostatic mechanisms that affect the proliferation and survival of autoreactive T- and B-cells, regulate the production of cytokines, and modulate inflammatory and immune reactions.[39] Comorbidity may be due to independent genetic causes. In such cases, the clinical picture of the patient reflects the realization of the pathological genotype according to several included genes. For example, we previously described a patient who had mutations in the *HFE and ATP7B* genes associated with the development of two independent monogenic pathologies – hemochromatosis and Wilson's disease.[50]

At the same time, the complex genetic architecture of multifactorial diseases presupposes the implementation against the background of many environmental factors, the contribution of which is difficult to assess. It is believed that exposure to xenobiotics can act as environmental triggers for the loss of autotolerance to autoantigens in individuals genetically predisposed to the development of autoimmune hepatitis.[39] In this regard, it is possible to discuss the effects of doses, duration of administration and the risk of hepatotoxicity of non-steroidal anti-inflammatory drugs, which the patient took against the background of the development of arthrosis.[51] However, we did not set a goal in this work to find out and present the genetic nature of comorbidity in the described patient. Such research requires a different design and technological solutions. Given the multifactorial nature of the described pathologies, assessment of their genetic landscape will probably, but not necessarily, be able to identify candidate genes or genes and may be a continuation of this project.

Considering the patient's comorbidity, during the administration of budesonide for the prevention of osteoporosis, it is suggested to maintain a high calcium intake, from food and supplements – 1500 mg/day, vitamin D – 800 IU/day.[48] Moderate physical activity without strain and lifting heavy objects is recommended. Also, considering the presence of vitiligo and chronic recurrent urticaria in the patient, regular supervision by a family doctor, gastroenterologist, traumatologist, and allergist is recommended. Since thyroid dysfunction and other endocrine aspects may be observed in patients with autoimmune hepatitis and given the burdened family history of autoimmune thyroiditis, consultation with an endocrinologist may be useful. The patient also received dietary recommendations.

The limiting factor in assessing the effectiveness of therapy is the patient's awareness and commitment to follow the instructions not only regarding the drug regimen, but also the diet, activities, daily routine and other components. This problem is international and depends on many factors. At the same time, modern models of specialist–patient partnership involve combining various ways of patient involvement, including care experience and mediation in health care, therapeutic patient education and patient education pathways, as well as patient–professional partnership.[52]

Conclusion

The clinical case demonstrates the appropriateness of personalized therapy in the example of autoimmune hepatitis, taking into account the genetic characteristics of patients that affect metabolism in the context of ademetionine therapy. The appropriateness of involving specialists of various specialties and a complex of research methods for etiopathogenetic treatment is sub-

stantiated. Genotyping an individual for rs3733890 allows specifying the ways of correcting one–carbon metabolism.

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Author contributions

Conceptualization, T. B. and O. F.; Methodology, T. B. and O. F.; Software, S. D. and V. D.; Validation, S. D. and V. D.; Formal Analysis, S. D. and V. D.; Investigation, T. B., V. B. and O. F.; Resources, T. B.; Data Curation, S. D. and V. D.; Writing – Original Draft Preparation, V. D.; Writing – Review & Editing, T. B., V. B., O. F., S. D. and V. D.; Visualization, T. B. and V. B.; Supervision, T. B., V. B. and O. F.; Project Administration, T. B.; Funding Acquisition, no

Conflicts of interest

All author declare have no conflict of interest.

Data availability

Not applicable.

Ethical approval

Patient signed informed consent was taken regarding publishing their data. And the study was approved by the Institutional Ethics Committee.

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CASE REPORT

Open rings of demyelination – a rare case of tumefactive multiple sclerosis

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ABSTRACT

Introduction and aim. Tumefactive multiple sclerosis (MS), a rare and atypical subtype of MS, presents with large demyelinating lesions that can mimic acute stroke, leading to diagnostic uncertainty. Stroke-like symptoms in such cases require a thorough neuroimaging. We describe the case of a 35-year-old woman who presented with acute onset of right-sided hemiparesis and hemisensory loss, along with facial weakness of the left upper motor neuron facial weakness and focal seizures. Initially suspected to be a cerebrovascular event, the condition was later diagnosed as tumefactive multiple sclerosis.

Description of the case. Comprehensive neurological assessment with neuroimaging and magnetic resonance peduncle, and bilateral cerebral hemispheres, raising suspicion of a demyelinating process. A differential diagnosis, including neoplastic, infectious, and inflammatory conditions was carefully evaluated before confirming tumefactive MS. The patient's stroke-like deficits improved significantly with high-dose intravenous methylprednisolone therapy. Follow-up imaging demonstrated the resolution of the enhancing lesions, strengthening the diagnosis. The dramatic response to steroids and the absence of progressive deterioration helped differentiate tumefactive MS from gliomas or infectious abscesses.

Conclusion. This case highlights the importance of considering tumefactive MS in acute neurological deficits with ring-enhancing lesions. Advanced imaging techniques are crucial for accurate differentiation that allows for timely and appropriate treatment.

Keywords. case report, demyelination, neuroimaging, open ring-enhancing lesion, stroke mimic, tumefactive MS

Introduction

Ring-enhancing lesions are frequently encountered in neuroimaging and are typically present as isointense or hypointense on noncontrast computed tomography scans. On contrast imaging, a ring or plate of enhancement occurs within the area of hypointensity or isointensity, which may be encircled by vasogenic edema. The differential considerations for ring-enhancing lesions are extensive, including tuberculomas, abscesses, metastases, subacute infarction, and radiation necrosis.[1] An open ring or crescent-shaped pattern of white matter enhancement is rare and often suggestive of a demyelinating or neoplastic lesion, posing a significant diagnostic challenge for healthcare professionals.

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Tumefactive multiple sclerosis (TMS) is a rare form of multiple sclerosis, with a reported prevalence ranging from 1.4% to 8.2% among patients with multiple sclerosis. The incidence of this rare subtype is approximately 0.3 per 100,000 individuals.[2] The initial treatment for tumefactive demyelinating lesions involves intravenous corticosteroids for 3 to 5 days, followed by a gradual reduction in oral corticosteroid dosage. If steroids are contraindicated or ineffective, alternative therapies, including plasmapheresis (PLEX), cyclophosphamide, and rituximab, are considered.[3] The prognosis of tumefactive MS depends on a range of factors, such as the size and areas of the lesions, as well as the individual's response to treatment.

Aim

We present a report on a woman who presented with symptoms resembling a cerebrovascular accident. Further investigations confirmed a diagnosis of tumefactive multiple sclerosis, and treatment with corticosteroid therapy was initiated, leading to total remission.

Table 1. Laboratory parameters

Test	Parameter	Result	Normal values
Complete hemogram	Hemoglobin	12.3 g/dL	12.1–15.1 g/dL
	White blood cell	5500 cells/µL	4,500–11,000 cells/µL
	Platelet count	265000 cells/µL	150,000–450,000 cells/µL
	Hematocrit	42%	36.1–44.3%
	Mean corpuscular volume	86 fL	80–100 fL
Liver chemical Panel	Alanine aminotransferase	32 U/L	7–56 U/L
	Aspartate aminotransferase	23 U/L	10–40 U/L
	Alkaline phosphatase	52 U/L	44–147 U/L
	Total bilirubin	0.8 mg/dL	0.1–1.2 mg/dL
	Albumin	3.7 g/dL	3.5–5.0 g/dL
	Total protein	6 g/dL	6.3–7.9 g/dL
Renal function analysis	Blood urea nitrogen	18 mg/dL	7–20 mg/dL
	Serum creatinine	0.6 mg/dL	0.59–1.04 mg/dL
	Glomerular filtration rate	124 mL/min/1.73 m^2	≥90 mL/min/1.73 m^2
Random blood glucose	Glucose	88 mg/dL	70–140 mg/dL
Lipid profile	Total Cholesterol	145 mg/dL	<200 mg/dL
	Low-density lipoprotein	87 mg/dL	<100 mg/dL
	High-density lipoprotein	42 mg/dL	>50 mg/dL
	Triglycerides	64 mg/dL	<150 mg/dL
Vasculitis profile	Extractable nuclear antigen	Negative	

Description of the case

A 35-year-old woman was admitted with seizures involving the right upper and lower limbs, which occurred upon waking in the morning. She experienced 1–2 episodes daily over the past 6 days, accompanied by progressive weakness in the right upper and lower limbs, involving both proximal and distal muscle groups. This was associated with sensory loss on the right side and deviation of the angle of the mouth to the right. There was no history of recent respiratory infections, diarrheal disease, travel, dog bites, or toxin exposure.

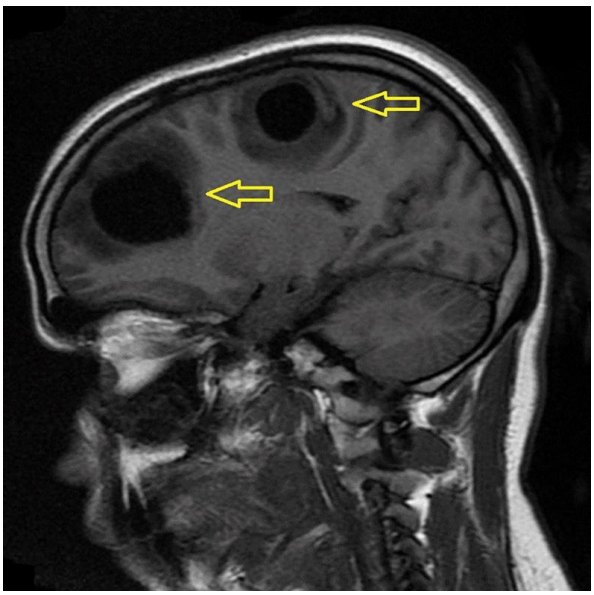

Fig. 1. Sagittal section of T_1 weighted MRI brain plain illustrating well-circumscribed `T_1 hypointense lesions with perilesional edema in the frontal and parietal lobe of the white matter of the cerebral hemisphere

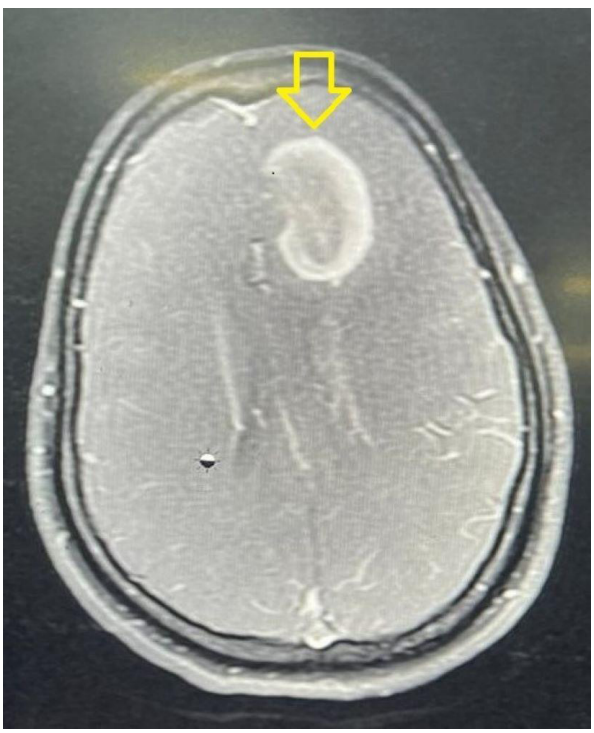

Fig. 2. Magnetic resonance brain imaging with contrast was done, which revealed these ring-enhancing lesions to be broken, classically opening towards the grey matter in the frontal lobe; termed as an open ring-enhancing lesion

On examination, the patient was conscious and well-oriented, with normal vital signs. Carotid pulses were equally palpable. Higher mental functions were in-

tact. Notably, there was a deviation of the angle of the mouth to the right, which clinically suggested left upper motor neuron facial palsy. The assessment of motor function revealed a power of 2/5 in both the right upper and lower limbs, with spasticity and brisk reflexes. Sensory examination showed diminished sensation on the right, particularly for pain and temperature. The clinical presentation resembled a stroke, affecting structures such as the left corticospinal tract, left spinothalamic tract, and left medial lemniscus (Fig. 1).

Complete hemogram, liver function analysis, renal function analysis, random blood glucose levels, lipid profile, and vasculitis profile were all within normal limits (Table 1).

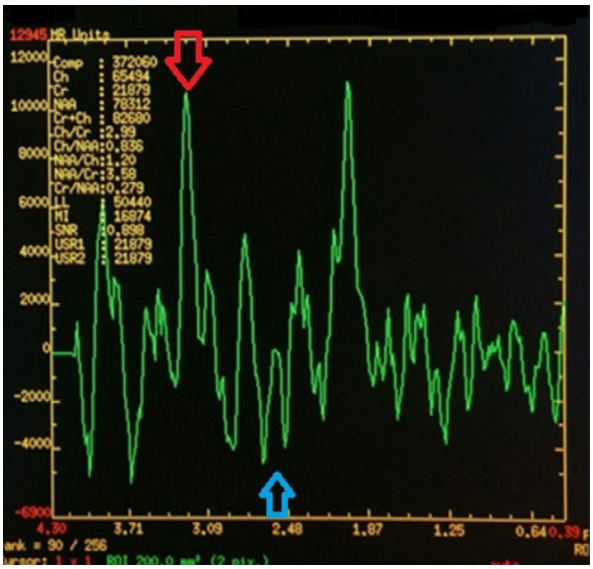

Fig. 3. Magnetic resonance spectroscopy further revealed to have a demyelinating pattern which indicates increased choline-containing peaks (red arrow) and decreased N-acetyl aspartate activity (blue arrow)

Discussion

Central nervous system demyelinating disorders are relatively common; among them, MS is the most prevalent. MS is an autoimmune disease characterized by dissemination in both time and space. Tumefactive multiple sclerosis is an uncommon and atypical form of MS, often presenting initially as a clinically isolated syndrome, which may later progress to develop MS several years afterward.[4] Tumefactive MS can onset at any age, with peak incidence occurring between 20 and 40 years, and it is more prevalent in women.[5] Tumefactive MS can present as an initial presentation of the disease or develop during the course of MS. It can also occur following the discontinuation of fingolimod therapy in MS patients.[6] MS lesions are typically small, numerous, and ovoid. In comparison, tumefactive MS lesions are larger (>2 cm) with focal areas of demyelination, which may or may not exhibit associated edema or mass effect.[7] Although the exact pathology of tumefactive TMS is not fully understood, a large multicenter retrospective study of tumefactive TMS patients found that approximately 10% had concomitant autoimmune disorders.[8]

The clinical presentation of tumefactive MS is often multifocal and varies with the specific areas of central nervous system involvement. The supratentorial areas, predominantly the frontal and parietal lobes, are most commonly involved.[9,10] Motor disturbances are the most common symptoms, followed by sensory, visual, and cognitive disturbances. The atypical symptoms reported in tumefactive MS can, at least in part, be explained by cortical grey matter involvement or by the effects of surrounding edema.[6]

The radiological approach is considered the gold standard for diagnosing tumefactive MS. On magnetic resonance imaging, tumefactive MS lesions typically appear hyperintense on T_2-weighted images and relatively hypointense on T_1-weighted and fluid-attenuated inversion recovery sequences.[11] Ring enhancement with gadolinium is a hallmark of tumefactive demyelinating lesions, observed in 95 to 100% of cases. This ring enhancement may present in various patterns, including open or closed rings, diffuse, homogeneous, punctate, or concentric configurations. The presence of an open-ring pattern, with the open segment connecting to the gray matter of the cortex or deep nuclei, is highly suggestive of a demyelinating disorder (Fig. 2). The enhancing component of the ring is considered an advancing front of demyelination, while the non-enhancing core at the center signifies a more chronic inflammatory process.[9]

Magnetic resonance spectroscopy demonstrated high levels of choline-containing compounds (Fig. 3), indicative of inflammation and the release of choline-containing membrane lipids resulting from active myelin and cell membrane breakdown. Concurrently, reduced levels of N-acetyl aspartate are observed, reflecting neuronal destruction and axonal damage. When combined with conventional magnetic resonance imaging, the Cho/NAA ratio has been demonstrated to enhance diagnostic accuracy.[10]

Histopathological features of tumefactive demyelination are consistent with active inflammatory demyelinating disease and typically include demyelinated areas with relative axonal preservation, infiltration of foamy macrophages, reactive astrocytosis, and perivascular lymphocytic infiltration.[10] Brain biopsy should be reserved for cases where a definitive diagnosis remains unclear and uncertain despite a comprehensive diagnostic approach using multiple modalities.[12]

High-dose corticosteroid therapy is considered the primary therapeutic approach for patients with tumefactive multiple sclerosis; however, the response to corticosteroid therapy is not universal, as approximately 86% of patients exhibit clinical improvement.[8] The standard reg-

imen involves administering a high dose of methylprednisolone (e.g., 1 gram per day for 3 to 5 days) followed by a gradual taper with oral corticosteroids.[3] If steroids are contraindicated or ineffective, PLEX is regarded as the alternative second-line therapy.[6,9] In patients who are refractory to intravenous corticosteroids or plasma exchange, cyclophosphamide may be an effective alternative due to its immunomodulatory properties.[13] The role of disease-modifying therapies in patients with tumefactive MS remains unclear. Interferon-beta and glatiramer acetate are typically preferred; however, these treatments are generally not initiated until a second clinical relapse occurs.[8]

The long-term prognosis of tumefactive MS is variable. Approximately one-third of patients will not experience a subsequent demyelinating episode. Among those who do, two-thirds will exhibit a relapsing-remitting course.[6,8]

In our case, a positive response was observed with pulse methylprednisolone therapy, further supporting the definitive diagnosis of tumefactive MS, as evidenced by the presence of open ring-enhancing lesions.

Conclusion

Tumefactive MS, an uncommon variant of multiple sclerosis, can be difficult to diagnose due to its resemblance to the radiological and clinical features of an abscess, tumor, or stroke. Our findings highlight the importance of including demyelination in the differential diagnosis of ring-enhancing lesions, particularly with a positive response to corticosteroids, thereby minimizing the necessity for biopsy and broadening the spectrum of potential tumefactive MS presentations.

Declarations

Funding

No funding was received for this study.

Author contributions

Conceptualization, P.K. and N.S.M.; Methodology, P.K. and A.C.; Software, N.D.C.; Validation, N.D.C. and D.S.V.; Formal Analysis, D.S.V., K.J.S. and D.R.; Investigation, P.K. and D.S.V.; Resources, P.K.; Data Curation, N.D.C.; Writing – Original Draft Preparation, N.S.M, P.K. and N.D.C.; Writing – Review & Editing, N.D.C., D.S.V. and D.R.; Visualization, N.D.C. and A.C.; Supervision, P.K. and K.J.S.; Project Administration, P.K.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Data availability

All data generated or analyzed during this study are included in this published article. Additional data are available from the corresponding author upon reasonable request.

Ethics approval

Ethical approval was not required for this case report. Written informed consent was obtained from the patient for participation in this study.

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CORRIGENDUM

Corrigendum: The effect of different blood groups on visual evoked potentials

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A Corrigendum on

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In the published article, an error occurred in Abstract. In the Abstract, the fourth line originally read 'December', which was incorrect. The correct version should state 'November'. The corrected Abstract appears below.

The authors apologize for this error and confirm that it does not affect the scientific conclusions of the article in any way. The original article has been updated.

ABSTRACT

Introduction and aim. Purpose of the study is to determine whether it is required to use different standards when evaluating visual evoked potential (VEP) measurements of healthy individuals with different blood groups.
Material and methods. The study consisted of healthy individuals with different blood groups who have applied to the ophthalmology and neurology outpatient clinic of Düzce University Medical Faculty from January to November 2022. The patients went through detailed ophthalmologic examination and VEP test and only the ones with normal results were included to the study.
Results. The study consisted of 119 individuals, with a blood group distribution of 30 A, 29 B, 30 AB and 30 O. VEP latency and amplitude changes were compared and no significant difference was observed within 4 groups in terms of P100 and N70 latency and amplitudes. There was N70 latency prolongation in Rh- group and this difference was found to be statistically significant (p=0.009). Rh+ group was found to be high in terms of P100 amplitudes and this was considered statistically significant (both p=0.023).
Conclusion. There was no statistically significant difference in the VEP parameters of the individuals with the ABO blood groups hence same VEP normal values can be used for ABO blood groups.

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Instructions for Authors

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We reserve the right to reject a paper even after it has been accepted if it becomes apparent that there are serious problems with its scientific content, or our publishing policies have been violated.

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Authorship provides credit for a researcher's contributions to a study and carries accountability. Authors are expected to fulfil the criteria below (adapted from McNutt et al., Proceedings of the National Academy of Sciences, 2018, 201715374; DOI: 10.1073/pnas.1715374115):

— **Each author** is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it
— **AND** to have approved the submitted version (and any substantially modified version that involves the author's contribution to the study);
— **AND** to have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

The Eur J Clin Exp Med does not require all authors of a research paper to sign the cover letter upon submission, nor do they impose an order on the list of authors. Submission to the Eur J Clin Exp Med is taken by the publication to mean that all the listed authors have agreed to all of the contents. The corresponding (submitting) author is responsible for having ensured that this agreement has been reached, and for managing all communication between the publication and all co-authors, before and after publication.

Author contributions statements

Authors are required to include a statement of responsibility in the manuscript (at the end of the main text, before the 'References' section) that specifies the contribution of every author. For articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.".

Corresponding author – responsibilities

The corresponding (submitting) author is solely responsible for communicating with the Eur J Clin Exp Med and for managing communication between co-authors. Before submission, the corresponding author ensures that all authors are included in the author list, its order has been agreed by all authors, and that all authors are aware that the paper was submitted.

A confidential process

The Eur J Clin Exp Med treats the submitted manuscript and all communication with authors and referees as confidential. Authors must also treat communication with the Eur J Clin Exp Med as confidential: correspondence with the Eur J Clin Exp Med, referee reports and other confidential material must not be posted on any website or otherwise publicized without prior permission from the Eur J Clin Exp Med publishing team, regardless of whether or not the submission is eventually published. Our policies about posting preprints and post prints, and about previous communication of the work at conferences or as part of a personal blog or of an academic thesis, are described in the Confidentiality section.

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During the submission process, please suggest three potential reviewers (names and institutional e-mail addresses) with the appropriate expertise to review the manuscript, but please keep in mind that we are not obliged to follow these recommendations. The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may suggest reviewers from among the authors that you frequently cite in your paper. You may also name a limited number of scientists who should not review your paper (up to 3 named individuals or laboratories); these exclusions will be honored. The decision of the Editorial Board Member on the choice of referees is final.

Ethics, use of experimental animals, and human participants

For articles in the Eur J Clin Exp Med reporting experiments on live vertebrates and/or higher invertebrates, the methods section must include a statement: (i) identifying the institutional and/or licensing committee approving the experiments, including any relevant details; (ii) confirming that all experiments were performed in accordance with relevant guidelines and regulations.

For research involving human participants, authors must identify the committee that approved the research, confirm that all research was performed in accordance with relevant guidelines/regulations, and include in their manuscript a statement confirming that informed consent was obtained from all participants and/or their legal guardians.

Authors may be required to submit, on request, a statement from the research ethics committee or institutional review board indicating approval of the research.

Competing interests policy

In the interests of transparency and to help readers to form their own judgements of potential bias, authors must declare any competing financial and/or non-financial interests in relation to the work described. For the purposes of this policy, competing interests are defined as financial and non-financial interests that could directly undermine, or be perceived to undermine, the objectivity, integrity and value of a publication, through a potential influence on the judgements and actions of authors with regard to objective data presentation, analysis and interpretation. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Competing interests statement format guidelines

The statement included in the article file must be explicit and unambiguous, describing any potential competing interest (or lack thereof) for EACH contributing author.

Examples of declarations are:

— Competing interests: The author(s) declare no competing interests.
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The Eur J Clin Exp Med invites peer-reviewers to exclude themselves in cases where there is a significant conflict of interest, financial or otherwise. However, just as financial interests need not invalidate the conclusions of an article, nor do they automatically disqualify an individual from evaluating it. We ask peer-reviewers to inform the editors of any related interests, including financial interests as defined above that might be perceived as relevant. Editors will consider these statements when weighing peer-reviewers' recommendations.

Availability of materials and data

In order to maintain the integrity, transparency and reproducibility of research records, authors are encouraged to make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal.

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Data availability statement format guidelines

The statement should be provided as a separate section (titled 'Data Availability') at the end of the main text, before the 'References' section. Data availability statements should include, where applicable, accession codes, other unique identifiers and associated web links for publicly available datasets, and any conditions for access of non-publicly available datasets. Where figure source data are provided, statements confirming this should be included in data availability statements. Depending on the data described in the manuscript, data availability statements commonly take one of the following forms, or can be a composite of the statements below:

— The datasets generated during and/or analyzed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS].
— The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.
— All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).
— The datasets generated during and/or analyzed during the current study are not publicly available due to [REASON(S) WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.
— No datasets were generated or analyzed during the current study.
— The data that support the findings of this study are available from [THIRD PARTY NAME] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [THIRD PARTY NAME].

Correction and retraction policy

The Eur J Clin Exp Med operates the following policy for making corrections to its peer-reviewed content.

Publishable amendments must be represented by a formal online notice because they affect the publication record and/or the scientific accuracy of published information. Where these amendments concern peer-reviewed material, they fall

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Publisher Correction (formerly Erratum). Notification of an important error made by the journal that affects the publication record or the scientific integrity of the paper or the reputation of the authors or the journal.

Author Correction (formerly Corrigendum). Notification of an important error made by the author(s) that affects the publication record or the scientific integrity of the paper, or the reputation of the authors or the journal.

Retraction. Notification of invalid results. All co-authors must sign a Retraction specifying the error and stating briefly how the conclusions are affected, and submit it for publication. In cases where co-authors disagree, the in-house editors may seek advice from independent referees and impose the type of amendment that seems most appropriate, noting the dissenting author(s) in the text of the published version.

Addendum. Notification of additional information. Addenda are published when the in-house editors decide that the addendum is crucial to the reader's understanding of a significant part of the published contribution.

Peer-review process

Initial checks

Once submitted, your manuscript will be assigned to a member of our Editorial Board, who will read the paper and decide whether it is appropriate for the journal. Manuscripts that are within scope and seem, on initial assessment, to be technically sound and scientifically valid, will be sent to external reviewers. Copies of any papers containing similar or related work under consideration or in press at other journals must be included with the submission.

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Peer review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. Reviewers will be able to access your manuscript securely using our online system, whilst maintaining referee anonymity. A double-blind review is applied, where authors' identities are unknown to reviewers and vice versa. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

Editorial decision

After considering the reviewer reports the Editorial Board Member will make one of the following decisions:

— Accept outright,
— Request a minor revision, where authors revise their manuscript to address specific concerns,
— Request a major revision, where authors revise their manuscript to address significant concerns and perhaps undertake additional work,
— Reject outright.

The final decision is made by the Editor-in-Chief.

Revisions

In cases where the referees or Editorial Board Member has requested changes to the manuscript, you will be invited to prepare a revision. The decision letter will specify a deadline for submission of a revised manuscript. Once resubmitted, the manuscript may then be sent back to the original referees or to new referees, at the Editorial Board Member's discretion.

A revised manuscript should be submitted via the revision link provided in the decision letter, and not as a new manuscript. Authors should attach a cover letter to explain, *point by point*, the details of the revisions to the manuscript and responses to the referees' comments. Cover letters should not contain information that could identify the authors. The destination of the cover letter file in the submission system is 'Supplementary File for Review'. Please ensure that all issues raised have been addressed in the first round of revision. Where the authors disagree with a reviewer, they must provide a clear response.

Final submission and acceptance

When all editorial issues are resolved, your paper will be formally accepted for publication. Once accepted, the manuscript will undergo professional copy-editing, English editing, final corrections, pagination, and, publication on the http://www.ejcem.ur.edu.pl/. The Eur J Clin Exp Med reserves the right to make the final decision about matters of style and the size of figures.

Appeals

Even in cases where the Eur J Clin Exp Med does not invite resubmission of a manuscript, some authors may ask the Editorial Board to reconsider a rejection decision. These are considered appeals, which, by policy, must take second place to the normal workload. In practice, this means that decisions on appeals often take several weeks. Only one appeal is permitted for each manuscript, and appeals can only take place after peer review. Final decisions on appeals will be made by the Editorial Board Member handling the paper.

Decisions are reversed on appeal only if the relevant Editorial Board Member is convinced that the original decision was a serious mistake. Consideration of an appeal is merited if a referee made substantial errors of fact or showed evidence of bias, but only if a reversal of that referee's opinion would have changed the original decision.

Similarly, disputes on factual issues need not be resolved unless they were critical to the outcome.

If an appeal merits further consideration, the Editorial Board Member may send the authors' response and the revised paper out for further peer review.

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Cover letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

Accepted file formats

Authors must use Microsoft Word to prepare their manuscript. Please insert your tables, graphics (schemes, figures, etc.) in the main text after the paragraph of its first citation.

In most cases, we do not impose strict limits on word count or page number. However, we strongly recommend that you write concisely and stick to the following guidelines:

— We encourage not exceeding 20 pages for original and review papers, and 8 pages for case reports of standard computer text (1800 signs on a page).
— The main text should be no more than 4,500 words (not including Abstract, Methods, References and figure legends).
— The title should be no more than 20 words.
— The abstract should be no more than 250 words.
— Recommended font: Times New Roman, 12 points.
— Manuscript text should be double-spaced. Do not format text in multiple columns.

Types of Publications

Manuscripts submitted to the Eur J Clin Exp Med should neither be published previously nor be under consideration for publication in another journal. The main article types are as follows:

Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information.

Reviews. These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA guidelines.

The Eur J Clin Exp Med accepts also the following types of submissions: case reports, letters to the editor, commentaries, book reviews, and reports from scientific meetings and conferences.

Reporting guidelines

The guidelines listed below should be followed where appropriate. Please use these guidelines to structure your article. Completed applicable checklists, structured abstracts and flow diagrams should be uploaded with your submission; these will be published alongside the final version of your paper.

Please refer to existing guidelines for reporting methodology; e.g.:

— AGREE guidelines for clinical practice guidelines
— ARRIVE guidelines for *in vivo* animal studies
— CARE guidelines for clinical case reports
— CONSORT guidelines for clinical trials
— PRISMA guidelines for systematic reviews and meta-analyses
— SPIRIT for clinical trials
— STARD guidelines for studies of diagnostic accuracy
— STROBE guidelines for observational studies

Manuscript preparation

Your paper should consist of the following parts. Title page should be supplied as a **separate** file.

Research manuscripts should comprise:

— Title page: Title, Author list, Affiliations, Abstract, Keywords.
— Research manuscript sections: Introduction, Aim, Materials and Methods, Results, Discussion, Conclusions.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions,

Conflicts of Interest, Data Availability, Ethics Approval, References.

Research manuscript sections:

— *Introduction*

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

— *Material and methods*

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

— *Results*

Results should be clear and concise. The section may be divided into subsections, each with a concise subheading. Tables and figures central to the study should be included in the main paper. Do not use the term "significant" unless p-values are provided. Show p-values to 2 or 3 decimal places. The Results section should be written in past tense.

— *Discussion*

This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature.

— *Conclusions*

Summarize the work's findings, state their importance, and possibly recommend further research.

Review manuscripts should comprise:

— Title page: Title, Author list, Affiliations.
— Abstract, Keywords, Literature review sections.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions, Conflicts of Interest, Data Availability, References.

Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.

Case reports should comprise:

— Title page: Title, Author list, Affiliations.
— Abstract, Keywords. Case reports should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions, Conflicts of Interest, Data Availability, Ethics Approval, References.

Requirements for case reports submitted to Eur J Clin Exp Med:

— Patient ethnicity must be included in the Abstract under the Case Presentation section.
— Consent for publication is a mandatory journal requirement for all case reports. Written informed consent for publication must be obtained from the patient (or their parent or legal guardian in the case of children under 18, or from the next of kin if the patient has died).

Language Style

Manuscripts must be submitted in English (American or British usage is accepted, but not a mixture of these).

Title page

These sections should appear in all manuscript types:

Title: The title of your manuscript should be concise and informative. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.

Author list and affiliations: Authors' full first and last names must be provided. For each affiliation provide the details in the following order: department, institution, city, country. If available, the e-mail address of each author should also be provided. At least one author should be designated as *corresponding author*, and his or her email address and other details should be included at the end of the affiliation section.

Abstract: The abstract should be a total of about 250 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts: *Introduction and aim:* Place the question addressed in a broad context and highlight the purpose of the study; *Material and methods*: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. *Results:* Summarize the article's main findings; and *Conclusion*: Indicate the main conclusions or interpretations. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords: Three to six pertinent keywords need to be added after the abstract in alphabetical order. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Back matter

Supplementary materials: Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.

Acknowledgments: Thank all of the people who helped with the research but did not qualify for authorship. Acknowledge anyone who provided intellectual assistance, technical help, or special equipment or materials.

Funding statement: All sources of funding of the study should be disclosed.

Author contributions: Authors must supply an Author Contribution Statement as described in the *Author contributions statements* section.

Conflicts of interest: Authors must supply a competing interests statement. For more details please see *Competing interests policy.*

Data availability: Authors must include a Data Availability Statement in all submitted manuscripts; see *Availability of materials and data* section for more information.

Ethics approval: Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

References: References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, Reference Manager or Zotero to avoid typing mistakes and duplicated references.

References style

In-text citations and references should be prepared according to the American Medical Association (AMA) style. Each item should be listed in numerical order.

In-text citations

Each reference should be cited in the text using superscript arabic numerals. These superscript numbers should be outside periods. If you are citing sequential references, these should be indicated with a hyphen. Nonsequential references should be separated with commas. There should not be a space between numbers. For example: The degree of respiratory muscles fatigue depends on the applied exercise protocol and the research group's fitness level.[1,2] The greatest load with which a patient continues breathing for at least one minute is a measure of inspiratory muscles strength.[3] Diabetes mellitus is associated with a high risk of foot ulcers.[4-6]

Sample Reference

In listed references, the names of all authors should be given unless there are more than 6, in which case the names of the first 3 authors are used, followed by "et al.". If the source does not have any authors, the citation should begin with the title.

To find the proper abbreviation of journal go to the National Library of Medicine PubMed Journals Database at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Journals.

Page number(s) should be inserted in full (for example: use 111–112, not 111–2).

The following are examples of individual citations made according to the required rules of editing and punctuation:

— **Article from a journal, number of authors from 1 to 6**

Author AA, Author BB, Author CC. Title of article. *Accepted Abbreviated Journal Title*. Year;Volume(Issue):Page-Page. doi (if available)

Lee JC, Seo HG, Lee WH, Kim HC, Han TR, Oh BM. Computer-assisted detection of swallowing difficulty. *Comput Methods Programs Biomed*. 2016;134(2):72-78. doi: 10.1016/j.cmpb.2016.07.010

Morris A. New test for diabetes insipidus. *Nat Rev Endocrinol*. 2019;15(10):564-565. doi: 10.1038/s41574-019-0247-x

— ***Article from a journal, number of authors* more *than 6***

Author AA, Author BB, Author CC, et al. Title of article. *Accepted Abbreviated Journal Title*. Year;Volume(Issue):Page-Page. doi (if available)

Gonzalez ME, Martin EE, Anwar T, et al. Mesenchymal stem cell-induced DDR2 mediates stromal-breast cancer interactions and metastasis growth. *Cell Rep*. 2017;18:1215-1228. doi: 10.1016/j.celrep.2016.12.079

Jordan J, Toplak H, Grassi G, et al. Joint statement of the European Association for the Study of Obesity and the European Society of Hypertension: obesity and heart failure. *J Hypertens*. 2016;34:1678-1688. doi: 10.1097/HJH.0000000000001013

— **Websites**

Author AA (if indicated). Webpage title. Name of Website. URL. Published or Updated date. Accessed date.

Cholera in Haiti. Centers for Disease Control and Prevention Web site. http://www.cdc.gov/haiticholera/. Published October 22, 2010. Updated January 9, 2012. Accessed February 1, 2012.

Address double burden of malnutrition: WHO. World Health Organization site. http://www.searo.who.int/mediacentre/releases/2016/1636/en/. Accessed February 2, 2017.

— **Book**

Author AA, Author BB. *Title of Work*. Location: Publisher; Year:Page-Page

Doane GH, Varcoe C. *Family Nursing as Relational Inquiry: Developing Health– Promoting Practice*. Philadelphia, PA: Lippincott Williams & Wilkins; 2005:25-28.
London ML, Ladewig PW, Ball JW, et al. *Maternal & Child Nursing Care*. Upper Saddle River, NJ: Pearson Education; c2011:101-103.
— Chapter in a book
Chapter Author AA. Title of chapter. In: *Name of Book*. Edition Number. Editor AA, ed. Location: Name of Publisher; Year:Page-Page.
Grimsey E. An overview of the breast and breast cancer. In: *Breast Cancer Nursing Care and Management*. 2nd ed. Harmer V, ed. Chichester, UK: Wiley-Blackwell; 2011:35-42.
NOTE: The Editorial Board requires consistent and carefully made references prepared according to the above-mentioned AMA standards. Otherwise, the work will be sent back to the authors.

Preparing figures, schemes and tables

File for Figures and Schemes must be provided during submission and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption should be placed directly below the figure (not on the figure itself) or above the table. All figures, schemes, and tables should be numbered following their number of appearance (Figure 1, Scheme 1, Figure 2, Scheme 2, Table 1, etc.).

Tables should present new information rather than duplicating what is in the text. Readers should be able to interpret the table without reference to the text.

All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Tables must be provided in an editable format in appropriate place in the main text. Tables provided as jpeg/tiff files will not be accepted. Do not submit your tables in separate files.

Abbreviations

The journal requires using only standard abbreviations. Common abbreviations such as DNA and RNA do not require definitions. Abbreviations should be defined in parentheses the first time they appear in the abstract, main text and in figure or table captions and used consistently thereafter. Ensure consistency of abbreviations throughout the article. Use the following abbreviations for measurement units: gram (g), litre (L), milligram (mg), kilogram (kg), seconds (s), minutes (min), and hours (h). Do not add 's' to indicate plural forms of units. Keep abbreviations to a minimum.

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SI Units (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.