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ORIGINAL PAPER

Pre-operative diagnosis of suspicious thyroid nodules – an integrated multidisciplinary approach

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ABSTRACT

Introduction and aim. Thyroid nodules are common, though few are malignant. In lower-middle-income countries such as Sri Lanka, limited diagnostic resources make accurate preoperative assessment essential to avoid unnecessary surgery. While the Thyroid Imaging reporting and Data System (TIRADS) and Bethesda systems are widely used, both have limitations, particularly in differentiating follicular lesions. This study aimed to evaluate the potential value of immunocytochemistry (ICC) as an adjunct to preoperative diagnosis in suspicious thyroid nodules.

Material and methods. A prospective observational diagnostic accuracy study was conducted on 106 patients with TIRADS ≥ 3 nodules. Fine-needle aspiration cytology was classified using the Bethesda system, and cell blocks from 34 cases underwent ICC with HBME-1, CK-19, and CD-56. Final diagnoses were established using histopathology where available or a composite reference standard based on stable imaging follow-up over two years. Diagnostic performance was assessed using logistic regression, ROC curves, and confusion matrices.

Results. Bethesda showed sensitivity of 80.8% and specificity of 60.6% (AUC=0.73), while TIRADS showed sensitivity of 89.0% and specificity of 30.3% (AUC=0.65). Among the ICC-tested cases, specificity was 100%; sensitivities were 94.1% for HBME-1, 88.2% for CD-56, and 41.2% for CK-19. The HBME-1⁺/CD-56⁻ pattern was strongly associated with malignancy. The combined Bethesda+ICC model demonstrated the best predictive performance (lowest AIC). Papillary carcinoma was the most frequent malignancy.

Conclusion. In this cohort, ICC using HBME-1, CK-19, and CD-56 showed potential as an adjunct to the Bethesda classification for the preoperative assessment of thyroid nodules. These findings suggest a possible role for ICC in selected cases, although further studies with larger histologically confirmed cohorts are needed.

Keywords. Bethesda, immunocytochemistry, thyroid cancer, TIRADS

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Introduction

Thyroid nodules are a common clinical finding, although only a small proportion are malignant. Accurate preoperative diagnosis is therefore essential to ensure appropriate patient management and to avoid unnecessary surgical intervention.¹ With the global incidence of thyroid carcinoma continuing to rise, reliable methods for distinguishing benign from malignant thyroid nodules remain an important clinical priority.²

Ultrasonography (USS) and fine-needle aspiration cytology (FNAC) are the principal preoperative diagnostic tools used in the evaluation of thyroid nodules. USS findings are commonly reported using the Thyroid Imaging Reporting and Data System (TIRADS), while FNAC results are classified according to the Bethesda System for Reporting Thyroid Cytopathology.³ These approaches play a central role in risk stratification and surgical decision-making. However, both modalities have recognized limitations, particularly in the evaluation of follicular-patterned lesions, as they provide limited information regarding tissue architecture. As a result, distinguishing benign follicular adenomas from malignant follicular neoplasms may remain challenging, leading to diagnostic uncertainty and, in some cases, unnecessary surgical procedures.

This diagnostic challenge is particularly relevant in lower-middle-income countries such as Sri Lanka, where healthcare resources are limited and avoiding unnecessary surgery is an important clinical and economic consideration. Although histopathological examination remains the gold standard for the diagnosis of thyroid tumors, it requires surgical excision and therefore cannot be used as a preoperative diagnostic tool. Furthermore, both cytological and histological assessment of thyroid lesions may be complicated by the gland's predominantly follicular architecture and nodular growth patterns.⁴

To improve preoperative diagnostic accuracy, several immunomarkers have been investigated as adjuncts to conventional cytological assessment. While these markers are traditionally applied to surgically resected specimens, they can also be evaluated on FNAC-derived cell block preparations using immunocytochemistry (ICC). This approach utilizes material obtained during routine FNAC and may provide additional diagnostic information without requiring more invasive procedures. Therefore, ICC performed on FNAC-derived cell blocks has the potential to serve as a practical adjunct to existing diagnostic methods, particularly in resource-limited settings. This study aimed to assess the value of selected ICC markers in improving the preoperative diagnosis of suspicious thyroid nodules.

Aim

Accordingly, the present study aimed to assess the utility of immunocytochemistry as an adjunctive technique to

improve the accuracy of preoperative diagnosis in suspicious thyroid nodules. This study further explored the feasibility and potential diagnostic contribution of immunocytochemistry performed on FNAC-derived cell blocks alongside conventional TIRADS and Bethesda assessment in a Sri Lankan resource-limited setting, where access to advanced molecular diagnostic techniques is limited.

Material and methods

A prospective observational diagnostic accuracy study was carried out at the Departments of Radiology and Pathology of Colombo South Teaching Hospital (CSTH), Sri Lanka, and the Department of Pathology, Faculty of Medical Sciences (FMS), University of Sri Jayewardenepura (USJP). A total of 106 patients presenting with thyroid nodules radiologically suspected to be malignant (TIRADS category 3 or higher) on ultrasonography were recruited for the study. Patients with incomplete or inadequate radiological data were excluded. Ethical approval for the study was obtained from the Ethics Review Committee of the Faculty of Medical Sciences, USJP (Ref. No. 65/2019). Written informed consent was obtained from all study participants.

Ultrasonography (USS) examinations were performed and reported by a consultant radiologist according to the TIRADS classification. Ultrasound-guided fine-needle aspirations (FNAs) were subsequently obtained from the radiologically suspected malignant nodules by the same radiologist. Direct smears were prepared from the aspirated material, while the residual needle rinse was used for cell block preparation. Cell blocks were processed using the alcohol-acid-formalin (AAF) method. Both the direct smears and cell block sections were stained with hematoxylin and eosin (H&E). The stained preparations were independently examined by two consultant pathologists and categorized according to the Bethesda System for Reporting Thyroid Cytopathology.

ICC staining was performed on adequately cellular FNAC-derived cell block sections using a manual streptavidin–biotin peroxidase method. Heat-induced epitope retrieval was carried out in citrate buffer (pH 6.0) using a pressure cooker. The immunocytochemical panel consisted of HBME-1 (Invitrogen; diluted 1:24), CK-19 (Dako; ready-to-use), and CD56 (Dako; ready-to-use). Following antigen retrieval, the sections were incubated with the respective primary antibodies for one hour. A secondary antibody and 3,3'-diaminobenzidine (DAB) chromogen were subsequently applied, and the slides were counterstained with hematoxylin. Appropriate positive and negative controls were included in each staining batch. The stained slides were independently evaluated by two consultant pathologists who were blinded to the cytological diagnosis.

For interpretation, cytoplasmic and/or membranous staining patterns were assessed according to the specific marker characteristics. HBME-1 and CK-19 were considered positive when moderate to strong membranous or cytoplasmic staining was observed in more than 10% of tumor cells, whereas CD56 expression was interpreted as positive when diffuse membranous staining was detected in more than 10% of follicular cells. Loss of CD56 expression was considered supportive of malignancy. If any discrepancies were observed between the two pathologists, a joint slide review followed by a consensus discussion was undertaken to resolve the differences and ensure consistency and accuracy in the final interpretations.

All patients included in the study were prospectively followed over a period of two years to determine the final outcome of their thyroid nodules as benign or malignant. For those who underwent surgical excision during the follow-up period, the definitive diagnosis was established through histopathological examination of the resected specimen. Among patients who did not undergo surgery, nodules that were classified as TIRADS 3 or 4 on ultrasonography or reported as non-diagnostic on initial FNAC were re-evaluated with repeat FNAC. In addition, serial ultrasonographic assessments were performed at six-month intervals to monitor changes in nodule size, morphology, or the appearance of suspicious features. If, during the follow-up period, any ultrasound scan revealed a nodule categorized as TIRADS 3 or above, a repeat FNAC was carried out.

The reference standard for malignancy was based on a composite outcome. Nodules reported as Bethesda category II on cytological evaluation were considered benign and managed with surveillance. Surgical intervention was undertaken when indicated based on comprehensive clinical, radiological, and cytological assessment (e.g., suspicion of malignancy, tracheal compression, significant cosmetic concerns, or poor compliance with follow-up). In patients who underwent surgery, the final diagnosis was determined by histopathological examination.

In the absence of surgical indications, patients were followed for two years. Nodules that remained stable in size and imaging characteristics and did not require surgery during follow-up were classified as benign for this study.

Clinical management was undertaken through standard multidisciplinary evaluation involving surgeons, radiologists, and the treating team. The estimated risk of malignancy was communicated to patients, and decisions regarding surgical intervention were made following shared clinical discussions integrating imaging, cytological, and clinical findings.

Data analysis was performed using the R language (version 4.3.1). Descriptive statistics were presented as frequencies and proportions. Diagnostic accuracy cal-

culations were based on a composite reference standard consisting of histopathological diagnosis in surgically treated patients and two-year clinical and radiological follow-up in patients who did not undergo surgery. Fisher's exact test was employed to evaluate associations between categorical diagnostic variables. Logistic regression analysis was conducted to quantify the strength and direction of associations among relevant variables. The diagnostic performance of the tests was further assessed using the receiver operating characteristic (ROC) curve analysis. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated based on the confusion matrix. A *p*-value of less than 0.05 was considered statistically significant. Logistic regression was performed to evaluate the predictive performance of cytological, radiological, and immunohistochemical classification systems for malignancy. Six predefined models were assessed: (1) ICC×Bethesda, (2) ICC×TI-RADS, (3) ICC alone, (4) Bethesda×TI-RADS, (5) Bethesda alone, and (6) TI-RADS alone. Model performance was compared using the Akaike Information Criterion (AIC), with lower values indicating better relative model fit. AIC was used for comparative evaluation within the dataset and does not imply absolute predictive superiority. Models were constructed based on clinical relevance without automated variable selection.

Results

The majority of the participants in the study were females (*n*=84, 79.2%). The age of the enrolled participants ranged from 15 to 76 years, with a mean and standard deviation of 47.20 ± 12.90 , respectively.

All 106 radiologically malignancy-suspected patients were subjected to FNAC. Of them, 78 (73.58%) were diagnosed as cytologically benign, while 28 (26.41%) were diagnosed as malignant or suspicious of malignant. At the two-year follow-up, 24 of the 78 cytologically benign patients had undergone surgery. Of those 24 patients, 15 patients were histologically confirmed to have malignant nodules, while 9 had benign nodules. Of the 28 cytologically malignant or malignancy-suspected patients, only 21 had undergone surgery. Of those 21 patients, 18 were histologically confirmed to have malignant tumors, while 3 patients were confirmed to have benign nodules.

As only patients with radiologically suspected malignancies were enrolled in the study, all patients were classified in TIRADS 3 or above categories. The majority (*n*=80, 75.4%) of the nodules belonged to the TIRADS 4 category (TIRADS 4a: *n*=54, TIRADS 4b: *n*=8, TIRADS 4c: *n*=18). Furthermore, 21 nodules in the TIRADS 3 cohort and 5 in the TIRADS 5 cohort were also reported.

Although all 106 enrolled patients had radiologically malignant nodules, only 28 (26.4%) had cytological-

ly diagnosed with suspicion of malignant (Bethesda 5: n=20, 18.9%) or malignant (Bethesda 6: n=8, 7.5%) nodules. The majority (n=43, 40.6%) of the nodules belonged to the benign category (Bethesda 2). Furthermore, 15 Bethesda 3 nodules, 14 Bethesda 1 nodules, and 6 Bethesda 4 nodules were also reported (Fig 3). Of the 20 cytologically malignant-suspected nodules, the majority (n=10) belonged to the TIRADS 4c category. The other ten patients were classified into TIRADS 4a (n=6), TIRADS 3 (n=2), TIRADS 4b (n=1), and TIRADS 5 (n=1) categories. Of the 8 cytologically malignant patients, 6 were classified in TIRADS 4c, and the other 2 cases were distributed in one per each of TIRADS 4b and TIRADS 5 categories (Table 1).

Table 1. Association of TIRADS classification and FNAC results*

TIRADS category vs direct cytology cross tabulation								
	Direct cytology – Bethesda category						Total	
	1	2	3	4	5	6		
TIRADS Category	3	1	14	3	1	2	0	21
	4a	11	26	9	2	6	0	54
	4b	1	2	1	2	1	1	8
	4c	1	0	1	0	10	6	18
	5	0	1	1	1	1	1	5
Total		14	43	15	6	20	8	106

* Non-diagnostic or unsatisfactory – Bethesda 1, benign – Bethesda 2, atypia of undetermined significance or follicular lesion of undetermined significance – Bethesda 3, follicular neoplasm or suspicious of follicular neoplasm – Bethesda 4, suspicious for malignancy – Bethesda 5, malignant – Bethesda 6

Among the forty-five (n=45) patients who had undergone surgery, thirty-three (n=33) were histologically confirmed to have malignant tumors, while the remaining twelve (n=12) were confirmed to have benign nodules. Of the 33 confirmed malignant cases, only 18 were both radiologically and cytologically malignancy suspected/ malignant (papillary thyroid carcinoma: n=16, papillary microcarcinoma: n=02), while the other 15 cases were cytologically non-malignant (papillary thyroid carcinoma: n=10, follicular variant of papillary thyroid carcinoma: n=03, cystic papillary thyroid carcinoma: n=01 and papillary microcarcinoma: n=01). However, radiologically and cytologically malignancy suspected three cases (Chronic thyroiditis: n=2, hyperplastic nodule: n=1) were histologically confirmed to have benign nodules. Nine patients were radiologically, cytologically, and histologically confirmed to have benign nodules (chronic thyroiditis: n=3; multinodular goiter: n 3; hyperplastic nodule: n 2; and colloid cyst: n=1).

Among the forty-five patients who underwent surgery, papillary thyroid carcinoma was identified as the

most common type (n=26) of malignant tumor (TIRADS 4a=10, TIRADS 4c=9, TIRADS 4b=3, TIRADS 3 =3, TIRADS 5=1). In addition, 3 papillary microcarcinoma cases (TIRADS 3: n=1, TIRADS 4c: n=1, TIRADS 5: n=1), 3 follicular variants of papillary carcinoma (TIRADS 3: n=1, TIRADS 4a: n=1, TIRADS 4b: n=1), and one cystic papillary carcinoma case (TIRADS 5=1) were reported.

Of the twelve (n=12) patients who were histologically confirmed to have benign nodules, 5 had chronic thyroiditis (TIRADS 3: n=2, TIRADS 4c:n=2, TIRADS 4a: n=1), while three had hyperplastic nodules (TIRADS 4a =3), two had multinodular goiter with thyroiditis (TIRADS 4a n=2), and one had a colloid cyst (TIRADS 3).

At the two-year follow-up, seventy-three (n=73) patients had clinically benign outcomes (TIRADS 3: n=15, TIRADS 4a: n=38, TIRADS 4b: n=5, TIRADS 4c: n=18, TIRADS 5: n=5).

Of the 26 patients with confirmed papillary thyroid carcinoma (PTC), sixteen were cytologically malignancy suspected (Bethesda 5: n=10) or malignant (Bethesda 6: n=6), while 6 cases were in Bethesda 2, 2 cases in Bethesda 1, and 1 case each in Bethesda 3 and 4 categories. However, the three papillary microcarcinoma cases belonged to the Bethesda 2 (n=1), Bethesda 5 (n=1), and Bethesda 6 (n=1) categories.

All three (n=3) follicular variants of papillary thyroid carcinoma patients were in cytologically nonmalignant categories (Bethesda 2: n=1, Bethesda 3: n=1, Bethesda 4: n=1), while they were radiologically classified under TIRADS 3, 4a, and 4b categories. Furthermore, one (n=1) cystic papillary thyroid carcinoma case was reported, and it was classified as Bethesda category 5 and TIRADS category 5.

Standardized residual analysis demonstrated a significant association between TIRADS classifications ($\geq$ TIRADS 3) and Bethesda categories, indicating that nodules with higher TIRADS scores were more likely to align with higher Bethesda categories, particularly among radiologically suspected malignant nodules. A statistically significant correlation was observed between TIRADS classification ($\geq$ TIRADS 3) and Bethesda categories ($p=0.00009$). Additionally, Fisher’s exact test revealed a significant association between TIRADS classification and the benign-malignant status of patients at two-year follow-up ($p=0.046$). Notably, the TIRADS 4c category had a significant contribution to this association ($p=0.047$), with nodules in this category demonstrating a four-fold increased risk of malignancy compared to those classified as TIRADS 3 (Table 2). ROC curve analysis further indicated that the TIRADS system possessed a limited to modest discriminatory ability to differentiate between benign and malignant lesions, with an area under the curve (AUC) of 0.65 (Fig. 1).

Table 2. Logistic regression data analysis – TIRADS classification

TIRADS classification	Odd ratio	95% CI1	p
3	–	–	
4a	0.91	0.29, 3.24	0.900
4b	3.20	0.57, 18.9	0.200
4c	4.00	1.06, 16.9	0.047
5	2.13	0.23, 16.9	0.500

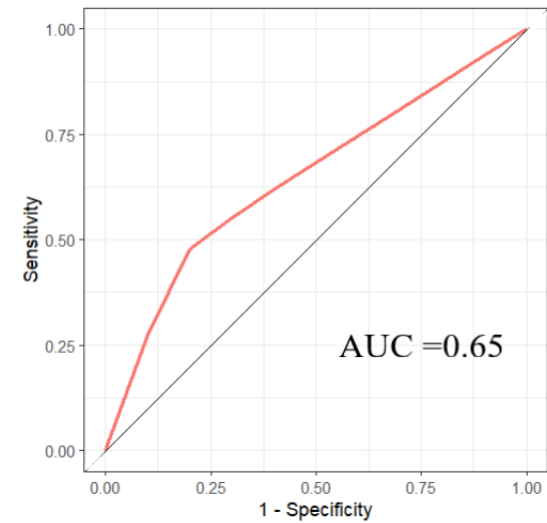


Fig. 1. ROC curve for the ultrasound scan findings (AUC=0.5: model performs like random guessing, AUC=1.0: model perfectly separates positive and negative cases, AUC > 0.5: model has some ability to predict correctly)

Furthermore, a statistically significant association was identified between the Bethesda classification and the benign-malignant status of patients at the two-year follow-up ($p=0.00143$). Logistic regression analysis revealed that patients classified as Bethesda category 6 had a significantly increased likelihood of malignancy, with an odds ratio of 13.1 (95% CI: 2.52–102; $p=0.004$), compared to those in Bethesda category 2. Similarly, individuals in Bethesda category 5 exhibited a fivefold higher risk of malignant outcomes (OR: 5.0; 95% CI: 1.70–18.0; $p=0.005$) (Table 3). The Bethesda system also demonstrated a moderate discriminatory capacity for distinguishing between benign and malignant lesions, as indicated by an AUC of 0.73 (Fig. 2)

Table 3. Logistic regression data analysis – Bethesda classification

Bethesda classification	Odd ratio	95% confidence interval	p
Bethesda 02	–	–	–
Bethesda 03	0.67	0.09, 4.99	0.600
Bethesda 04	4.38	0.70, 27.9	0.100
Bethesda 05	5.35	1.70, 18.0	0.005
Bethesda 06	13.1	2.52, 102	0.004

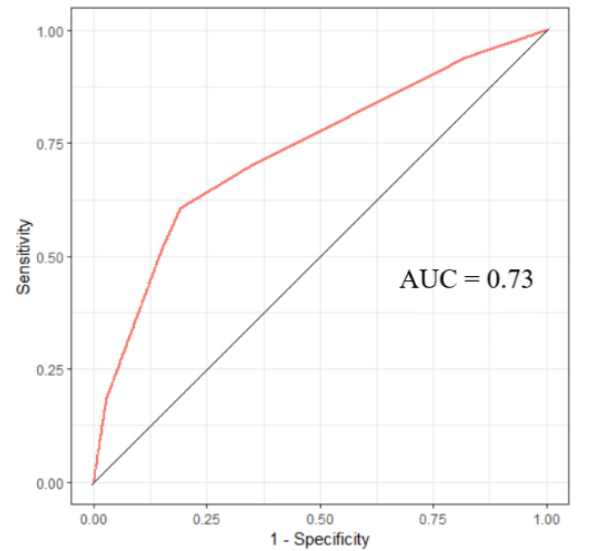


Fig. 2. ROC curve of FNAC findings compared to histology (AUC=0.5: model performs like random guessing, AUC=1.0: model perfectly separates positive and negative cases, AUC > 0.5: model has some ability to predict correctly)

Diagnostic performance was evaluated separately in the histology-verified subgroup and the entire cohort. In the histology-verified subgroup, where histopathology served as the gold standard, TIRADS ($\geq$ TR4) demonstrated a sensitivity of 94.3%, specificity of 22.2%, PPV of 77.3%, NPV of 57.1%, and accuracy of 75.0%. In comparison, Bethesda classification showed a sensitivity of 62.9%, specificity of 77.8%, PPV of 88.0%, NPV of 46.7%, and accuracy of 67.3%. In the entire cohort, using a composite reference standard, TIRADS showed a sensitivity of 89.04%, specificity of 30.30%, PPV of 73.86%, NPV of 55.56%, and accuracy of 70.75%, whereas Bethesda demonstrated a sensitivity of 80.82%, specificity of 60.61%, PPV of 87.94%, NPV of 58.82%, and accuracy of 74.53% (Fig. 3).

Table 4. Frequency of HBME-1, CK-19 and CD-56 immunomarker

	IHC_HBME1		IHC_CD56			Total
			Negative	Positive	Equivocal	
Negative	IHC_CK19	Negative		17		17
	Total			17		17
Positive	IHC_CK19	Negative	5	0		5
		Positive	7	0		7
		Focal positive	2	2		4
	Total		14	2		16
Focal Positive	IHC_CK19	Focal positive	1			1
	Total		1			1
Total	IHC_CK19	Negative	5	17	0	22
		Positive	7	0	0	7
		Focal positive	3	0	2	5
	Total		15	17	2	34

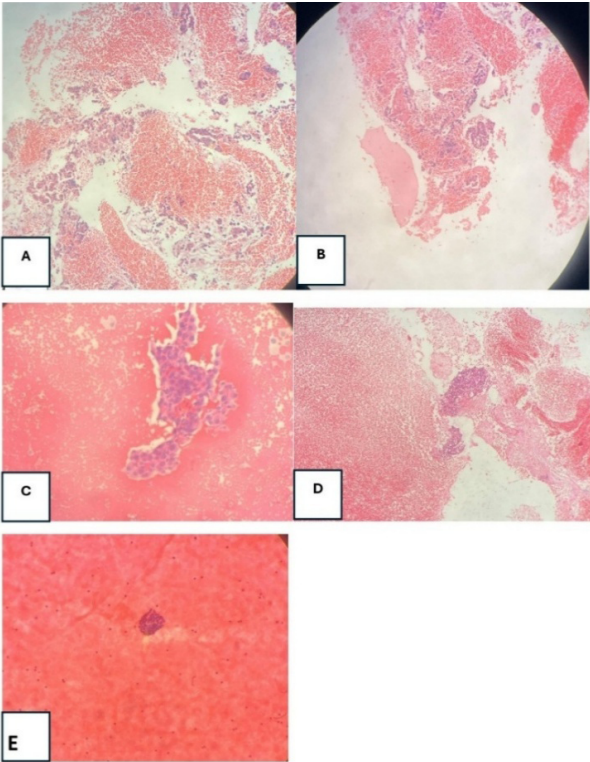


Fig. 3. Microscopic appearance of cell block sections and direct cytology smears, A: Cell block section showing papillary structures characteristic of papillary thyroid carcinoma (H&E stained, ×100 magnification), B: Cell block section showing nuclear pseudoinclusions typical of papillary thyroid carcinoma (H&E stained, ×100 magnification), C: Direct smear of papillary thyroid carcinoma displaying nuclear pseudoinclusions (H&E stained, ×200 magnification), D: Cell block section demonstrating hyperplastic benign nodules of a hyperplastic thyroid nodule (H&E stained, ×200 magnification), E: Direct smear showing benign follicular epithelial cells in a hyperplastic nodule (H&E stained, ×100 magnification)

ICC was performed on 34 FNAC-derived cell blocks with adequate cellularity. Of these, 17 (50.0%) cases were negative for HBME-1, 16 (47.1%) were positive, and one (2.9%) was focally positive. All HBME-1-negative cases were negative for CK-19 and positive for CD56. In contrast, among the HBME-1-positive or focally positive cases, the majority demonstrated positive or focally positive CK-19 expression together with loss of CD56 expression (Table 4) (Fig. 4).

A statistically significant association was observed between the expression patterns of HBME-1, CK-19, and CD56 and the final benign-malignant status of thyroid nodules at two-year follow-up ($p<0.001$). All three markers demonstrated 100% specificity. Sensitivity was highest for HBME-1 (94.11%), followed by CD56 (88.23%) and CK-19 (41.17%). ROC analysis yielded AUC values of 1.00 for HBME-1, 0.853 for CK-19, and 0.882 for CD56.

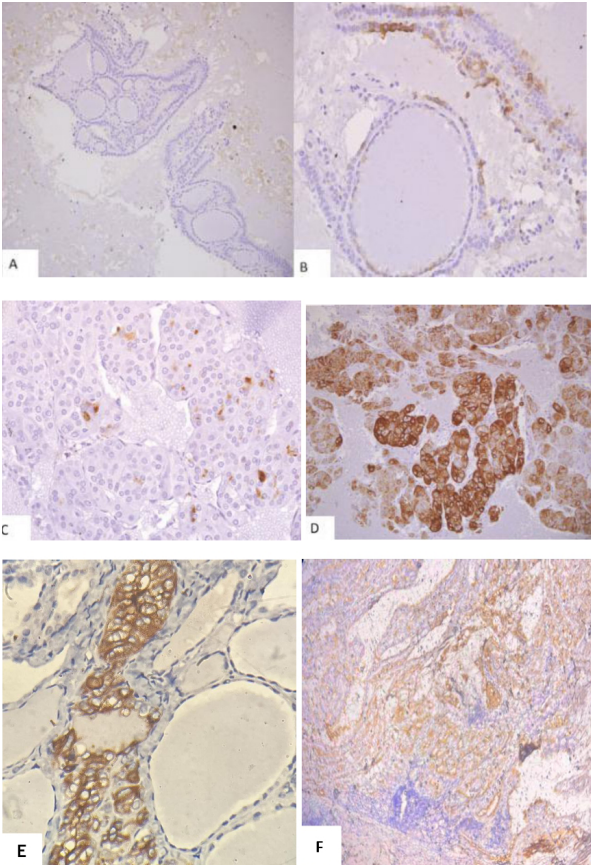


Fig. 4. IHC staining patterns observed in the study, A: Negative staining for HBME-1 (×40 magnification), B: Focal staining for CK-19 (×40 magnification), C: Focal staining for HBME-1 (×200 magnification), D: Positive staining for CK-19 (×200 magnification), E: Positive staining for HBME-1 (×200 magnification), F: Positive staining for CD-56 (×200 magnification)

Of the 34 cases evaluated by ICC, 24 had histopathological confirmation, while 10 were classified according to the two-year follow-up reference standard. The ten non-operated cases were negative for HBME-1 and CK-19 and positive for CD56 and demonstrated benign clinical outcomes throughout follow-up.

When ICC findings were compared with the final diagnosis, all nodules negative for HBME-1 and CK-19 but positive for CD56 were classified as benign. Conversely, all nodules demonstrating positive or focally positive HBME-1 staining were classified as malignant. Among the 17 malignant cases, CK-19 was positive in seven ($n=7$) cases, focally positive in five ($n=5$) cases, and negative in five ($n=5$) cases. CD56 expression was absent in 15 cases, while two ($n=2$) cases demonstrated equivocal staining.

PTC was the most common malignant lesion identified in the ICC subgroup, accounting for 12 of 34 cases (35.3%). All PTC cases demonstrated positive or focally positive HBME-1 staining. CK-19 expression was positive in four cases, focally positive in five cases, and neg-

active in three cases. CD56 expression was absent in 11 of 12 PTC cases, while one case demonstrated equivocal staining. No PTC case demonstrated the combination of HBME-1 negativity and CD56 positivity.

Among the papillary microcarcinoma cases ($n=2$), one showed positivity for both HBME-1 and CK-19 with loss of CD56 expression, while the other was positive for HBME-1, negative for CK-19, and negative for CD56. The cystic and follicular variants of papillary thyroid carcinoma each demonstrated positive HBME-1 and CK-19 staining with loss of CD56 expression. The multifocal PTC case was positive for HBME-1 and negative for both CK-19 and CD56.

All benign lesions, including chronic thyroiditis ($n=4$), colloid cyst ($n=1$), hyperplastic nodule ($n=1$), and multinodular goiter with thyroiditis ($n=1$), demonstrated the characteristic staining pattern of HBME-1 negativity, CK-19 negativity, and CD56 positivity.

The predictive performance of six logistic regression models was compared using the Akaike Information Criterion (AIC), with lower values indicating better relative model fit. The combined immunocytochemistry (ICC) and Bethesda classification model demonstrated the lowest AIC value ($AIC=4$), indicating the best relative fit among the evaluated models. The ICC×TIRADS model showed the second-lowest AIC value ($AIC=12$), followed by ICC alone ($AIC=14$), Bethesda×TIRADS ($AIC=18$), Bethesda alone ($AIC=20$), and TIRADS alone ($AIC=22$). Overall, models incorporating ICC showed lower AIC values than models based solely on TIRADS or Bethesda classifications. These findings suggest that the addition of ICC may improve the relative predictive performance of conventional classification systems in identifying malignant thyroid nodules within this study cohort.

Discussion

The observed female predominance and peak incidence in the fourth to fifth decades are consistent with established epidemiological patterns in thyroid neoplasia.^{6–8} These findings support previously reported associations between thyroid neoplasia and biological susceptibility factors, including hormonal influences and X-chromosome inactivation, although the exact mechanisms remain incompletely understood.⁹

The relatively higher false-negative rate observed in the cytology findings highlights recognized limitations of FNAC in routine clinical practice. While false-positive results were infrequent, false-negative findings may result from sampling inadequacy, operator-dependent factors, and overlapping cytomorphological features between benign and malignant lesions.¹⁰ In particular, atypical cyst lining cells, granular cytoplasm, cytoplasmic processes, small micronodules, and subtle nuclear features may contribute to diagnostic uncertainty

and misclassification.^{11,12} These findings reinforce the well-recognized challenges associated with the diagnosis of follicular-patterned lesions using cytology alone.

Misclassification of follicular variants of papillary thyroid carcinoma further illustrates the inherent limitations of cytology. Subtle nuclear alterations, such as nuclear enlargement, membrane irregularities, and chromatin clearing, may overlap with benign reactive changes when present in a limited number of cells, thereby reducing diagnostic specificity.¹² This reinforces the recognized difficulty in distinguishing follicular-patterned lesions using cytology alone.

The significant association between higher TIRADS categories (4c and 5) and malignancy supports the clinical utility of ultrasound-based risk stratification. However, the magnitude of risk observed in the present study was lower than that reported in previous studies, including an Indian study demonstrating higher risk estimates across TIRADS categories.¹³ Despite these differences, the progressive increase in malignancy risk with higher TIRADS categories supports its role as a stratification tool rather than a standalone diagnostic method.

Comparison of TIRADS and Bethesda classification demonstrated complementary diagnostic strengths. In the histology-verified subgroup, TIRADS showed higher sensitivity, supporting its role in reducing missed malignancies, whereas the Bethesda classification demonstrated higher specificity and positive predictive value. A similar pattern was observed in the overall cohort using the composite reference standard. These findings are broadly comparable to those reported by George et al., although differences in specificity and overall diagnostic performance may reflect variations in case selection, inclusion of indeterminate nodules, and the use of a composite reference standard.¹³ The comparatively lower diagnostic accuracy relative to larger cohorts¹⁴ further highlights the influence of case mix and methodological differences. Collectively, these findings support the complementary use of radiological and cytological assessment in the evaluation of thyroid nodules.

The observed differences in diagnostic performance between the histology-verified subgroup and the overall cohort may be explained by verification bias and the use of a composite reference standard. Histopathology-based evaluation provides a definitive diagnosis but is limited to surgically treated patients, who are more likely to have a higher risk of malignancy. In contrast, inclusion of non-operated cases in the overall cohort may introduce potential misclassification, particularly among patients managed conservatively. Therefore, these findings should be interpreted in light of the applied reference standards and the characteristics of the study population.

The present study demonstrates the added value of immunocytochemistry in improving diagnostic pre-

cision, particularly in indeterminate cases. The high specificity observed for HBME-1, CK-19, and CD-56 supports their utility as adjunctive markers.^{15,16} The superior performance of HBME-1 is consistent with its established association with papillary thyroid carcinoma,^{17,18} while lower sensitivities reported in other studies may reflect differences in tumour spectrum.^{19,20} Although CK-19 demonstrated lower sensitivity, its high specificity supports its role in excluding malignancy when negative.²¹ The absence of CD-56 expression in malignant nodules further reinforces its inverse association with malignancy.^{22–24}

The marker expression patterns observed in this study are consistent with the concept of panel-based interpretation. The combination of HBME-1 positivity, CK-19 positivity, and loss of CD-56 expression was associated with malignant lesions, whereas benign lesions typically demonstrated the opposite staining profile. Similar observations have been reported previously, supporting the potential value of combined marker assessment.²⁵ This approach may be particularly relevant in resource-limited settings where access to advanced molecular diagnostic techniques is limited.^{26–28} In addition, subtype-specific expression patterns observed in papillary thyroid carcinoma and its variants were broadly consistent with previous reports.^{29,30} However, given the relatively small number of cases evaluated by immunocytochemistry, these findings should be regarded as preliminary and require validation in larger cohorts before firm conclusions can be drawn.

Although markers such as galectin-3, BRAF V61, and TROP-2 have demonstrated diagnostic value in thyroid cytology, they were not included in the present study because they were not part of the routinely applied panel during the study period. Future studies incorporating broader immunocytochemical and molecular panels may provide additional diagnostic information.

In the present study, the AUC for TIRADS was 0.65, indicating limited to modest discriminatory ability. Although this suggests some capacity to differentiate between benign and malignant lesions, such performance does not reflect strong diagnostic accuracy. Therefore, TIRADS classification alone may be insufficient to guide definitive clinical decision-making, and its findings should be interpreted in conjunction with cytological, immunocytochemical, and clinical parameters.

Comparative AIC analysis suggested that the combined immunocytochemistry and Bethesda classification model provided the most favorable relative fit among the evaluated models. Models incorporating immunocytochemistry generally demonstrated lower AIC values than models based solely on TIRADS or Bethesda classifications. However, these findings should be interpreted cautiously. The relatively small number of cases available for immunocytochemistry increases the

risk of overfitting and limits the generalizability of model-based inferences. Consequently, the observed differences in AIC values should be regarded as exploratory indicators of relative model fit rather than evidence of superior predictive performance.

Several limitations should be considered when interpreting the findings of this study. Histopathological confirmation was available only for patients who underwent surgical excision, creating the potential for verification bias. Patients managed conservatively were classified according to cytological findings and imaging stability during two years of follow-up, which reflects routine clinical practice but may not completely exclude indolent malignancies. Furthermore, the relatively small number of malignant outcomes and immunocytochemistry-tested cases may have increased the risk of model overfitting and reduced the precision of multivariable estimates. The wide confidence intervals observed for several predictors further suggest uncertainty in some effect estimates. Therefore, the multivariable and model-comparison findings should be interpreted as exploratory and require confirmation in larger, independent cohorts.

Conclusion

This study demonstrates that while TIRADS and the Bethesda system are valuable tools in the preoperative evaluation of thyroid nodules, each has inherent limitations, particularly in the assessment of follicular-patterned lesions. The findings suggest that immunocytochemistry using a panel of HBME-1, CK-19, and CD-56 may provide useful additional diagnostic information when used as an adjunct to conventional radiological and cytological assessment. The combined use of immunocytochemistry with Bethesda classification showed improved predictive performance within the study cohort and may help reduce diagnostic uncertainty in selected cases. These findings indicate a potential role for immunocytochemistry in supporting preoperative decision-making, particularly in resource-limited settings where access to advanced molecular diagnostics is restricted. However, given the relatively small number of cases evaluated by immunocytochemistry, further validation in larger, independent cohorts is required before its routine clinical application can be recommended.

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Declarations

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Author contributions

Conceptualization, H.K., B.S., N.F., A.P., B.G. and A.S.; Methodology, H.K., B.S., A.P. and B.G.; Software, H.K.; Validation, H.K., B.S., N.F., A.P., and B.G.; Formal Analysis, H.K. and B.S.; Investigation, H.K., B.S., N.F., A.P., and B.G.; Resources, B.S. and N.F.; Data Curation, H.K., B.S., N.F., A.P., B.G. and A.S.; Writing – Original Draft Preparation, H.K.; Writing – Review & Editing, H.K., B.S., N.F., A.P., B.G. and A.S.; Visualization, H.K.; Supervision, B.S., N.F., A.P., B.G. and A.S.; Project Administration, B.S., N.F., A.P., B.G. and A.S.; Funding Acquisition, B.S. and N.F.

Conflicts of interest

The authors have no financial or proprietary interest in any material discussed in this article.

Data availability

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

Ethics approval

Ethical approval for the study was obtained from the ethics review committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura (65/19) and informed written consent was taken from every participant of the study.

Use of AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT to correct the language. After using this tool/service, the author(s) reviewed and edited the content as needed and take/s full responsibility for the content of the published article.

References

- Mazeh H, Mizrahi I, Halle D, et al. Development of a microRNA-based molecular assay for the detection of papillary thyroid carcinoma in aspiration biopsy samples. *Thyroid*. 2011;21(2):111-118. doi:10.1089/thy.2010.0356
- Jayarajah U, Fernando A, Prabashani S, Fernando EA, Seneviratne SA. Incidence and histological patterns of thyroid cancer in Sri Lanka 2001-2010: an analysis of national cancer registry data. *BMC Cancer*. 2018;18(1):163. doi:10.1186/s12885-018-4083-5
- Grant EG, Tessler FN, Hoang JK, et al. Thyroid Ultrasound Reporting Lexicon: White Paper of the ACR Thyroid Imaging, Reporting and Data System (TIRADS) Committee. *J Am Coll Radiol*. 2015;12(12PtA):1272-1279. doi:10.1016/j.jacr.2015.07.011
- Liu H, Lin F. Application of immunohistochemistry in thyroid pathology. *Arch Pathol Lab Med*. 2015;139(1):67-82. doi:10.5858/arpa.2014-0056-RA
- Baloch Z, Mete O, Asa SL. Immunohistochemical biomarkers in thyroid pathology. *Endocr Pathol*. 2018;29(2):91-112. doi:10.1007/s12022-018-9526-4
- Dabbagh AA, Ibraheem MM. Clinical, cytological and histological correlation of thyroid nodule(s): an observational study. *J Med Sci Clin Res*. 2019;7(1):61-70.
- Krišto B, Krželj IV, Krželj A, Perković R. Ultrasound-guided fine needle aspiration cytology (FNAC): an assessment of the diagnostic potential in histologically proven thyroid nodules. *Acta Clin Croat*. 2022;61(1):184-188.
- Kumar SK, Seetharamaiah T, Rampure D, Ramakrishna C, Devi RY. Thyroid nodule: cytohistological correlation. *Sch J App Med Sci*. 2013;1(6):745-747.
- Brix TH, Knudsen GP, Kristiansen M, Kyvik KO, Ørstavik KH, Hegedüs L. High frequency of skewed X-chromosome inactivation in females with autoimmune thyroid disease: a possible explanation for the female predisposition to thyroid autoimmunity. *J Clin Endocrinol Metab*. 2005;90(11):5949-5953. doi:10.1210/jc.2005-1366
- Haberal AN, Toru S, Özen Ö, Arat Z, Bilezikçi B. Diagnostic pitfalls in the evaluation of fine needle aspiration cytology of the thyroid: correlation with histopathology in 260 cases. *Cytopathology*. 2009;20(2):103-108. doi:10.1111/j.1365-2303.2008.00594.x
- Kumar S, Aqil S, Dahar A. Role of fine needle aspiration cytology in thyroid diseases. *J Surg Pak (Int)*. 2008;13(1):23-26.
- Malheiros DC, Canberk S, Poller DN, Schmitt F. Thyroid FNAC: causes of false-positive results. *Cytopathology*. 2018;29(5):407-417. doi:10.1111/cyt.12560
- George NA, Suresh S, Jiji V, et al. Correlation of TIRADS and Bethesda scoring systems with final histopathology of thyroid nodules: an institutional experience. *Indian J Otolaryngol Head Neck Surg*. 2022;74(3):5753-5758. doi:10.1007/s12070-021-02380-8
- Horvath E, Majlis S, Rossi R, et al. An ultrasonogram reporting system for thyroid nodules stratifying cancer risk for clinical management. *J Clin Endocrinol Metab*. 2009;94(5):1748-1751. doi:10.1210/jc.2008-1724
- Cha H, Pyo JY, Hong SW. The usefulness of immunocytochemistry of CD56 in determining malignancy from indeterminate thyroid fine-needle aspiration cytology. *J Pathol Transl Med*. 2018;52(6):404-410. doi:10.4132/jptm.2018.09.20
- Margari N, Giovannopoulos I, Pouliakis A, et al. Application of immunocytochemistry on cell block sections for the investigation of thyroid lesions. *Acta Cytol*. 2018;62(2):137-144. doi:10.1159/000485824
- Chen YJ, Zhao RM, Zhao Q, et al. Diagnostic significance of elevated expression of HBME-1 in papillary thyroid carcinoma. *Tumour Biol*. 2016;37(7):8715-8720. doi:10.1007/s13277-015-4169-5

18. Ozolins A, Narbutis Z, Strumfa I. Diagnostic value of HBME-1, galectin-3, and cytokeratin 19 in thyroid pathology. *Proc Latv Acad Sci Sect B Nat Exact Appl Sci*. 2010;64(5-6):200-206.
19. Xiong Y, Luo D, Cheng S, Dong Y, Liu Y. Diagnostic value of immunomarkers in thyroid tumors: a comparative study. *Endocr Pathol*. 2021;32(2):161-168.
20. Sanuvada R, Nandyala R, Chowhan AK. Utility of HBME-1 and galectin-3 in differentiating benign and malignant follicular-patterned lesions of the thyroid. *J Lab Physicians*. 2020;12(3):199-205. doi:10.1055/s-0040-1716607
21. de Matos PS, Ferreira AP, de Oliveira Facuri F, Assumpção LV, Metze K, Ward LS. Usefulness of HBME-1, cytokeratin 19 and galectin-3 immunostaining in the diagnosis of thyroid malignancy. *Histopathology*. 2005;47(4):391-401. doi:10.1111/j.1365-2559.2005.02221.x
22. Kyung MS, Jeong HS, Kim BM. Diagnostic utility of CD56 in papillary thyroid carcinoma. *Korean J Pathol*. 2011;45(6):573-578.
23. Pyo JS, Kim DH, Yang J. Diagnostic value of CD56 immunohistochemistry in thyroid lesions. *Int J Biol Markers*. 2018;33(2):161-167. doi:10.1177/1724600818754620
24. El Demellawy D, Nasr AL, Babay S, Alowami S. Diagnostic utility of CD56 immunohistochemistry in papillary carcinoma of the thyroid. *Pathol Res Pract*. 2009;205(5):303-309. doi:10.1016/j.prp.2008.11.007
25. Nasr M, Mukhopadhyay S, Zhang S, et al. Immunohistochemical markers in diagnosis of papillary thyroid carcinoma: utility of HBME1 combined with CK19 immunostaining. *Mod Pathol*. 2006;19(12):1631-1637. doi:10.1038/modpathol.3800705
26. Agrawal SJ, Patil SB, Umap PS, Oberoi JS, Agrawal SS. Diagnostic utility of fine needle aspiration cytology and cell block technique in thyroid lesions. *J Med Sci Clin Res*. 2020;8(7). doi:10.18535/jmscr/v8i7.72
27. Cochand-Priollet B, Giannini C, Dupuy R, Vénissac N, Alwan Z, Vielh P. Immunocytochemistry in the diagnostic work-up of thyroid nodules: a panel of three markers on liquid-based cytology. *Diagn Cytopathol*. 2011;39(10):730-736. doi:10.1002/dc.21493
28. Mai KT, Landry DC, Thomas J, Yazdi HM. The application of immunostains in the cytologic diagnosis of thyroid neoplasms. *Diagn Cytopathol*. 2000;23(3):187-191.
29. Song JY, Kim SY, Ahn S. Immunohistochemical expression of CK19 and HBME-1 in papillary thyroid carcinoma and other thyroid lesions. *Ann Diagn Pathol*. 2019;41:75-80. doi:10.1016/j.anndiagpath.2019.05.003
30. Barut F, Onak Kandemir N, Bilezikçi B. Comparative expression of HBME-1, galectin-3, and cytokeratin 19 in the diagnosis of thyroid neoplasms. *Turk Patoloji Derg*. 2010;26(1):60-67.



ORIGINAL PAPER

Exploratory mediation analysis of associations between serum biomarkers of neuronal injury, astrocyte reactivity, neuroinflammation, and neurotrophic support and fatigue in Parkinson's disease

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ABSTRACT

Introduction and aim. Fatigue is a debilitating non-motor symptom in Parkinson's disease (PD). This exploratory cross-sectional mediation analysis investigated whether specific serum biomarkers mediate the relationship between PD and fatigue severity.

Material and methods. Ninety PD patients and 45 matched controls were enrolled in the study. Fatigue was assessed using the Fibro-Fatigue scale (FF). Serum levels of ten biomarkers (neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), brain-derived neurotrophic factor (BDNF), β -amyloid-42, α -synuclein, ubiquitin carboxy-terminal hydrolase L1 (UCHL1), high-mobility group box 1 (HMGB1), R-spondin 1 (RSPO1), Dickkopf-1 (DKK1), and sclerostin) were quantified by enzyme-linked immunosorbent assay. Mediation analysis (PROCESS Macro, Version 4.2, Model 4) tested whether these biomarkers mediated the relationship between PD status and FF score.

Results. The overall model predicting FF scores was highly significant ($F(26,108)=54.08$, $p<0.001$, $R^2=0.929$). Mediation analysis revealed significant indirect effects from PD status to fatigue via GFAP, HMGB1, BDNF, and NSE. Moderation analysis indicated that the biomarker fatigue relationship was significantly modified by PD status only for HMGB1 and BDNF. For GFAP and NSE, the interactions were non-significant. Bootstrapped analyses confirmed significant indirect effects of PD on fatigue through elevated levels of NSE (effect=1.743), HMGB1 (effect=1.207), GFAP (effect=1.101), and BDNF (effect=0.921).

Conclusion. PD-related fatigue is significantly mediated by neuroinflammation (HMGB1), astroglial activation (GFAP), neuronal injury (NSE), and a paradoxical BDNF response. Disease-specific moderation was confirmed only for HMGB1 and BDNF.

Keywords. fibro-fatigue, mediation study, neuronal damage biomarkers, Parkinson's disease, Wnt pathway biomarkers

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder pathologically defined by the degeneration of dopaminergic neurons in the substantia nigra and the accumulation of α -synuclein in Lewy bodies, leading to profound dopamine deficiency in the striatum.^{1,2} The

cardinal motor symptoms, bradykinesia, tremor, rigidity, and postural instability form the basis of the clinical diagnosis.^{3,4} Non-motor symptoms are increasingly recognized for their devastating impact on quality of life.⁵ Among these, chronic fatigue stands out as one of the most prevalent and disabling symptoms, affecting

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over 50% of patients and severely impairing daily functioning. However, its underlying pathophysiological mechanisms remain poorly understood.^{6,7} A pathway critically implicated in PD pathogenesis is the canonical Wnt/ β -catenin signaling cascade. This pathway is essential for the development, maintenance, and survival of midbrain dopaminergic neurons.^{8,9} Its activation supports neurogenesis and synaptic plasticity and protects neurons by improving mitochondrial function and reducing oxidative stress.^{10,11} Endogenous agonists and antagonists govern the precise modulation of this pathway. For instance, Dickkopf-1 (DKK1) is a potent antagonist whose upregulation is linked to neuronal damage, while R-spondin 1 (RSPO1) acts as an agonist that potentiates Wnt signaling and can attenuate DKK1-mediated inhibition.^{12,13} Other relevant modulators include sclerostin, a Wnt antagonist,¹⁴ and high-mobility group box 1 (HMGB1), a mediator of neuroinflammation with elevated levels in PD that can interact with α -synuclein to potentiate neurodegeneration.^{15,16} Dysregulation of this central pathway is recognized as a contributor to neurodegeneration.^{12,13,17} In parallel, a panel of circulating biomarkers provides insight into underlying neurodegenerative processes. Proteins such as α -synuclein and β -amyloid-42 are being explored for their diagnostic potential, as they reflect core pathological hallmarks.^{18,19} Furthermore, biomarkers indicative of active neuronal damage and glial response have emerged. Neuron-specific enolase (NSE) and ubiquitin carboxy-terminal hydrolase L1 (UCHL1) are elevated following neuronal injury.²⁰ Recently, Al-Hakeim and colleagues²¹ reported that serum neuronal damage markers (NSE, GFAP, S100B) correlate with affective and chronic fatigue symptoms in a cohort of 70 PD patients. However, that study did not examine a broader panel of inflammatory and astroglial markers, nor did it test for mediation or disease-specific conditional effects. Conversely, brain-derived neurotrophic factor (BDNF) supports the survival of dopaminergic neurons, although its utility as a clear biomarker is debated.^{22,23} Glial fibrillary acidic protein (GFAP), released into the blood upon astrocytic activation, shows promise for monitoring disease progression in PD.^{24,25} Despite these advances, a critical gap persists. While the role of Wnt signaling in neuronal survival is well established, its potential effects on specific non-motor manifestations, particularly fatigue, remain largely unexplored. Furthermore, it is unclear whether the subjective experience of fatigue is reflected in quantifiable changes in circulating biomarkers of neuronal damage, neuroinflammation, and glial activation. Thus, the specific novelty of the present study is twofold. First, rather than reporting simple associations between biomarkers and fatigue, we formally test a statistical mediation model in which circulating biomarkers mediate the relationship between PD and fatigue.

Second, we examine whether these mediation effects are disease-specific, i.e., whether the link between biomarker elevation and fatigue severity differs between PD patients and controls. To the best of our knowledge, this exploratory mediation approach has not previously been applied to fatigue in PD. It is important to emphasize that this analysis tests statistical indirect effects consistent with a causal model; however, causality cannot be established from this cross-sectional design, and all findings are hypothesis-generating. We hypothesized that fatigue severity in PD might be associated with altered levels of biomarkers indicative of neuronal damage (NSE, UCHL1), astrocytic activation (GFAP), neuroinflammation (HMGB1), and neurodegeneration (α -synuclein and β -amyloid-42). Given the established role of Wnt signaling in neuronal survival, we also explored whether Wnt pathway modulators (DKK1, RSPO1, sclerostin) were associated with fatigue, though these analyses were considered exploratory.

Aim

This exploratory cross-sectional study aimed to investigate whether circulating levels of selected biomarkers (NSE, GFAP, BDNF, HMGB1, α synuclein, β amyloid 42, UCHL1, DKK1, RSPO1, and sclerostin) mediate the relationship between PD status and fatigue severity. A secondary aim was to test whether the biomarker–fatigue association differs between PD patients and controls (disease specific moderation). Given the exploratory nature of the analysis, all findings are intended to generate hypotheses for future longitudinal mechanistic studies, not to establish biological causality or clinical recommendations.

Material and methods

Study design and participants

This case-control study enrolled ninety patients with a confirmed diagnosis of PD and forty-five age- and sex-matched healthy controls. Participants were recruited from Al-Sadr Medical City, Al-Najaf Teaching Hospital, and Al-Furat Al-Awsat Center for Neurosciences in Najaf, Iraq, between February and May 2025. The study protocol was approved by the Institutional Ethics Committee of the University of Kufa (MEC-110/2025) and was conducted in accordance with the World Medical Association's Declaration of Helsinki. All participants provided written informed consent before enrollment.

PD diagnosis was established using the UK Parkinson's Disease Society Brain Bank Clinical Diagnostic Criteria.²⁶ All patients presented with bradykinesia plus at least one other cardinal feature (resting tremor, rigidity, or postural instability) and exhibited no signs suggestive of atypical Parkinsonism. To rule out confounding conditions, individuals with concurrent liver or kidney disease were excluded. Furthermore, to con-

trol for systemic inflammation, only participants with serum C-reactive protein (CRP) levels below 6 mg/L were included.²⁷

Clinical assessments

An experienced neurologist conducted a semi-structured interview to collect sociodemographic and clinical information from both control subjects and patients. Motor and non-motor symptoms associated with PD were assessed using the Movement Disorders Society Revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS), as previously outlined.²⁸ The MDS-UPDRS is divided into four parts: Part I (non-motor Experiences of Daily Living, nM-EDL), Part II (motor Experiences of Daily Living, M-EDL), Part III (motor examination), and Part IV (motor complications).²⁸ Neurologists conducted the MDS-UPDRS assessment in patients, and ratings from the four domains were used for statistical analysis. The Hoehn and Yahr staging method was also used to rate PD severity.²⁹ The severity of chronic fatigue syndrome (CFS) and fibromyalgia was assessed by a senior psychiatrist using the Fibro-Fatigue (FF) scale.³⁰ The FF scale assesses both mental and physical aspects of central fatigue, including exhaustion, lack of energy, and cognitive fatigue, without directly measuring pain or fibromyalgia tender points. However, fatigue in PD may overlap with depressive symptoms, sleep disturbances (e.g., insomnia, REM sleep behavior disorder), or medication effects (e.g., dopaminergic agents, sedatives). While we did not systematically exclude or separately measure these factors, our statistical model included key covariates (age, sex, BMI, exercise, and tobacco use disorder (TUD)) to reduce potential confounding. Nevertheless, the possibility that some variance in FF scores reflects depression, sleep dysfunction, or medication side effects cannot be excluded, and this is addressed as a limitation.

Assays

Fasting venous blood samples (5 mL) were collected from all participants at approximately 9:00 a.m. After clotting, samples were centrifuged at 1200×g for 5 minutes at room temperature. The resulting serum was aliquoted into three Eppendorf tubes, and any hemolyzed samples were discarded. Aliquots were stored at -80 °C until analysis. Serum concentrations of key biomarkers were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits from Wuhan USCN Business Co., Ltd. (China). The key biomarkers included α -synuclein (Catalog E3002h; range 0.1–15 ng/mL), β -amyloid-42 (Catalog E1706h; range 1–60 pg/mL), BDNF (Catalog E0115h; range 15–2000 pg/mL), DKK1 (Catalog E1672h; range 31.2–2000 pg/mL), GFAP (Catalog E0186h; range 0.1–20 ng/mL), HMGB1 (Catalog E0722h; range 0.05–15 ng/mL),

NSE (Catalog E0999h; range 0.5–50 ng/mL), RSPO1 (Catalog E3921h; range 0.1–10 ng/mL), Sclerostin (Catalog E2845h; range 15.6–1000 pg/mL), and UCHL1 (Catalog E3156h; range 0.1–12 ng/mL). The inter- and intra-assay coefficients of variation for all ELISAs were below 10%. Each sample was run in duplicate, and the mean optical density was used to calculate concentration. All assays were performed according to the manufacturer's protocols, and samples with high analyte concentrations were diluted 1:5 with the provided diluent.

Statistical analysis

An a priori power analysis using G*Power 3.1.9.7 indicated that a total sample size of 134 participants would provide 90% power to detect a medium effect size ($f=0.28$) at an α level of 0.05. Data normality was assessed using the Kolmogorov-Smirnov test. Normally distributed continuous data were presented as mean $\pm$ standard deviation (SD) and compared using one-way analysis of variance (ANOVA). Non-normally distributed data are reported as medians (25th–75th percentiles) and were compared using the Mann-Whitney U test. Categorical variables were analyzed using chi-square (χ^2) tests.

Mediation and moderation were tested separately, not as a single moderated mediation model. First, moderation was tested using linear regression with an interaction term (PD status $\times$ biomarker) to determine whether the biomarker–fatigue relationship differed between PD patients and controls. Second, simple mediation was tested using PROCESS Macro (Version 4.2, Model 4; Hayes, 2022) with PD status as the independent variable, fatigue as the dependent variable, and all ten biomarkers entered as parallel mediators. Covariates (age, sex, BMI, exercise, TUD) were included in all models. The indirect effect of PD on fatigue via each biomarker was considered significant if the bias-corrected bootstrap 95% confidence interval (5,000 resamples) did not cross zero.

The significance of the indirect effects was determined using bias-corrected bootstrap confidence intervals (CIs) based on 5,000 bootstrap samples. An indirect effect was considered statistically significant if the 95% CI did not include zero. Variance inflation factors (VIFs) were calculated for all predictors in each mediation model. All VIFs were <2.5 (range 1.12–2.31), indicating no problematic multicollinearity. Bootstrapped standard errors (5,000 resamples) were used for all indirect effects. Confidence intervals were stable across repeated resampling. Residuals were approximately normal for all models, and Breusch-Pagan tests were non-significant ($p>0.05$ for all models). All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 28.0, with a two-tailed significance level of $p<0.05$.

Results

Sociodemographic and clinical data

The sociodemographic and clinical characteristics of the study participants are summarized in Table 1. No significant differences were observed between the PD patient group and the healthy control group in age, BMI, sex distribution, residency, physical activity levels, TUD, or marital status, indicating successful matching across these baseline variables. However, significant intergroup differences were identified in several key areas. Patients with PD had significantly lower levels of formal education ($p<0.05$). Employment status also differed markedly, with a significantly higher proportion of PD patients unemployed or retired, likely reflecting the disease’s functional impact. Furthermore, a positive family history of PD was significantly more prevalent in the patient group compared to controls ($p<0.05$). Clinically, the PD cohort exhibited moderate disease severity, as indicated by a mean Hoehn and Yahr stage of 2.77 ± 0.87 . Motor symptoms were quantified using the Unified Parkinson’s Disease Rating Scale (UPDRS). The mean score for Part III (Motor Examination) was 54.27 ± 11.38 , and for Part IV (Motor Complications) was 16.77 ± 4.11 , confirming substantial motor impairment. Regarding pharmacological management, 70.0% (63/90) of patients were on levodopa therapy. A subset of patients was also treated with other common antiparkinsonian agents, including procyclidine (Kemadrin; 27.8%, 25/90) and carbido-
pa-levodopa (Sinemet; 25.6%, 23/90).

Table 1. Demographic and clinical parameters in patients with PD and healthy controls*

Parameter	Healthy controls	PD Patients	F/ χ^2	df	p
Age (years)	61.311 $\pm$ 5.351	63.722 $\pm$ 13.154	1.392	1/133	0.240
Sex (female/male)	19/26	40/50	0.061	1	0.806
BMI (kg/m ²)	27.019 $\pm$ 2.27	26.797 $\pm$ 4.131	0.113	1/133	0.737
TUD, no/yes	31/14	72/18	2.046	1	0.152
Exercise, no/yes	32/13	70/20	0.722	1	0.369
Employment, no/yes	12/33	16/74	1.438	1	0.023
Rural/Urban	11/34	17/73	0.563	1	0.453
Family history, no/yes	44/1	61/29	15.921	1	<0.001
Age of onset (years)	–	58.811 $\pm$ 13.164	–	–	–
Total-FF	11.467 $\pm$ 2.634	40.289 $\pm$ 7.312	–	–	–
TOTAL-m-EDL	–	29.189 $\pm$ 7.55	–	–	–
TOTAL-PARTIII	–	54.267 $\pm$ 11.38	–	–	–
TOTAL-Part IV	–	16.767 $\pm$ 4.105	–	–	–
Hoehn & Yahr stage	–	2.772 $\pm$ 0.868	–	–	–
TOTAL-nM-EDL	–	29.111 $\pm$ 7.102	–	–	–
Levodopa, no/yes	–	27/63	–	–	–
Kemadrin, no/yes	–	65/25	–	–	–
Sinemet, no/yes	–	67/23	–	–	–

* BMI – body mass index, TUD – tobacco use disorder

Results of the mediation study

A multiple mediation analysis was conducted using Hayes’ PROCESS Macro (Version 4.2, Model 4) to

examine whether a panel of ten biomarkers mediated the relationship between PD status and FF score, while controlling for sex, TUD, exercise, age, and BMI. The analysis was performed on a sample of 135 participants.

Effects of PD on biomarker levels

A series of linear regression models was first estimated to examine the effect of PD status on each of the ten biomarkers. As summarized in Table 2, PD was a significant positive predictor of eight biomarkers. Specifically, patients with PD exhibited significantly elevated levels of NSE ($\beta=1.379$, $p<0.001$), DKK1 ($\beta=14.011$, $p<0.001$), HMGB1 ($\beta=1018.094$, $p=0.003$), GFAP ($\beta=0.798$, $p=0.002$), BDNF ($\beta=8.143$, $p=0.018$), β -Amyloid-42 ($\beta=9.942$, $p=0.048$), α -Synuclein ($\beta=5.620$, $p=0.033$), and UCHL1 ($\beta=0.545$, $p=0.012$). No significant effects were found for RSPO1 or sclerostin.

Mediation model for FF scores

A comprehensive regression model predicting FF score from PD status, all ten biomarkers, their interactions with disease status, and the covariates was highly significant ($F(26, 108)=54.08$, $p<0.001$), accounting for 92.9% of the variance in FF ($R^2=0.929$) as seen in Table 2. The test of highest-order unconditional interactions revealed significant moderation effects for two biomarkers. This high R^2 value indicates well-fitting model, and the significant F-test confirms that the overall model has strong predictive power. Specifically, the interactions between PD status and both HMGB1 (R^2 -change=0.005, $\beta=0.001$, $p=0.010$) and BDNF (R^2 -change=0.003, $\beta=0.114$, $p=0.033$) were statistically significant. The nature of these interactions is detailed in the conditional effects section below and visualized in Figure 1.

Table 2. Associations between Parkinson’s disease status (X) and circulating biomarker mediators (M) after controlling for age, BMI, exercise, sex, and TUD. This table shows the regression models with each biomarker as the outcome variable

Biomarker (M)	Effect of X on M (coefficient)	SE	t-value	p	R ²	Model p
NSE	1.379	0.209	6.603	<0.001	0.283	<0.001
DKK1	14.011	2.287	6.127	<0.001	0.255	<0.001
GFAP	0.798	0.251	3.173	0.002	0.108	0.022
HMGB1	1018.094	335.348	3.036	0.003	0.098	0.037
α -Synuclein	5.620	2.601	2.161	0.033	0.085	0.072
Sclerostin	-0.442	1.589	-0.278	0.781	0.080	0.095
BDNF	8.143	3.385	2.406	0.018	0.065	0.188
UCHL1	0.545	0.215	2.534	0.012	0.058	0.257
β -Amyloid-42	9.942	4.969	2.001	0.048	0.057	0.271
RSPO1	24.835	22.339	1.112	0.268	0.026	0.754

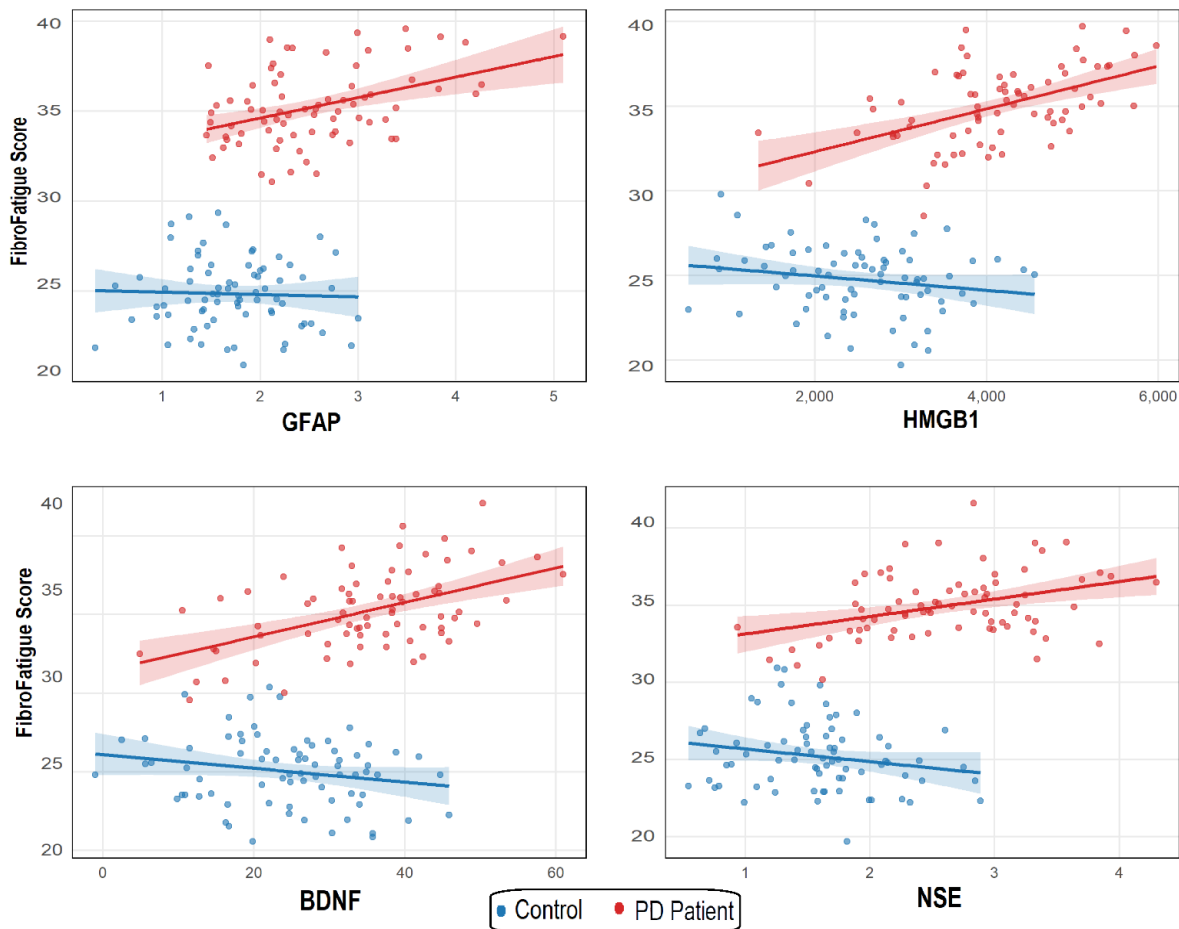


Fig. 1. Moderating effect of PD status on biomarker–fatigue relationships. Significant interactions ($p < 0.05$) were observed for HMGB1 and BDNF, with associations present only in PD patients. For GFAP and NSE, interactions were non-significant ($p > 0.05$), indicating no disease-specific moderation

Table 3. Interaction effects between Parkinson’s disease status and biomarkers on fatigue scores: results of simple regression analyses. The moderation effects represent the $X \times M$ interaction on FF score, X =Parkinson’s disease status, and M =proposed biomarker mediators (M); the conditional effect of M on Y in controls ($X=0$) is not significant*

Biomarker (M)	PD status $\times$ Biomarker interaction p-value	Effect of M on FF in PD patients (SE) [95% CI]	Effect of M on FF in controls (SE) [95% CI]	Significant interaction
HMGB1	0.01	0.001 (<0.001) [0.001-0.002]	-0.001 (0.001) [-0.003 to 0.001]	Yes
BDNF	0.033	0.113 (0.028) [0.057-0.169]	-0.001 (0.018) [-0.037 to 0.035]	Yes
GFAP	0.469	1.380 (0.332) [0.722-2.037]	0.915 (0.876) [-0.822 to 2.652]	No
NSE	0.264	1.263 (0.442) [0.387-2.140]	0.447 (0.654) [-0.849 to 1.743]	No
α -Synuclein	0.445	0.063 (0.033) [-0.002 to 0.128]	0.031 (0.040) [-0.049 to 0.111]	No

* Conditional effects are derived from regression models including all covariates (age, sex, BMI, exercise, TUD), a significant interaction indicates that the biomarker’s effect on fatigue differs between PD patients and controls

Conditional direct effects of biomarkers on FF scores
The interaction effects between PD status (X) and the biomarkers (M) on FF scores (Y) are presented in Table 3. A significant interaction (PD status $\times$ biomarker) was observed for HMGB1 ($p=0.010$) and BDNF ($p=0.033$), indicating that the associations between these two biomarkers and fatigue differ between PD patients and controls. The interactions were not significant for GFAP ($p=0.469$) and NSE ($p=0.264$), suggesting that their associations with fatigue do not significantly differ by PD status. Other biomarkers (α -synuclein, etc.) also showed non-significant interactions.

Direct, indirect, and total effects
The direct, indirect, and total effects of the biomarkers on the FF score in PD are presented in Table 4. The bootstrapped analysis of indirect effects revealed four significant mediation pathways through which PD status correlates with FF scores. The pure natural direct effect, accounting for all mediators, remained significant ($\beta=22.598$, $SE=1.114$, $p<0.001$, 95% CI [20.391–24.806]). The results in Table 4 present the bootstrap

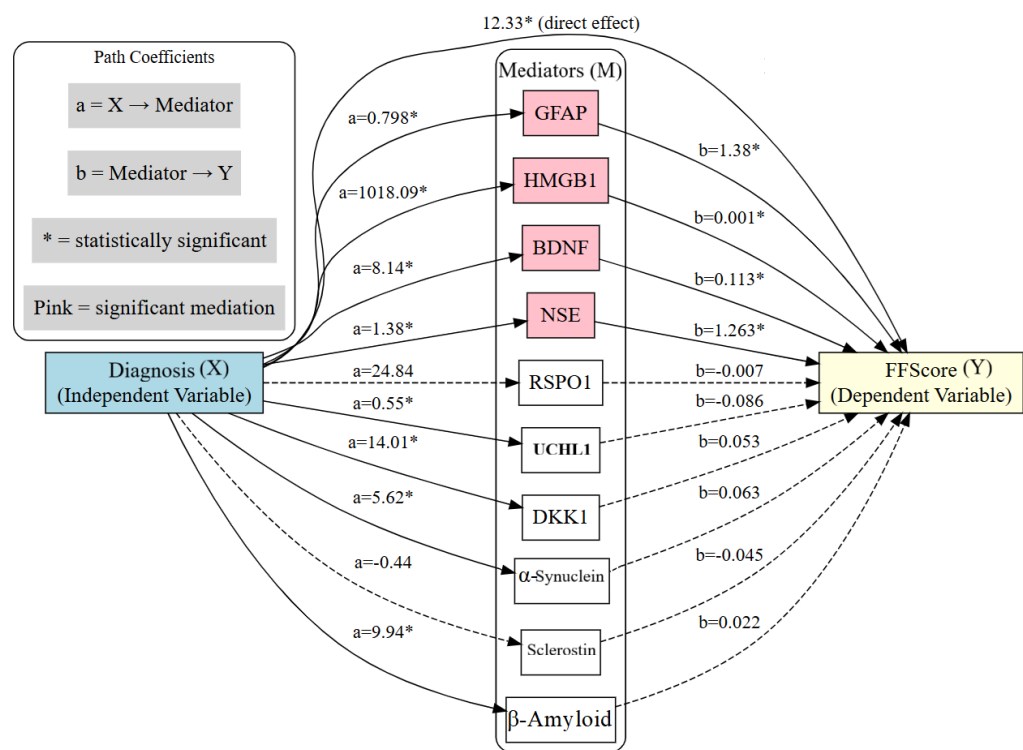


Fig. 2. Graphical diagram of the final model with key mediation and moderation pathways of fatigue in Parkinson's disease

confidence intervals for specific indirect effects, indicating significant mediation via four biomarkers.

Table 4. Bootstrapped estimates of indirect associations between Parkinson's disease status and fatigue scores via biomarkers*

Indirect pathway	Effect (Boot)	BootSE	BootLLCI	BootULCI	p
PD → NSE → FF	1.743	0.735	0.366	3.293	<0.05
PD → HMGB1 → FF	1.207	0.543	0.266	2.38	<0.05
PD → GFAP → FF	1.101	0.371	0.464	1.936	<0.05
PD → BDNF → FF	0.921	0.445	0.14	1.869	<0.05
PD → DKK1 → FF	0.738	0.502	-0.292	1.692	>0.05
PD → α -Synuclein → FF	0.355	0.252	-0.156	0.844	>0.05
PD → β -Amyloid-42 → FF	0.223	0.273	-0.191	0.88	>0.05
PD → Sclerostin → FF	0.02	0.14	-0.296	0.315	>0.05
PD → UCHL1 → FF	-0.047	0.196	-0.407	0.384	>0.05
PD → RSPO1 → FF	-0.164	0.186	-0.598	0.125	>0.05
Total indirect effect	6.095	-	-	-	-
Pure direct effect	22.598	1.114	20.391	24.806	<0.001
Total effect	28.693	0.939	26.828	30.524	<0.001

* Bootstrapped 95% confidence intervals (5,000 resamples) that do not cross zero indicate significant indirect effects, models controlled for age, sex, BMI, exercise, and TUD, LLCI – lower level confidence interval, ULCI – upper level confidence interval

A statistically significant indirect effect of PD on fatigue was observed through elevated levels of NSE (Effect=1.743, 95% BootCI [0.366-3.293]), HMGB1 (Effect=1.207, 95% BootCI [0.266-2.380]), GFAP (Effect=1.101, 95% BootCI [0.464-1.936]), and BDNF (Ef-

fect=0.921, 95% BootCI [0.140-1.869]). The indirect effects through the remaining biomarkers were not statistically significant. The direct and indirect effects of diagnosis (presence of PD) on functional score through blood biomarkers are presented in Figure 2. The total effect of PD on FF was significant ($b=28.693$, BootSE=0.939, 95% BootCI [26.828, 30.524]).

Discussion

The primary finding of this exploratory cross-sectional mediation analysis is that GFAP, HMGB1, BDNF, and NSE each show significant indirect (mediated) effects from PD status to fatigue. Moderation analysis revealed that the biomarker-fatigue relationship was disease-specific (i.e., differed significantly between PD patients and controls) only for HMGB1 and BDNF, suggesting that the PD pathophysiological environment may be required for their association with fatigue. For GFAP and NSE, the non-significant interactions suggest that their mediating roles did not differ significantly between groups. These findings are associational only; causality cannot be inferred from this cross-sectional design.

The indirect effects observed through GFAP, HMGB1, BDNF, and NSE are statistically consistent with the hypothesis that dysregulation within these molecular pathways may be associated with fatigue severity in PD. However, cross-sectional mediation does not establish causation. Interestingly, Table 3 shows that only HMGB1 and BDNF had significant interactions with PD status, meaning their relationships with fatigue dif-

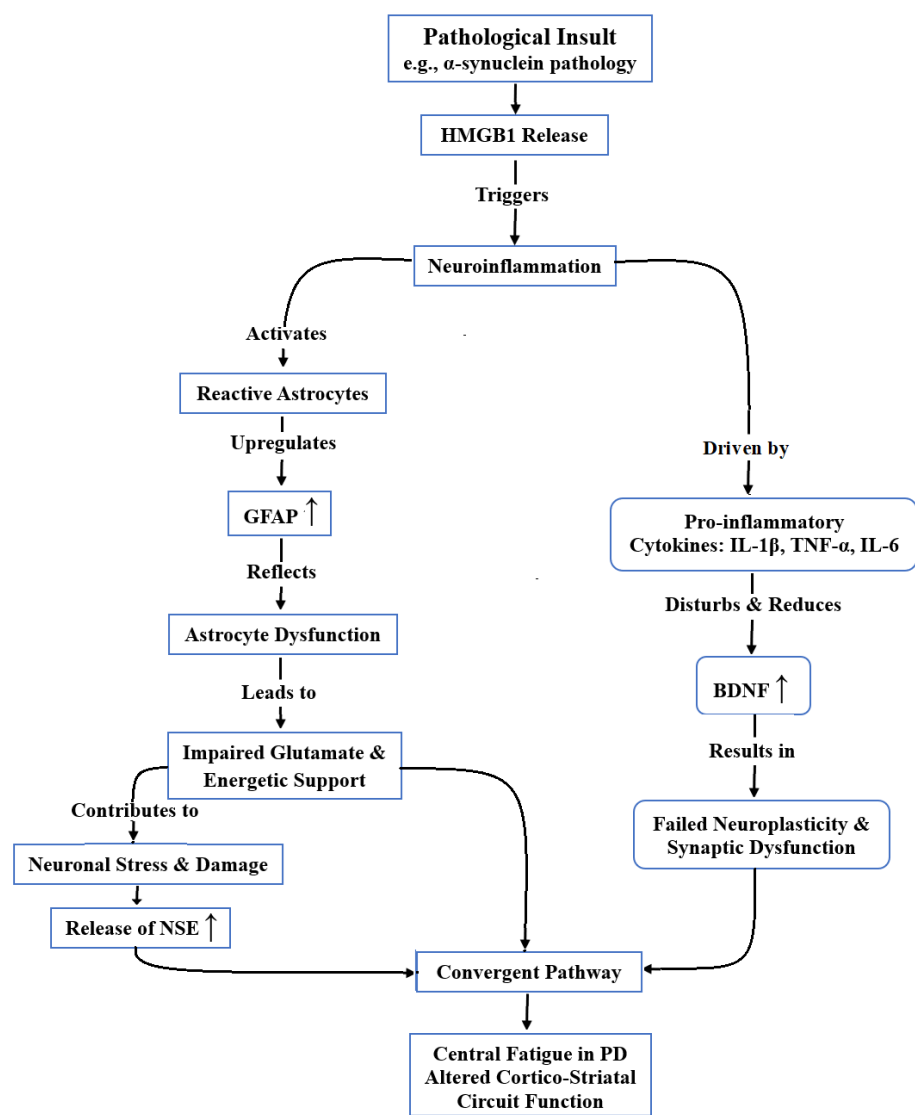


Fig. 3. Hypothetical integrative pathway linking GFAP, HMGB1, BDNF, and NSE to fatigue in Parkinson's disease

fer between PD patients and controls. GFAP, a structural protein released during astrocytic activation and reactive gliosis,^{31,32} was significantly elevated in PD patients compared to controls and statistically mediated the PD-fatigue association. Previous studies reported that serum GFAP levels are elevated in PD and correlate with cognitive decline,^{24,33} indicating astrocytic remodeling as a response to neurodegeneration.^{33,34} The present results extend these observations by suggesting that astroglial activation may also be statistically linked to non-motor symptoms, specifically fatigue.

HMGB1 plays a central role in orchestrating inflammatory cascades upon release, driving microglial activation and progressive neurodegeneration, thereby establishing a feed-forward cycle of damage.^{35,36} The role of HMGB1, a significant damage-associated molecular pattern (DAMP) and key regulator of neuroinflammation, highlights the essential involvement of innate immune activation in this process.^{35,37} Elevated HMGB1 levels were associated with higher fatigue scores only among PD patients (condition-

al $\beta=0.001$, $p=0.001$). This aligns with previous work linking HMGB1 to chronic neuroinflammation in PD,^{15,35} and extends it by associating HMGB1 with a specific non-motor symptom. HMGB1-induced microglial activation and mitochondrial dysfunction could, in theory, be associated with a perceived energy deficit, a core feature of fatigue.³⁸ Nevertheless, serum HMGB1 may also originate from peripheral sources.

NSE was elevated in PD and showed a significant indirect (mediated) effect on fatigue (Table 4). However, the PD status $\times$ NSE interaction was not significant ($p=0.264$, Table 3), indicating that the association between NSE and fatigue did not differ significantly between PD patients and controls. Elevated NSE is well documented as a marker of acute or ongoing neuronal injury in various neurological disorders.^{20,32} Thus, NSE may reflect a general fatigue mechanism rather than a PD-specific pathway.

BDNF, typically considered neuroprotective and supportive of dopaminergic neuron survival,³⁹ was un-

expectedly elevated in PD patients and positively associated with fatigue severity, with a conditional effect present only in the PD group ($\beta=0.113$, $p<0.001$). This finding appears paradoxical, given BDNF's established role in neuronal health.^{23,39} Several speculative explanations exist, none of which can be confirmed by the present data. First, serum BDNF may reflect peripheral (not central) sources and could be influenced by medication effects, disease stage, or comorbid conditions, none of which were fully controlled in this exploratory analysis. Second, if the finding is replicable, speculative explanations could include a maladaptive compensatory response or context-dependent signaling in inflamed neural tissue. However, longitudinal studies measuring central and peripheral BDNF are needed before any mechanistic conclusions can be drawn.

The overall findings are consistent with a hypothesized model in which central fatigue in PD may be related to neuroinflammation and neuronal-astroglial dysfunction. However, causality is not established. Figure 3 illustrates the hypothesized integrative pathway of GFAP, HMGB1, BDNF, and NSE in PD fatigue. This cascade is proposed to disrupt critical brain regions involved in motivation, reward, and the perception of effort, including the basal ganglia and prefrontal cortex.

While α -synuclein, β -amyloid-42, UCHL1, RSPO1, DKK1, and sclerostin may be involved in PD pathogenesis or progression, they did not statistically mediate the relationship between PD and fatigue in our model. This dissociation suggests that different non-motor symptoms may be associated with distinct biomarker profiles. For example, β -amyloid-42 has been linked to dopa-resistant gait disturbances and oxidative stress, rather than fatigue.^{40,41} Similarly, Wnt pathway modulators (DKK1, RSPO1, sclerostin) may be more relevant to neuronal survival and motor progression than to fatigue perception. These null findings should be interpreted cautiously, given the sample size and the exploratory nature of the analysis.

Study limitations

Several limitations should be acknowledged. First, the cross-sectional design precludes conclusions about causality, directionality, reverse causation, or unmeasured confounding. Second, biomarkers were measured in blood, not cerebrospinal fluid or brain tissue, and we lack neuroimaging to confirm central origin. Third, the FF scale does not fully distinguish fatigue from depression, apathy, sleep disturbances, or medication effects, and residual confounding cannot be excluded. Fourth, the case-control design and single-country sample (Iraq) limit generalizability. Finally, although Hoehn and Yahr stage was recorded, it was not included in the mediation model; future studies should examine whether disease stage modifies these effects.

Conclusion

The results of the present study are consistent with a statistical mediation model in which PD is associated with fatigue indirectly through elevated levels of specific biomarkers of neuroinflammation (HMGB1), astroglial activation (GFAP), neuronal injury (NSE), and a paradoxical BDNF response. However, causality cannot be established from this cross-sectional design. The disease-specific moderation effects (biomarkers predicting fatigue only in PD for HMGB1 and BDNF) generate testable hypotheses for future longitudinal and mechanistic studies. These findings identify candidate biomarkers that warrant further investigation in independent cohorts.

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Declarations

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Author contributions

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

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The datasets used during the current study are available from the corresponding author upon reasonable request

Ethics approval

The study was approved by the Institutional Ethics Committee of the University of Kufa (MEC-110/2025).

Use of AI and AI-assisted technologies in the writing process

No artificial intelligence (AI) tools or AI-assisted technologies were used in the writing, editing, or preparation of this manuscript.

References

- Mhyre TR, Boyd JT, Hamill RW, Maguire-Zeiss KA. Parkinson's disease. *Subcell Biochem.* 2012;65:389-455. doi:10.1007/978-94-007-5416-4_16
- Tong J. The pathogenesis and treatment of Parkinson's disease. *Theoretical and Natural Science.* 2024;47:15-19. doi:10.54254/2753-8818/47/2024PJ0101
- Tarakad A. Motor features of Parkinson's disease. *Neurol Clin.* 2025;43(2):279-289. doi:10.1016/j.ncl.2024.12.007
- Heo JY, Nam MH, Yoon HH, et al. Aberrant tonic inhibition of dopaminergic neuronal activity causes motor symptoms in animal models of Parkinson's disease. *Curr Biol.* 2020;30(2):276-291.e279. doi:10.1016/j.cub.2019.11.079
- Peña-Zelayeta L, Delgado-Minjares KM, Villegas-Rojas MM, et al. Redefining non-motor symptoms in Parkinson's disease. *J Pers Med.* 2025;15(5):172. doi:10.3390/jpm15050172
- Müller B, Assmus J, Herlofson K, Larsen JP, Tysnes OB. Importance of motor vs. non-motor symptoms for health-related quality of life in early Parkinson's disease. *Parkinsonism Relat Disord.* 2013;19(11):1027-1032. doi:10.1016/j.parkreldis.2013.07.010
- Siciliano M, Trojano L, Santangelo G, De Micco R, Tedeschi G, Tessitore A. Fatigue in Parkinson's disease: a systematic review and meta-analysis. *Mov Disord.* 2018;33(11):1712-1723. doi:10.1002/mds.27461
- Arenas E, Saltó C, Villaescusa C. WNT signaling in midbrain dopaminergic neuron development and cell replacement therapies for Parkinson's disease. *Springer Plus.* 2015;4(1):L49. doi:10.1186/2193-1801-4-S1-L49
- Dai TL, Zhang C, Peng F, et al. Depletion of canonical Wnt signaling components has a neuroprotective effect on midbrain dopaminergic neurons in an MPTP-induced mouse model of Parkinson's disease. *Exp Ther Med.* 2014;8(2):384-390. doi:10.3892/etm.2014.1745
- Vallée A, Vallée JN, Lecarpentier Y. Potential role of cannabidiol in Parkinson's disease by targeting the WNT/ β -catenin pathway, oxidative stress and inflammation. *Aging.* 2021;13(7):10796-10813. doi:10.18632/aging.202951
- Marchetti B, Tirolo C, L'Episcopo F, et al. Parkinson's disease, aging and adult neurogenesis: Wnt/ β -catenin signalling as the key to unlock the mystery of endogenous brain repair. *Aging cell.* 2020;19(3):e13101. doi:10.1111/accel.13101
- Jacobs BM, Belete D, Bestwick J, et al. Parkinson's disease determinants, prediction and gene-environment interactions in the UK Biobank. *J Neurol Neurosurg Psychiatry.* 2020;91(10):1046-1054. doi:10.1136/jnnp-2020-323646
- Huang Y, Liu L, Liu A. Dickkopf-1: Current knowledge and related diseases. *Life Sci.* 2018;209:249-254. doi:10.1016/j.lfs.2018.08.019
- Drake MT, Khosla S. Hormonal and systemic regulation of sclerostin. *Bone.* 2017;96:8-17. doi:10.1016/j.bone.2016.12.004
- Angelopoulou E, Piperi C, Papavassiliou AG. High-mobility group box 1 in Parkinson's disease: from pathogenesis to therapeutic approaches. *J Neurochem.* 2018;146(3):211-218. doi:10.1111/jnc.14450
- Liang K, Li X, Guo Q, et al. Structural changes in the retina and serum HMGB1 levels are associated with decreased cognitive function in patients with Parkinson's disease. *Neurobiol Dis.* 2024;190:106379. doi:10.1016/j.nbd.2023.106379
- Anand AA, Khan M, V M, Kar D. The molecular basis of Wnt/ β -Catenin signaling pathways in neurodegenerative diseases. *Int J Cell Biol.* 2023;2023:9296092. doi:10.1155/2023/9296092
- Das S, Ramteke H. A Comprehensive review of the role of biomarkers in early diagnosis of Parkinson's disease. *Cureus.* 2024;16(2):e54337. doi:10.7759/cureus.54337
- Soni R, Mathur K, Shah J. An update on new-age potential biomarkers for Parkinson's disease. *Ageing Res Rev.* 2024;94:102208. doi:10.1016/j.arr.2024.102208
- Han Y, Wang Y, Zou X, Guo H. The serum proteomic profile in patients with migraine. *Front Mol Neurosci.* 2025;18:1460403. doi:10.3389/fnmol.2025.1460403
- Al-Hakeim HK, Khudhair HN, Ranaei-Siadat SO, et al. Affective and chronic fatigue symptoms are associated with serum neuronal damage markers in Parkinson's disease. *Sci Rep.* 2025;15(1):20647. doi:10.1038/s41598-025-07735-7
- Wolf D, Ayon-Olivas M, Sendtner M. BDNF-Regulated modulation of striatal circuits and implications for Parkinson's disease and dystonia. *Biomedicines.* 2024;12(8). doi:10.3390/biomedicines12081761
- Ali NH, Al-Kuraishy HM, Al-Gareeb AI, et al. BDNF/TrkB activators in Parkinson's disease: a new therapeutic strategy. *J Cell Mol Med.* 2024;28(10):e18368. doi:10.1111/jcmm.18368
- Lin J, Ou R, Li C, et al. Plasma glial fibrillary acidic protein as a biomarker of disease progression in Parkinson's disease: a prospective cohort study. *BMC Med.* 2023;21(1):420. doi:10.1186/s12916-023-03120-1
- Liddel SA, Barres BA. Reactive astrocytes: production, function, and therapeutic potential. *Immunity.* 2017;46(6):957-967. doi:10.1016/j.immuni.2017.06.006
- Postuma RB, Poewe W, Litvan I, et al. Validation of the MDS clinical diagnostic criteria for Parkinson's disease. *Mov Disord.* 2018;33(10):1601-1608. doi:10.1002/mds.27362
- Al-Hakeim HK, Al-Rammahi DA, Al-Dujaili AH. IL-6, IL-18, sIL-2R, and TNF α proinflammatory markers in depression and schizophrenia patients who are free of overt inflammation. *J Affect Disord.* 2015;182:106-114. doi:10.1016/j.jad.2015.04.044
- Goetz CG, Tilley BC, Shaftman SR, et al. Movement disorder society-sponsored revision of the unified Parkinson's disease rating scale (MDS-UPDRS): scale presentation and clinimetric testing results. *Mov Disord.* 2008;23(15):2129-2170. doi:10.1002/mds.22340

29. Hoehn MM, Yahr MD. Parkinsonism: onset, progression and mortality. *Neurology*. 1967;17(5):427-442. doi: 10.1212/wnl.17.5.427
30. Zachrisson O, Regland B, Jahreskog M, Kron M, Gottfries CG. A rating scale for fibromyalgia and chronic fatigue syndrome (the FibroFatigue scale). *J Psychosom Res*. 2002;52(6):501-509. doi:10.1016/s0022-3999(01)00315-4
31. Gogishvili D, Honey MIJ, Verberk IMW, et al. The GFAP proteoform puzzle: how to advance GFAP as a fluid biomarker in neurological diseases. *J Neurochem*. 2025;169(1):e16226. doi:10.1111/jnc.16226
32. Godeiro Coelho LM, Teixeira FJP, Koru-Sengul T, et al. Predictive value of nervous cell injury biomarkers in moderate-to-severe traumatic brain injury: a network meta-analysis. *Neurology*. 2025;105(5):e213997. doi:10.1212/wnl.00000000000213997
33. Tang Y, Han L, Li S, et al. Plasma GFAP in Parkinson's disease with cognitive impairment and its potential to predict conversion to dementia. *NPJ Parkinsons Dis*. 2023;9(1):23. doi:10.1038/s41531-023-00447-7
34. Su W, Chen HB, Li SH, Wu DY. Correlational study of the serum levels of the glial fibrillary acidic protein and neurofilament proteins in Parkinson's disease patients. *Clin Neurol Neurosurg*. 2012;114(4):372-375. doi:10.1016/j.clin-neuro.2011.11.002
35. Gao HM, Zhou H, Zhang F, Wilson BC, Kam W, Hong JS. HMGB1 acts on microglia Mac1 to mediate chronic neuroinflammation that drives progressive neurodegeneration. *J Neurosci*. 2011;31(3):1081-1092. doi:10.1523/jneurosci.3732-10.2011
36. Li J, Wu N, Xiao Y, Xia Y. High mobility group box 1 and its post-translational modifications: Molecular mechanisms underlying neurodegenerative disease pathogenesis. *Neural Regen Res*. 2025. doi:10.4103/nrr.Nrr-d-25-01091
37. Razali K, Mohamed WMY. High-mobility group box 1 (HMGB1) protein in Parkinson's disease research: a 10-year bibliometric analysis. *J Integr Neurosci*. 2023;22(4):87. doi:10.31083/j.jin2204087
38. Goyal A, Agrawal A, Dubey N, Verma A. High mobility group box 1 protein: a plausible therapeutic molecular target in Parkinson's disease. *Curr Pharm Biotechnol*. 2024;25(8):937-943. doi:10.2174/1389201025666230905092218
39. Palasz E, Wysocka A, Gasiorowska A, Chalimoniuk M, Niewiadomski W, Niewiadomska G. BDNF as a promising therapeutic agent in Parkinson's disease. *Int J Mol Sci*. 2020;21(3). doi:10.3390/ijms21031170
40. Rochester L, Galna B, Lord S, et al. Decrease in A β 42 predicts dopa-resistant gait progression in early Parkinson disease. *Neurology*. 2017;88(16):1501-1511. doi:10.1212/wnl.00000000000003840
41. Butterfield DA, Perluigi M, Sultana R. Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics. *Eur J Pharmacol*. 2006;545(1):39-50. doi:10.1016/j.ejphar.2006.06.026



ORIGINAL PAPER

Neurofilament light chain and nerve growth factor as biomarkers of axonal injury and neurotrophic dysfunction in diabetic neuropathy

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ABSTRACT

Introduction and aim. Diabetic peripheral neuropathy (DPN) is the most common complication of diabetes and is often diagnosed at an advanced stage when nerve damage becomes irreversible. Neurofilament light chain (NfL), a neuroaxonal injury marker, and nerve growth factor (NGF), a neurotrophin, are potential biomarkers for evaluating axonal damage and neurotrophic support in DPN. This study aimed to evaluate the efficacy of serum NfL and NGF as concurrent biomarkers of axonal injury and impaired neurotrophic support in a single cohort of patients with DPN, exploring their relationship with clinical and electrophysiological parameters.

Material and methods. A case-control study was conducted, including 30 patients with type 2 diabetes mellitus (T2DM) and DPN, 30 patients with T2DM without neuropathy (diabetic controls), and 60 healthy control subjects. Serum NfL and NGF concentrations were measured by enzyme-linked immunosorbent assay (ELISA). The primary outcome was the receiver operating characteristic (ROC) performance of serum NfL for discriminating patients with DPN from healthy controls.

Results. Serum NfL levels were significantly elevated in patients with DPN (13.9 ± 1.20 ng/mL) compared with diabetic controls (9.83 ± 2.08 ng/mL) and healthy controls (5.60 ± 2.53 ng/mL) ($p < 0.0001$). Conversely, NGF levels were significantly lower in both diabetic groups than in healthy controls ($p < 0.0001$). In this case-control sample, ROC analysis showed that serum NfL discriminated against DPN from healthy controls with an area under the curve (AUC) of 0.994 (sensitivity 96.6%, specificity 100.0%), though this exceptionally high performance reflects discrimination against healthy individuals rather than diabetic controls.

Conclusion. Serum NfL and NGF represent promising candidate biomarkers of axonal injury and impaired neurotrophic support in DPN, respectively, and were correlated with glycemic control and disease duration in this cohort. However, given the small, single-center, case-control design, further validation in larger, longitudinal, and duration-matched cohorts is required before clinical implementation can be considered.

Keywords. diabetic peripheral neuropathy, nerve growth factor, neurofilament light chain

Introduction

Diabetes mellitus is a growing global health problem. According to the International Diabetes Federation, 589 million adults were living with diabetes in 2024, and this burden is expected to increase further.¹ Diabetic peripheral neuropathy (DPN) is among the most com-

mon chronic complications of diabetes mellitus, causing pain, sensory loss, and substantial impairment in quality of life.² DPN arises from chronic hyperglycemia, oxidative stress, and neuroinflammation, which together drive progressive nerve injury, demyelination, and ultimately neuronal apoptosis.^{3,4}

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A critical issue in the clinical management of DPN is its often-delayed diagnosis, which typically occurs at an advanced stage when significant and irreversible nerve damage has already occurred.^{5,6} This diagnostic latency underscores the urgent need for sensitive and specific biomarkers that can facilitate early detection and intervention in this patient population. In this context, two molecular candidates have garnered considerable attention: neurofilament light chain (NfL) and nerve growth factor (NGF). NfL is a 68-kDa structural protein that is an integral component of the neuronal cytoskeleton and is essential for maintaining axonal caliber and structural integrity.⁷ Upon neuroaxonal injury, NfL is released from damaged neurons into the extracellular space and subsequently into the peripheral bloodstream, where its concentration reflects the magnitude of ongoing axonal damage. Elevated circulating NfL levels have been consistently demonstrated across a wide range of neurodegenerative and peripheral nerve disorders, and emerging evidence supports its role as a dynamic and sensitive biomarker for DPN.^{8,9}

Conversely, nerve growth factor is a member of the neurotrophin family and is indispensable for the development, survival, differentiation, and long-term maintenance of peripheral sensory and sympathetic neurons. Its biological effects are mediated primarily through the high-affinity tropomyosin receptor kinase A (TrkA), which promotes neuronal survival and axonal regeneration. A growing body of evidence indicates that a neurotrophic deficit, characterized by reduced NGF synthesis and impaired TrkA signaling, contributes to DPN and renders neurons increasingly vulnerable to hyperglycemia-induced injury.^{10,11} While previous studies have independently investigated NfL and NGF in diabetic neuropathy, research evaluating both markers in the same cohort remains limited. Their ability to distinguish diabetic participants with neuropathy from those without neuropathy, and their relationships with glycemic exposure and disease duration, therefore merit further investigation.

Aim

Accordingly, this study aimed to evaluate serum NfL and NGF as individual and concurrent biomarkers of axonal injury and neurotrophic dysfunction in DPN, and to explore their associations with clinical and electrophysiological parameters. The primary novelty of this study lies in the simultaneous assessment of both serum NfL and NGF within the same cohort of patients with DPN, which allows for a direct comparison of axonal damage and neurotrophic support. However, this novelty statement is made cautiously, as individual assessments of these biomarkers have been reported previously. The analysis of DPN severity was considered exploratory.

Material and methods

Study design and ethical considerations

This case-control study was conducted at Al-Imameen Al-Kadhimayn Medical City in Baghdad, Iraq, over a duration of 5 months, from September 2025 to January 2026. The study protocol was developed in accordance with ethical principles for medical research involving human subjects as outlined by the Declaration of Helsinki. Ethical approval was obtained from the Institutional Review Board of the College of Medicine, Babylon University, Iraq (Approval Number: MB5-69, date: 18/9/2025). Participants were given a full explanation of the study purposes and procedures, and written informed consent was obtained prior to study enrollment.

Participant recruitment and group allocation

A total of 120 participants aged 41–77 years were recruited for this study. The sample size was determined based on previous literature evaluating biomarker variations in DPN, ensuring sufficient power (80%) to detect a significant difference at an alpha level of 0.05. The principal analytic focus was the discrimination of participants with DPN from healthy controls, whereas severity subgroup comparisons were planned as exploratory analyses. Potential confounding factors such as age, metabolic status (BMI), and renal function were considered during patient selection and data interpretation, as these variables may influence circulating NfL and NGF levels. Participants were allocated to one of three distinct cohorts:

- Group 1 (DPN patients, n=30): This group comprised patients with a pre-existing diagnosis of type 2 diabetes mellitus and a clinically and electrophysiologically confirmed diagnosis of diabetic peripheral neuropathy.
- Group 2 (diabetic controls, n=30): This group comprised patients with a diagnosis of type 2 diabetes mellitus but with no clinical or electrophysiological evidence of peripheral neuropathy.
- Group 3 (healthy controls, n=60): This group included apparently healthy individuals with no history of diabetes, neurological disorders, or other chronic illnesses that could potentially interfere with the study's endpoints.

The inclusion criteria for the diabetic groups were a confirmed diagnosis of type 2 diabetes mellitus. The exclusion criteria for all groups included a history of significant renal or hepatic disease, active malignancy, acute inflammatory conditions, or other known causes of peripheral neuropathy.

Clinical and demographic data collection

A standardized case report form was used to collect detailed information from all participants. This included demographic data (age and sex), comprehensive med-

ical history, and duration of diabetes for participants in Groups 1 and 2. Anthropometric measurements, including height and weight, were used to calculate the Body Mass Index (BMI) for each participant, quantified as kg/m^2 .¹²

Assessment of peripheral neuropathy

All participants in the diabetic cohorts (groups 1 and 2) underwent comprehensive nerve conduction studies (NCS) to objectively assess the functional status of their peripheral nerves. The examinations were performed by an experienced neurophysiologist using a Nihon Kohden electrophysiology system (Nihon Kohden, Japan). All studies were conducted under standardized, temperature-controlled conditions to ensure the reliability and consistency of the results. The NCS protocol included the assessment of both sensory and motor nerves to confirm the diagnosis of DPN and grade its severity. The severity of DPN was stratified into mild, moderate, or severe categories based on a standardized composite score derived from NCS parameters. Mild DPN was defined as a reduction in sensory nerve action potential (SNAP) amplitude ($<50\%$ of the lower limit of normal, LLN) in at least two sensory nerves (sural or superficial peroneal) with normal motor conduction. Moderate DPN was defined as abnormal sensory conduction combined with mild-to-moderate reductions in motor conduction velocity ($<10\%$ below LLN) or compound muscle action potential (CMAP) amplitudes in the common peroneal or tibial nerves. Severe DPN was characterized by the complete absence of recordable SNAPs in the lower extremities, accompanied by severe motor conduction velocity slowing ($>20\%$ below LLN) or a marked reduction in CMAP amplitudes ($<50\%$ of LLN).¹³

Biochemical analyses

Sample collection and processing

Fasting venous blood samples were collected from all participants in the morning. Samples were harvested in serum separator tubes and allowed to clot at room temperature. After that, the samples were centrifuged at 3000 rpm for 15 min to obtain the serum. Further, the serum obtained was aliquoted into cryovials and stored at -80°C until analysis to maintain stability of all biomarkers.

Immunoassays

Serum levels of NfL and NGF were measured using commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (Bioassay Technology Laboratory, Shanghai, China; lot no of NfL: E4467Hu and NGF E2102Hu), strictly adhering to the manufacturer's instructions. The assay procedure was as follows: 1) Standards and samples were prepared and diluted as recommended by manufacturer; 2) 50 μL of standard or sample was added to the appropriate wells; 3) 50 μL

of biotinylated antibody was added to each well and incubated; 4) Wells were washed, and streptavidin-HRP was added; 5) After further washing, substrate solution was added for color development; 6) The reaction was stopped, and optical density was measured at 450 nm. All samples were measured in duplicate to ensure accuracy. The analytical sensitivity for the NfL assay was 0.054 ng/mL with a standard curve range of 0.1–35 ng/mL. The analytical sensitivity for the NGF assay was 3.48 pg/mL with a detection range 7–1500 pg/mL. For both assays, the intra-assay coefficient of variation (CV) was $<8\%$, and the inter-assay CV was $<10\%$.

Statistical analysis

Statistical analysis of the data obtained was performed using the SPSS software package (version 26.0, IBM, Armonk, NY, USA). Summary statistics of quantitative variables are expressed as mean $\pm$ standard deviation (SD). Differences between the three study groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. For categorical variables, numbers and percentages are shown, and comparisons between groups were made with the chi-square (χ^2) test. The prespecified primary outcome was the area under the receiver operating characteristic (ROC) curve for serum NfL in discriminating participants with DPN from healthy controls. Secondary analyses included between-group comparisons for NfL and NGF, bivariate correlations, and exploratory comparisons according to DPN severity. ROC curve analysis was used to evaluate diagnostic accuracy, and the AUC, optimal cut-off values, sensitivity, and specificity were calculated from the ROC curves. No multivariable regression model or combined biomarker ROC model was generated in the present dataset. Statistical significance was defined as $p < 0.05$.

Results

Demographic and clinical data of the 120 participants are described in Table 1. The mean glycated hemoglobin (HbA1c) level was significantly higher in patients with DPN compared to those without neuropathy ($8.33 \pm 2.36\%$ vs. $6.71 \pm 1.13\%$; $p < 0.0001$). The duration of diabetes was significantly longer in the DPN group compared to the non-DPN group (16.9 ± 6.77 vs. 2.5 ± 1.35 years; $p = 0.003$). A significant difference in glycemic control (HbA1c) was observed among the three groups ($p < 0.0001$), with the highest HbA1c level found in patients with DPN ($8.33 \pm 2.36\%$). Diabetic cohorts demonstrated significantly elevated levels of NfL concentrations compared to healthy controls ($p < 0.001$), and patients with DPN had the highest mean level at 13.9 ± 1.20 ng/mL, which was significantly higher than in diabetic controls (9.83 ± 2.08 ng/mL, $p < 0.001$). On the other hand, NGF concentrations were markedly de-

creased in both diabetic groups compared to healthy controls ($p<0.0001$) and especially low in diabetic patients with neuropathy (202.6 ± 21.3 pg/mL), although the difference between DPN patients and diabetic controls did not reach statistical significance ($p>0.05$).

Table 1. Demographic characteristics of the population under study*

Characteristic	Diabetes patients with neuropathy (n=30)	Diabetes patients without neuropathy (n=30)	Healthy control (n=60)	p
Age (years)				
Mean±SD	56.66±6.67	53.26±8.63	54.69±8.78	0.086
Range	(42.0–70.0)	(43.0–73.0)	(41.0–77.0)	
Sex				
Male, n (%)	20 (66.7%)	18 (60.0%)	30 (50.0%)	0.1128
Female, n (%)	10 (33.3%)	12 (40.0%)	30 (50.0%)	
Duration of disease (years)				
Mean±SD	16.9±6.77	2.5±1.35	—	0.003
Severity of disease				
Mild, n (%)	10 (33.3%)	—	—	N/A
Moderate, n (%)	11 (36.7%)	—	—	
Severe, n (%)	9 (30.0%)	—	—	
HbA1c (%)				
Mean±SD	8.33±2.36 ^A	6.71±1.13 ^B	5.14±0.53 ^B	<0.0001
NfL (ng/mL)				
Mean±SD	13.9±1.20 ^A	9.83±2.08 ^B	5.60±2.53 ^C	<0.0001
NGF (pg/mL)				
Mean±SD	202.6±21.3 ^A	212.3±26.2 ^A	311.2±32.2 ^B	<0.0001

* n – number of cases, capital letters A, B and C indicate the level of significance following Tukey’s multiple comparisons test; similar letters indicate no significant difference, whereas different letters indicate significant difference

A statistically significant positive correlation was observed between HbA1c and serum NfL levels ($r=0.574$, $p<0.001$) and a significant negative correlation was found between HbA1c and serum NGF levels ($r=-0.570$, $p<0.001$). Serum NfL was positively correlated with diabetes duration ($r=0.733$, $p<0.001$). NGF demonstrated a strong inverse correlation with NfL ($r=-0.613$, $p<0.001$). The correlation of NGF with diabetes duration was not statistically significant ($r=0.059$, $p=0.667$).

Table 2. Correlation matrix of investigated biomarkers and clinical variables

Variables	AGE	BMI	Duration	HbA1c	NfL	NGF
NfL	R	0.221	-0.15	0.733	0.574	1
	p	0.046	0.16	<0.001	<0.001	<0.001
	n	60	60	60	60	60
NGF	R	-0.071	0.10	0.059	-0.570	-0.613
	p	0.524	0.37	0.667	<0.001	<0.001
	n	60	60	60	60	60

The ROC curve analysis results are presented in Table 3. Both biomarkers showed good discrimination between diabetic groups and healthy controls. NfL demonstrated an AUC of 0.886, with a sensitivity of 73.1% and specificity of 99.1%, at a cut-off value of >5.93 ng/mL in distinguishing diabetic patients without neuropathy from control subjects ($p<0.001$). For differentiating DPN patients from healthy controls, NfL demonstrated an AUC of 0.994 with 96.6% sensitivity and 100% specificity at a cut-off value of >11.9 ng/mL. NGF also showed good discriminative power, with an AUC of 0.85 ($p<0.001$) for differentiating diabetic subjects without neuropathy from healthy controls (cut-off: ≤226.2 pg/mL, sensitivity: 92.3%, specificity: 88.5%) and an AUC of 0.87 ($p<0.001$) for differentiating DPN patients from healthy controls (cut-off: ≤227.8 pg/mL, sensitivity: 96.7%, specificity: 88.0%). Importantly, to address the more clinically relevant distinction, we performed an exploratory ROC analysis comparing patients with DPN directly against diabetic controls (T2DM without neuropathy). For this comparison, serum NfL demonstrated a high discriminative capacity with an AUC of 0.892 (95% CI: 0.79–0.99, $p<0.001$) at an optimal cut-off of >11.8 ng/mL, yielding a sensitivity of 90.0% and a specificity of 83.3%. In contrast, serum NGF exhibited poor discriminative power between the two diabetic cohorts, with an AUC of 0.584 (95% CI: 0.43–0.74, $p=0.26$) at a cut-off of ≤208.5 pg/mL (sensitivity: 63.3%, specificity: 56.7%), which reflects the substantial overlap in NGF levels between diabetic patients with and without neuropathy.

Table 3. Diagnostic performance of NfL and NGF

Marker	Comparison Groups	Cut-off	Sensitivity	Specificity	AUC (95% CI)	p
NfL (ng/mL)	DM without neuropathy vs healthy control	>5.93	73.1 %	99.1%	0.886 (0.78–0.98)	<0.001
	DM with neuropathy vs healthy control	>11.9	96.6%	100%	0.994 (0.96–1.00)	<0.001
	DM with neuropathy vs DM without neuropathy	>11.8	90.0%	83.3%	0.892 (0.79–0.99)	<0.001
NGF (pg/mL)	DM without neuropathy vs healthy control	≤226.2	92.3%	88.5%	0.85 (0.73–0.96)	<0.001
	DM with neuropathy vs healthy control	≤227.8	96.7%	88%	0.87 (0.76–0.97)	<0.001
	DM with neuropathy vs DM without neuropathy	≤208.5	63.3%	56.7%	0.584 (0.43–0.74)	0.260

An analysis was conducted to compare the levels of biochemical parameters according to the severity of DPN (Table 4). The mean serum NfL concentrations were 13.9 ± 1.10 ng/mL in the mild group, 13.52 ± 1.3 ng/mL in the moderate group, and 13.61 ± 2.3 ng/mL in the severe group. The mean serum NGF concentra-

tions were 206.2±14.1 pg/mL, 196.1±13.2 pg/mL, and 211.3±24.4 pg/mL in the mild, moderate, and severe groups, respectively. Despite numerical variations, there were no statistically significant differences in the mean levels of either NfL ($p=0.87$) or NGF ($p=0.31$) across the three DPN severity stages.

Table 4. Comparison of biochemical parameters according to the severity of disease

Characteristic	Mild n=10	Moderate n=11	Severe n=9	p
NfL (ng/mL, mean±SD)	13.9±1.10	13.52±1.3	13.61±2.3	0.87
NGF (pg/mL, mean±SD)	206.2±14.1	196.1±13.2	211.3±24.4	0.31

Discussion

The present study evaluated serum NfL and NGF levels in patients with type 2 diabetes mellitus (T2DM) with and without peripheral neuropathy. We observed significantly higher serum NfL and lower serum NGF levels in patients with DPN compared to healthy controls, reflecting progressive neuroaxonal injury and impaired neurotrophic support.^{14,15}

Our observation that serum NfL levels were highest in patients with DPN (13.9±1.20 ng/mL), intermediate in diabetic controls (9.83±2.08 ng/mL), and lowest in healthy controls (5.60±2.53 ng/mL) supports the role of NfL as a sensitive marker of axonal damage. This graded elevation suggests that neuroaxonal injury is a continuous process in diabetes, potentially starting subclinically before the clinical onset of neuropathy. This is consistent with findings by Nasr-Eldin et al., who reported elevated serum NfL in diabetic patients regardless of neuropathy status, with the highest levels in those with confirmed DPN.¹⁶ This indicates that diabetes may induce a state of subclinical neural injury that can be detected by NfL.

The strong positive correlation between serum NfL and diabetes duration ($r=0.733$, $p<0.001$) as well as HbA1c ($r=0.574$, $p<0.001$) suggests that cumulative glycemic exposure and metabolic stress drive progressive axonal degeneration. This is in agreement with prior longitudinal analyses,^{14,17} although our cross-sectional design prevents causal inferences.

In terms of diagnostic performance, the exceptionally high AUC of 0.994 for NfL in distinguishing patients with DPN from healthy controls must be interpreted with caution, as case-control studies comparing diseased cohorts with healthy individuals typically overstate diagnostic accuracy. Indeed, when we performed the more clinically relevant comparison between DPN patients and diabetic controls, the AUC for NfL decreased to 0.892. While still reflecting good diagnostic performance, this indicates that the biomarker’s utility is more modest in clinical settings where the

goal is to differentiate neuropathy from diabetes-related changes. This is consistent with Chen et al., who reported an AUC of 0.829 for NfL alone in distinguishing DPN from non-DPN diabetic patients.¹⁵ Therefore, serum NfL should be regarded as a promising candidate biomarker requiring further validation, rather than a tool ready for routine clinical use.

Interestingly, our study did not find a significant association between NfL levels and clinical severity of DPN (mild, moderate, severe). This finding should be interpreted cautiously because the severity subgroups were small, making the analysis exploratory and underpowered. Nevertheless, the absence of a clear gradient is consistent with recent reports suggesting that serum NfL may reflect axonal injury more directly than symptom-based severity phenotypes.^{18,19}

Our study identified significantly lower serum NGF levels in patients with DM, with the most pronounced reduction in the DPN group. This supports the neurotrophic deficit hypothesis, which posits that a deficiency in essential growth factors, such as NGF, contributes to neuronal vulnerability and degeneration in DPN.^{3,4} The significant negative correlation between NGF and HbA1c ($r=-0.570$, $p<0.001$) in our cohort reinforces the link between poor glycemic control and impaired neurotrophic support.

A comprehensive review by Massimino et al. explains that disruption of the NGF/proNGF axis is a key element in DPN pathophysiology.²⁰ Our findings are also directionally consistent with prior clinical work by Sun et al., who reported lower serum NGF levels in patients with DPN and suggested diagnostic utility for this marker.¹⁰ These external data should be interpreted as contextual support rather than confirmation of our single-center cross-sectional observations.

The structural and functional alterations observed in DPN, reflected by altered NfL and NGF levels, are fundamentally driven by chronic hyperglycemia-induced oxidative stress and neuroinflammation. Recent experimental evidence highlights the potential of targeting these pathways to mitigate nerve injury. For instance, a recent study by Ari et al. investigated the protective effects of dichloroacetic acid (DCA) in a rat model of diabetic neuropathy. The authors demonstrated that DCA administration significantly alleviated neuropathic alterations by reducing lipid peroxidation and inflammation, leading to improved nerve conduction and motor function.²¹ Such mechanistic insights underscore the critical role of oxidative stress and inflammation in the pathogenesis of DPN and provide a vital context for our biomarker findings, suggesting that therapeutic interventions targeting these pathways may help preserve neuroaxonal integrity and neurotrophic support.

The diagnostic performance of NGF in our study (AUC=0.87) was good, although not as high as that of

NfL. This is consistent with the general understanding that structural damage markers NfL often exhibit higher diagnostic accuracy than markers of regulatory pathways or trophic factors (NGF).²² NGF may be more reflective of the underlying state of neurotrophic dysregulation than a direct measure of the extent of axonal breakdown.

The inverse correlation between NfL and NGF ($r = -0.613$, $p < 0.001$) suggests that increasing neuroaxonal injury may coexist with declining neurotrophic support. This conceptual framework is biologically appealing; however, we did not construct a combined biomarker model, compare a combined ROC curve with NfL alone, or test incremental discrimination. Therefore, the potential added value of using both markers together should be considered hypothesis-generating rather than established.

It is important to acknowledge that the statistical analyses employed in this study, namely one-way ANOVA, bivariate Pearson correlation, and ROC curve analysis, do not account for the potential confounding effects of variables such as age, BMI, and renal function on biomarker levels. Although age and sex were not significantly different between groups, multivariable regression analysis would have provided a more rigorous assessment of the independent association of NfL and NGF with DPN status. In addition, renal function was addressed through exclusion criteria rather than direct adjustment with creatinine or eGFR values, which warrants caution because circulating NfL may be influenced by kidney clearance.

Study limitations

Several limitations of this study should be acknowledged. First, the relatively small sample from a single center may limit generalizability. Second, the cross-sectional design precludes causal inference. Third, although major comorbid conditions were excluded, renal function was not incorporated as a measured covariate, and unmeasured subclinical renal impairment or systemic inflammation may have affected biomarker levels. Fourth, no multivariable regression, confidence intervals, standardized effect sizes, or combined biomarker models were generated, limiting inference about independent or additive diagnostic value. Fifth, the severity analysis was underpowered because the DPN subgroup sizes were small (10/11/9) and should therefore be regarded as exploratory. Finally, the ROC analyses were performed against healthy controls, and the exceptionally high diagnostic accuracy observed for NfL requires validation in larger cohorts that include clinically relevant diabetic comparison groups. Additionally, there was a significant difference in the duration of diabetes between the DPN group and the non-neuropathy diabetic group. Because both NfL and NGF levels may be influenced by cumulative glycemic exposure over time, this disparity makes it difficult to definitively determine whether the observed

changes in biomarker levels are directly attributable to the presence of neuropathy or simply reflect the longer duration of diabetes. Future studies with duration-matched cohorts are required to clarify this.

Conclusion

In conclusion, serum NfL levels were significantly elevated and serum NGF levels were significantly reduced in patients with DPN compared to healthy controls. These findings support the potential of serum NfL and NGF as promising candidate biomarkers of axonal injury and impaired neurotrophic support in DPN, respectively. However, given the small, single-center, cross-sectional case-control design, lack of multivariable adjustment, and the reduced diagnostic performance of NfL in distinguishing DPN from diabetic controls, these results must be considered preliminary. Further validation in larger, longitudinal, and duration-matched cohorts is strictly required before these biomarkers can be considered for clinical implementation or routine diagnostic monitoring.

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Declarations

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Author contributions

Conceptualization: R.A.A.K. and T.M.J.; Methodology: R.A.A.K. and T.M.J.; Validation: R.A.A.K. and T.M.J.; Formal Analysis: R.A.A.K.; Investigation: R.A.A.K. and T.M.J.; Resources: R.A.A.K.; Data Curation: R.A.A.K.; Writing – Original Draft Preparation: R.A.A.K. and T.M.J.; Writing – Review & Editing: R.A.A.K. and T.M.J.; Visualization: R.A.A.K.; Supervision: R.A.A.K.; Project Administration: TMJ; Funding Acquisition: R.A.A.K. and T.M.J..

Conflicts of interest

The authors declare that they have no competing interests.

Data availability

The datasets produced and examined in the present study can be obtained from the corresponding author

upon a reasonable request, adhering to ethical and privacy considerations.

Ethics approval

This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the College of Medicine, Babylon University (Approval Number: MB5-69, date: 18/9/2025).

Use of AI and AI-assisted technologies in the writing process

AI tools (PaperPal) were used only for language editing and grammar improvement. All scientific content, data analyses, and conclusions are the authors' own.

References

- Duncan BB, Magliano DJ, Boyko EJ. IDF Diabetes Atlas 11th edition 2025: global prevalence and projections for 2050. *Nephrol Dial Transplant*. 2025;41(1):7-9. doi:10.1093/ndt/gfaf177
- Singh I, Issar T, Poynten AM, Milner KL, Krishnan A V, Dhanapalaratnam R. Evaluation of the impact of advanced glycation end-products on peripheral neuropathy outcomes in type 2 diabetes. *Biomedicines*. 2024;12(11):2518. doi:10.3390/biomedicines12112518
- Feldman EL, Callaghan BC, Pop-Busui R, et al. Diabetic neuropathy. *Nat Rev Dis Prim*. 2019;5(1):41. doi:10.1038/s41572-019-0092-1
- Vinik AI, Nevoret ML, Casellini C, Parson H. Diabetic neuropathy. *Endocrinol Metab Clin North Am*. 2013;42(4):747-787. doi:10.1016/j.ecl.2013.06.001
- Fujita Y, Murakami T, Nakamura A. Recent advances in biomarkers and regenerative medicine for diabetic neuropathy. *Int J Mol Sci*. 2021;22(5):2301. doi:10.3390/ijms22052301
- Morgenstern J, Groener JB, Jende JME, et al. Neuron-specific biomarkers predict hypo- and hyperalgesia in individuals with diabetic peripheral neuropathy. *Diabetologia*. 2021;64(12):2843-2855. doi:10.1007/s00125-021-05557-6
- Daponte A, Koros C, Skarlis C, et al. Neurofilament biomarkers in neurology: from neuroinflammation to neurodegeneration, bridging established and novel analytical advances with clinical practice. *Int J Mol Sci*. 2025;26(19):9739. doi:10.3390/ijms26199739
- Maalmi H, Strom A, Petrera A, et al. Serum neurofilament light chain: a novel biomarker for early diabetic sensorimotor polyneuropathy. *Diabetologia*. 2023;66:579-589. doi:10.1007/s00125-022-05846-8
- Määttä LL, Andersen ST, Parkner T, et al. Serum neurofilament light chain – a potential biomarker for polyneuropathy in type 2 diabetes? *Diabetes Res Clin Pract*. 2023;205:110988. doi:10.1016/j.diabres.2023.110988
- Sun Q, Tang DD, Yin EG, et al. Diagnostic significance of serum levels of nerve growth factor and brain derived neurotrophic factor in diabetic peripheral neuropathy. *Med Sci Monit Int Med J Exp Clin Res*. 2018;24:5943-5950. doi:10.12659/MSM.909449
- Pittenger G, Vinik A. Nerve growth factor and diabetic neuropathy. *Exp Diabetes Res*. 2003;4(4):271-285. doi:10.1155/EDR.2003.271
- Nuttall FQ. Body mass index: obesity, BMI, and health: a critical review. *Nutr Today*. 2015;50(3):117-128. doi:10.1097/NT.0000000000000092
- Kamiya H, Shibata Y, Himeno T, et al. Point-of-care nerve conduction device predicts the severity of diabetic polyneuropathy: a quantitative, but easy-to-use, prediction model. *J Diabetes Investig*. 2021;12(4):583-591. doi:10.1111/jdi.13386
- Fridman V, Sillau S, Ritchie A, et al. Plasma neurofilament light chain concentrations are elevated in youth-onset type 2 diabetes and associated with neuropathy. *J Peripher Nerv Syst*. 2023;28(3):460-470. doi:10.1111/jns.12575
- Chen Y, Huang S, Cai W, Song J, Sui C. Combined serum neurofilament light chain and Tau as biomarkers of diabetic peripheral neuropathy. *Front Endocrinol (Lausanne)*. 2026;17:1705881. doi:10.3389/fendo.2026.1705881
- Nasr-Eldin YK, Cartwright MS, Hamed A, Ali LH, Abdel-Nasser AM. Evaluation of serum neurofilament light chain and nerve ultrasound in diabetic neuropathy. *J Ultrasound Med*. 2024;43(4):683-696. doi:10.1002/jum.16393
- Määttä LL, Andersen ST, Parkner T, et al. Longitudinal change in serum neurofilament light chain in type 2 diabetes and early diabetic polyneuropathy: ADDITION-Denmark. *Diabetes Care*. 2024;47(6):986-994. doi:10.2337/dc23-2208
- Määttä LL, Andersen ST, Parkner T, et al. Serum neurofilament light chain and structural and functional nerve fiber loss in painful and painless diabetic polyneuropathy. *Diabetes Res Clin Pract*. 2025;223:112098. doi:10.1016/j.diabres.2025.112098
- Kim AS, Lee JM. The limited role of serum neurofilament light chain in predicting pain severity of patients with diabetic polyneuropathy. *Sci Rep*. 2024;14(1):15612. doi:10.1038/s41598-024-66444-9
- Massimino M, Rubino M, Natale MR, et al. Nerve growth factor in diabetes mellitus: pathophysiological mechanisms, biomarkers and therapeutic opportunities. *Pharmaceuticals*. 2025;18(12):1805. doi:10.3390/ph18121805
- Arı M, Erdogan MA, Erbaş O. Investigation of the protective effects of dichloroacetic acid in a rat model of diabetic neuropathy. *BMC Pharmacol Toxicol*. 2025;26(1):15. doi:10.1186/s40360-025-00849-8
- Fundaun J, Kolski M, Molina-Álvarez M, Baskozos G, Schmid AB. Types and concentrations of blood-based biomarkers in adults with peripheral neuropathies: a systematic review and meta-analysis. *JAMA Netw Open*. 2022;5(12):e2248593-e2248593. doi:10.1001/jamanetworkopen.2022.48593



ORIGINAL PAPER

Selected determinants of health-related behaviors of nurses – a cross-sectional study

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ABSTRACT

Introduction and aim. Health behaviors include all actions that may affect health status. This research was conducted to assess the level of health-related behaviors among professionally active nurses.

Material and methods. 322 nurses took part in the study. The work used the method of cross-sectional study, the questionnaire technique, and the research tool was the author's Personal Information Form (PIF), and Health Behavior Inventory (HBI).

Results. In the studied group of nurses, the level of the health behavior index was 78.9 points. Rural residents have a slightly worse mental attitude and are slightly more likely to show a low level of health behavior. Nurses with chronic illness present a higher level of health behavior than their healthy colleagues. Age and seniority do not affect the health behavior of the respondents.

Conclusion. The level of health behavior in the studied group of active nurses was moderate. The results obtained indicate the need to undertake targeted preventive and educational activities, especially among groups of nurses with lower health behaviors.

Keywords. health, health behaviors, nurses

Introduction

Nurses constitute the most feminized and numerous professional group in the healthcare system in the world.¹ Their role goes far beyond performing medical procedures and includes educational, preventive and health promotion activities. At the same time, it is a professional group particularly exposed to physical and mental overload. Numerous studies confirm that high workload, time pressure, professional responsibility, staff shortages and stress related to caring for patients in life-threatening conditions significantly affect the health and well-being of nurses.²⁻⁴

Shift work, including night shifts and irregular work rhythms, is a particularly important risk factor. It disrupts the body's natural circadian rhythm, leading to sleep problems, chronic fatigue, and an increased risk of metabolic and cardiovascular diseases.^{3,4} The literature also indicates a link between shift work and an increased risk of obesity, mood disorders and burnout.⁵⁻⁸ Long-term exposure to occupational stress can further lead to a decrease in the quality of life and deterioration of the over-all health of nursing staff.²

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Despite the numerous burdens, working with a patient can be a source of professional satisfaction and a sense of meaning in the duties performed.^{9,10} However, this satisfaction often co-occurs with a high level of stress, which creates a complex picture of the professional functioning of nurses. In the context of growing staff short-ages and the ageing of the nursing population, the issue of nurses' health and well-being is of particular systemic importance.¹

A healthy lifestyle and taking appropriate health behaviors are a key element of maintaining good health and a high quality of life.^{11,12} Health behaviors are defined as all actions of an individual that affect their health – both in a beneficial way (health-promoting behavior) and in an unfavorable way (anti-health behavior).¹³ These range from responses to health-related situations as well as entrenched habits and conscious actions taken to protect it.¹⁴

The most frequently analyzed health-promoting behaviors includes regular physical activity, rational nutrition, avoidance of psychoactive substances, attention to sleep hygiene and skillful stress management. These behaviors have both short-term (improved well-being, increased energy levels) and long-term (prevention of chronic diseases, prolongation of life).¹⁵ Wellbeing research emphasizes that health status is one of the key components of quality of life and strongly correlates with subjective life satisfaction.¹⁶ Health behaviors affect well-being both directly and indirectly – by improving physical and mental health. People leading a health-promoting lifestyle show a higher level of mental well-being, greater emotional resilience and lower levels of stress.¹⁷ In a holistic approach, health is treated as a multidimensional construct, including physical, mental and social components, the level of which directly affects the overall quality of life.^{13,18,19}

In recent years, more and more attention has been paid to the well-being of nursing staff as a factor determining not only the quality of life of nurses themselves, but also the quality of patient care.^{10,12} Good health and high life satisfaction are conducive to greater professional engagement, lower levels of burnout and higher work efficiency.²⁰ On the other hand, chronic stress, lack of organizational support and work overload significantly increase the risk of burnout and reduced quality of care.^{2,21} From a systemic perspective, maintaining a high level of nurses' well-being translates into patient safety, fewer medical errors and higher satisfaction with healthcare.^{2,22} At the same time, nurses are expected to act as models of health-promoting behavior, which means that their own health attitudes can influence the credibility and effectiveness of educational activities.²³

Despite numerous studies on the workload and burnout of nurses, relatively less attention has been paid to the analysis of the level of their health behavior and

the factors that determine it. In particular, there is a limited number of studies analyzing the relationship between health behaviors, selected sociodemographic and occupational factors (including shift work) and well-being indicators. An in-depth understanding of these relationships can be the basis for the development of targeted support programs, modification of work organization and the implementation of systemic health promotion activities in the nursing community.

Aim

The aim of this study was to assess the level of health-related behaviors among professionally active nurses and to analyze selected sociodemographic and occupational factors, including the nature of shift work and chronic diseases occurrence. The following research questions were formulated: What is the level of health behavior in the studied group of nurses? Do health behaviors vary depending on selected sociodemographic variables? Is there a relationship between health behavior and occupational factors, including shift work? The novelty of this study lies in the assessment of health-related behaviors and occupational characteristics.

Material and methods

Study design

It was a descriptive cross-sectional study aimed at assessing the level of health behavior of nurses and their relationship with selected sociodemographic and occupational factors.

Participants and settings

The sample was selected using a convenience sampling method. The study involved 322 nurses employed in two hospitals in south-eastern Poland. The inclusion criteria included: age over 18 years, active employment as a nurse, minimum one year's work experience in the nursing profession and voluntary participation in the study. People who do not meet the above criteria are excluded. A total of 322 completed forms were included in the analysis due to missing data, some analyses were conducted on reduced subsamples; therefore, the number of observations varies across tables.

Ethical considerations

The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki and received approval from the Bioethics Committee at the University of Rzeszow from 09/05/2019 (Resolution No. 30/05/2019). Prior to the commencement of the study, participants were informed about the aims and design of the project and were advised that participation was anonymous and entirely voluntary. They were then asked to provide informed verbal consent to participate. No incentives were offered for participa-

tion. Nurses who provided consent were subsequently invited to complete the questionnaire.

Data collections

The questionnaires were distributed in two hospitals in south-eastern Poland between May and June 2019. Respondents received clear instructions on how to complete the survey. After filling out the questionnaires, they were deposited in a special urn located on the premises of the hospital.

Research instruments

Personal Information Form (PIF)

The PIF author’s questionnaire included questions on socio-demographic and occupational characteristics, including age, place of residence, length of service, work system, occurrence of chronic diseases.

Health Behavior Inventory (HBI)

The level of health behavior was assessed using the HBI by Zygfryd Juczyński.²⁴ The questionnaire consists of 24 statements relating to health-promoting behaviors. Respondents determined the frequency of undertaking individual behaviors over the past year, using a five-point Likert scale (1 – almost never, 2 – rarely, 3 – occasionally, 4 – often, 5 – almost always). On the basis of the data obtained, the overall rate of health-related behaviors undertaken by respondents and the level of severity in four categories of health behaviors were calculated: proper eating habits, preventive behaviors, positive mental attitude and health practices. The value of the General Health Behavior Index (GI) ranges from 24 to 120 points, with a higher value indicating a higher level of declared health-promoting behaviors. According to the summary classification of the HBI scale, the results are converted into sten units, on the basis of which three levels of health behavior are distinguished: low (1–4 sten), average (5–6 sten) and high (7–10 sten). The HBI is a standardized tool with established psychometric properties. In the original validation study, the overall internal consistency was good (Cronbach’s $\alpha=0.85$), while the four subscales showed reliability coefficients ranging from 0.60 to 0.65.²⁴

Statistical analysis

The statistical analysis was carried out in the Statistica 13.3 (TIBCO Software Inc. Palo Alto, CA, USA) program. Quantitative variables are described using the arithmetic mean (M), standard deviation (SD), median, and range of values. Qualitative variables are presented as numbers (n) and percentages (%). The normality of the distribution of quantitative variables was assessed using the Shapiro-Wilk test. Due to the non-normal distribution of the analyzed variables, the

non-parametric Mann-Whitney and Kruskal-Wallis tests were used. The chi-square test of independence was applied for qualitative variables. The level of statistical significance was assumed to be $p<0.05$.

Results

Study group characteristics

The study group consisted of 322 nurses. Only women participated in the study. The analysis of sociodemographic characteristics showed that over 40% of respondents were under 30 years of age, and the largest percentage had 1 to 5 years of work experience in the profession. The majority of participants lived in the countryside 57.8%, while 42.2% came from the city. The dominant work system was shift work, i.e. 80.1% of the respondents, while 19.9% worked in a one-shift system. One in five nurses (23%) confirmed a chronic disease (Table 1).

Table 1. Sociodemographic characteristics of respondents (n=322)

Variables		n	%
Age (in years)	<30	130	40.3
	30-49	128	39.7
	>50	61	19.0
Place of residence	city	135	41.9
	village	187	58.1
Seniority (in years)	>5	130	40.3
	5-25	97	30.2
	>25	95	29.5
System of work	shift work	258	80.1
	single-shift work	64	19.9
Chronic disease	yes	74	23.0
	not	248	77.0

Level of health behavior according to HBI

The table below summarizes the distribution of health behavior levels across the four domains assessed using the HBI, as well as the overall index. In the study group, the mean value of the overall HBI was 78.9 points (Me=79), which corresponds to a average level of health behaviors according to the HBI classification (Table 2).

Table 2. Level of health behaviors according to HBI

Level of health-related behaviors	$\bar{x}$	Me	s	c25	c75	min	max
Healthy dietary habits	3.39	3.33	0.68	3.00	3.83	1.50	5.00
Preventive behaviors	3.38	3.50	0.71	2.83	3.83	1.17	4.83
Positive psychological attitude	3.37	3.33	0.68	3.00	3.83	1.17	5.00
Health practices	3.01	3.00	0.64	2.67	3.50	1.00	5.00
HBI	78.9	79	13.2	71	88	35	117

The majority of nurses present a low (44.7%) or average (41.6%) level of health behavior, and only 13.7% a high level (Table 3).

Table 3. Level of health-related behaviors

Level of health-related behaviors	Frequency	Percentage
Low	144	44.7%
Average	134	41.6%
High	44	13.7%

The influence of selected socio-demographic factors on psychometric measures

In the next part, the impact of selected factors – independent variables – related to professional status, but also demographic characteristics and social status – on the level of health behavior was analyzed. The calculations were based on the numerical values of the HBI measures, as well as the classification made on this basis.

Selected socio-demographic factors and the level of health behavior

The analysis did not show statistically significant differences in the level of health behavior depending on the age of the respondents ($p>0.05$). The values of the medians of the individual components of the HBI and the general index were similar in the analyzed age groups. However, a slight upward trend was observed, consisting in obtaining slightly higher values of the HBI index and selected categories of health behaviors in the oldest group of respondents (>50 years).

The place of residence showed a certain tendency to differentiate the level of health behavior. Respondents living in cities scored slightly higher in terms of positive mental attitude compared to rural residents, with this difference being close to the level of statistical significance ($p=0.058$). A similar trend was observed for the overall HBI index.

Similarly, the length of service in the profession did not significantly differentiate the level of health behavior ($p>0.05$). Although the longest-serving people scored slightly higher median in some categories of health behaviors, these differences did not reach the level of statistical significance. There were also no statistically significant differences in the level of health behavior between people working in shifts and in a one-shift system ($p>0.05$).

A statistically significant relationship was observed in relation to the occurrence of chronic disease. People declaring a chronic disease showed a higher level of proper eating habits compared to respondents without chronic diseases ($p=0.015$). In addition, slightly higher values of the overall HBI index were recorded in this group, with this difference being close to the level of statistical significance ($p=0.077$), (Table 4).

The analysis did not show statistically significant relationships between the level of health behavior and the age of respondents ($p=0.1443$) or length of service ($p=0.2832$). In each of the analyzed groups, a low level of health behavior prevailed, although with age there

Table 4. The influence of selected socio-demographic factors on psychometric measures

Level of health-related behaviors	Age [years] – Kruskal-Wallis test									p
	<30			30–49			>50			
	$\bar{x}$	Me	s	$\bar{x}$	Me	s	$\bar{x}$	Me	s	
Proper dietary habits	3.36	3.33	0.62	3.36	3.50	0.72	3.52	3.67	0.68	0.2413
Preventive behaviors	3.38	3.42	0.61	3.40	3.50	0.79	3.39	3.50	0.70	0.7902
Positive mental attitude	3.38	3.33	0.55	3.31	3.33	0.76	3.47	3.67	0.67	0.3192
Health practices	3.01	3.00	0.55	2.98	3.00	0.72	3.11	3.17	0.64	0.5165
Overall HBI score	78.8	77.0	10.5	78.3	80.0	15.3	80.9	84.0	13.0	0.3366
Level of health-related behaviors	Seniority in profession [years], Kruskal-Wallis test									p
	<5			5–24			>25			
	$\bar{x}$	Me	s	$\bar{x}$	Me	s	$\bar{x}$	Me	s	
Proper dietary habits	3.37	3.33	0.62	3.33	3.42	0.74	3.45	3.50	0.66	0.4760
Preventive behaviors	3.41	3.50	0.61	3.39	3.50	0.75	3.38	3.50	0.74	0.9903
Positive mental attitude	3.38	3.33	0.59	3.34	3.33	0.67	3.43	3.50	0.70	0.4674
Health practices	2.99	3.00	0.54	2.97	3.00	0.71	3.10	3.00	0.66	0.4335
Overall HBI score	78.8	77.0	11.1	78.1	80.0	14.1	80.1	81.0	13.5	0.5212
Level of health-related behaviors	Place of residence						p			
	City			Village						
	$\bar{x}$	Me	s	$\bar{x}$	Me	s				
Proper dietary habits	3.43	3.50	0.72	3.35	3.33	0.64	0.2605			
Preventive behaviors	3.40	3.50	0.73	3.37	3.42	0.71	0.5115			
Positive mental attitude	3.44	3.50	0.69	3.32	3.33	0.66	0.0581			
Health Practices	3.05	3.00	0.67	2.99	3.00	0.61	0.5933			
Overall HBI score	79.9	82.0	14.0	78.2	78.0	12.6	0.1113			
Level of health-related behaviors	Work system (Mann-Whitney test)						p			
	Shift work			Single-shift work						
	$\bar{x}$	Me	s	$\bar{x}$	Me	s				
Proper dietary habits	3.37	3.33	0.65	3.43	3.33	0.79	0.6767			
Preventive behaviors	3.39	3.50	0.72	3.35	3.50	0.68	0.6500			
Positive mental attitude	3.34	3.33	0.68	3.48	3.50	0.67	0.1896			
Health Practices	2.99	3.00	0.64	3.11	3.00	0.63	0.1198			
Overall HBI score	78.5	79.0	13.3	80.2	79.0	12.9	0.3715			
Level of health-related behaviors	Presence of chronic disease (Mann-Whitney test)						p			
	Yes			No						
	$\bar{x}$	Me	s	$\bar{x}$	Me	s				
Proper dietary habits	3.55	3.67	0.71	3.34	3.33	0.65	0.0154*			
Preventive behaviors	3.40	3.50	0.75	3.39	3.50	0.69	0.4749			
Positive mental attitude	3.41	3.42	0.71	3.36	3.33	0.66	0.4365			
Health Practices	3.08	3.00	0.71	3.00	3.00	0.61	0.3076			
Overall HBI score	80.6	81.5	14.9	78.5	79.0	12.4	0.0775			

was a tendency toward a lower a lower proportion of respondents presenting low levels of health behavior. A tendency toward a relationship between place of residence and health behaviors was observed; however, the result did not reach statistical significance ($p=0.0849$). Respondents living in rural areas more often presented a low level of health behavior, while city dwellers more often declared a high level of health-promoting behavior. There was also no significant relationship between the level of health behavior and the work system ($p=0.4166$). A statistically significant association was noted in the case of chronic disease occurrence ($p=0.0240$). Respondents with chronic disease were more likely to present a high level of health behaviors

and less likely to present a low level of these behaviors compared to respondents without chronic diseases (Table 5).

Table 5. The influence of selected socio-demographic factors on the level of health behaviors*

Level of health-related behaviors	Variables			Total (n#)
	Age [years] (p=0.1443)			
	<30	30–49	>50	
Low	66 (50.8%)	56 (43.8%)	20 (32.8%)	142
Average	51 (39.2%)	51 (39.8%)	31 (50.8%)	133
High	13 (10.0%)	21 (16.4%)	10 (16.4%)	44
Total	130	128	61	319
Level of health-related behaviors	Seniority in profession [years], (p=0.2832)			Total
	<5	5–25	>25	
Low	66 (51.6%)	37 (41.1%)	36 (37.9%)	139
Average	49 (38.3%)	40 (44.4%)	44 (46.3%)	133
High	13 (10.2%)	13 (14.4%)	15 (15.8%)	41
Total	128	90	95	313
Level of health-related behaviors	Place of residence (p=0.0849)		Total	
	City	Village		
Low	52 (38.5%)	91 (48.9%)	143	
Average	59 (43.7%)	75 (40.3%)	134	
High	24 (17.8%)	20 (10.8%)	44	
Total	135	186	321	
Level of health-related behaviors	Work system (p=0.4166)		Total	
	Shift work	Single-shift work		
Low	117 (45.3%)	27 (42.2%)	144	
Average	109 (42.2%)	25 (39.1%)	134	
High	32 (12.4%)	12 (18.8%)	44	
Total	258	64	322	
Level of health-related behaviors	Presence of chronic disease (p=0.0240*)		Total	
	Yes	No		
Low	27 (36.5%)	116 (47.0%)	143	
Average	30 (40.5%)	104 (42.1%)	134	
High	17 (23.0%)	27 (10.9%)	44	
Total	74	247	321	

* # – n may vary due to missing responses, p – probability value calculated using the chi-square test of independence

Discussion

The aim of this study was to assess the level of health behavior of professionally active nurses. The results obtained indicate that the average level of health behavior measured by the HBI was 78.9 points, which classifies the surveyed people in the range of moderate to high level of health-promoting habits. This result can be interpreted as, especially in the context of the professional burden char-

acteristic of the nursing profession.²⁵ At the same time, the structure of the results indicates that the majority of respondents presented a low or average level of health behavior, and only 13.7% reached a high level, which suggests significant space for intervention activities.

A moderate mean level of health-promoting behavior may be related to the specific characteristic of the nursing profession, including a high level of health knowledge, medical education and daily contact with patients. According to the World Health Organization (WHO)¹, nurses are a key professional group in the health care system and play an important role in health promotion. Theoretical and practical knowledge may promote greater awareness of the importance of prevention; however previous studies showed that health awareness does not always translate into consistent implementation of health behaviors in everyday life.^{23,26,27}

The results of this study are similar to the observations of Kilar et al.,²⁸ who obtained an average HBI score of 80.52 points. In that study, the family played a key role in the nurses' value system, along with health and professional work, and the level of health behavior was described as average. The authors also pointed to the importance of educational and environmental factors – higher education correlated with a higher level of health-promoting behaviors. The literature emphasizes that education increases health competences and promotes rational health decisions.¹⁴ Similar findings have been reported in studies conducted among Polish nurses, which indicate that health-related behaviors are associated not only with sociodemographic factors but also with psychological traits and occupational conditions.²⁹

In the study by Trojanowska et al. on a group of pediatric nurses, the HBI score was 80.7 points, a higher level of health behavior was presented by older respondents and those with higher professional experience, which is consistent with our results.³⁰

International studies conducted in China, Nepal, Qatar, Iran and South Korea also indicate that nurses commonly present low or moderate levels of health-related behaviors, although the results vary depending on the measurement tools and cultural context. However, comparisons between studies should be interpreted cautiously due to the use of different measurement tools and scoring systems.^{31–35}

The findings of the present study are consistent with recent research indicating that nurses generally present a moderate level of health-related behaviors, although important deficits remain in specific domains.³⁶ Moreover, contemporary studies emphasize that nurses' health and well-being are closely associated with their work performance and quality of care, highlighting the importance of supporting this professional group at both individual and organizational levels.^{37,38}

The analysis of socio-demographic factors did not reveal any statistically significant relationships between the level of health behavior and age, length of service or the work system. However, there was a noticeable upward trend with age – the percentage of people with low levels of health behavior decreased in older age groups. This phenomenon may be associated with an increase in health maturity, a change in life priorities and greater awareness of the health consequences of incorrect habits.^{16,30}

An interesting result is the relationship between the occurrence of chronic disease and the level of health behavior. Nurses with chronic diseases showed a significantly higher level of proper eating habits and were more likely to achieve a high level of health behavior. This can be interpreted as the result of increased motivation to check health and greater internalization of medical recommendations. The literature indicates that the experience of the disease can be a strong stimulus to lifestyle modification.¹⁵

However, it remains worrying that despite high health knowledge, only a small percentage of nurses achieve a high level of health behavior. Numerous studies emphasize the impact of occupational stress, work overload, shift work and the risk of burnout on the deterioration of the well-being and quality of life of nursing staff.^{2,20,21} Previous research also suggests that work-family conflict and sleep disturbances may negatively affect mental health among nursing personnel, further limiting engagement in health-promoting behaviors.³⁹ In turn, participation in preventive programs and effective coping with occupational stress may support healthier behaviors among nurses.⁴⁰

Long-term stress can lead to sleep disorders, reduced physical activity and incorrect eating habits.^{3,4,8} As a consequence, there is a discrepancy between the role of the nurse as a health promoter and her own health behavior.²³

The results of this study fit into the broader context of research on the well-being and quality of life of healthcare workers.^{10,12} According to the approach to health as a multidimensional construct, encompassing physical, mental and social components,¹³ health behaviors are important, but are not the only determinant of well-being. Organizational factors, social support, and workplace culture can strengthen or weaken individual health resources.^{17,18}

In summary, the study indicates that nurses predominantly present low or moderate levels of health-related behaviors despite their professional knowledge and health awareness. The findings suggest a potential role of occupational and organizational factors in shaping these behaviors; however, the descriptive nature of the study does not allow causal interpretation or identification of independent predictors. These results highlight the importance of targeted workplace health promotion and

further research using multivariate approaches however, the findings should be interpreted in light of several limitations of the study. The study is limited by the use of univariate analyses without controlling for potential confounding variables. In addition, the use of a convenience sampling method and inclusion of participants from only two hospitals may limit the representativeness of the sample and restrict the generalizability of the findings to the broader population of nurses.

The data on health behaviors are based solely on respondents' declarations and have not been otherwise verified, which may introduce self-report bias. Moreover, the study did not control for psychosocial factors that may influence health behaviors, such as perceived stress, social support or other contextual determinants. Workload was not directly measured, and the study did not include information on lifestyle behaviors outside the workplace, which may also have influenced the observed results. In addition, other potentially relevant factors, such as quality of life, were not assessed. Another limitation is that effect sizes and confidence intervals were not reported, which may limit the interpretation of the magnitude and precision of the observed associations.

Despite these limitations, the results provide valuable insights into the level of health behavior among nurses and the demographic and health factors associated with their health-promoting practices.

Conclusion

Professionally active nurses demonstrate low or average level of health behaviors. No statistically significant associations were observed between the level of health-related behaviors and age, professional experience, and work system. A tendency toward an association between place of residence and health-related behaviors was observed. Nurses with chronic disease was associated with healthier eating habits and a higher overall Health Behavior Index score. These findings highlight the need for targeted health promotion initiatives aimed at nurses, including both educational and organizational interventions to supporting healthy lifestyles and well-being.

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Declarations

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Author contributions

Conceptualization, A.N. and M.N.; Methodology, A.N., Ka.K. and M.N.; Software, A.N., Ka.K. and M.N.; Validation, A.N., Ka.K., and M.N.; Formal analysis,

A.N., and M.N.; Investigation, A.N., Ka.K., and M.N.; Resources, A.N., Ka.K., Kr.K. and M.N.; Data curation, A.N., and M.N.; Writing - Original Draft Preparation, A.N., Ka.K., Kr.K. and M.N.; Writing - Review & Editing, A.N., and M.N.; Visualization, A.N., Ka.K., Kr.K. and M.N.; Supervision, A.N., and M.N.; Project Administration, A.N., and M.N.; Funding Acquisition, M.N.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The data presented in this study are available on request from the corresponding author.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Bioethics Committee at the University of Rzeszow from 09/05/2019 (Resolution No. 30/05/2019).

References

- World Health Organization. Nursing and midwifery. <https://www.who.int/news-room/fact-sheets/detail/nursing-and-midwifery>. Accessed January 7, 2026.
- Babapour AR, Gahassab-Mozaffari N, Fathnezhad-Kazemi A. Nurses' job stress and its impact on quality of life and caring behaviors: a cross-sectional study. *BMC Nurs*. 2022;21(1):75. doi:10.1186/s12912-022-00852-y
- Saedpanah K, Ghasemi M, Akbari H, Adibzadeh A, Akbari H. Effects of workload and job stress on the shift work disorders among nurses: PLS SEM modeling. *Eur J Transl Myol*. 2022;33(1):10909. doi:10.4081/ejtm.2022.10909
- Bennaroch K, Shochat T. Psychobiological risk factors for insomnia and depressed mood among hospital female nurses working shifts. *Front Sleep*. 2023; 2:1206101. doi:10.3389/frsle.2023.1206101
- Uchmanowicz I, Manulik S, Lomper K, et al. Life satisfaction, job satisfaction, life orientation and occupational burnout among nurses and midwives in Poland: a cross-sectional study. *BMJ Open*. 2019;9:e023231. doi:10.1136/bmjopen-2018-024296
- Phiri LP, Draper CE, Lambert EV, Kolbe-Alexander TL. Nurses' lifestyle behaviours, health priorities and barriers to living a healthy lifestyle: a qualitative descriptive study. *BMC Nurs*. 2014;13:38. doi:10.1186/s12912-014-0038-6
- Guo X, Gong S, Chen Y, et al. Lifestyle behaviors and stress are risk factors for overweight and obesity in healthcare workers: a cross-sectional survey. *BMC Public Health*. 2023;23:1791. doi:10.1186/s12889-023-16673-w
- Vitale E, Lupo R, Artioli G, et al. How shift work influences anxiety, depression, stress and insomnia in Italian nurses: an exploratory study. *Acta Biomed*. 2023;94:e2023102. doi:10.23750/abm.v94i2.14230
- Ayalew F, Kibwana S, Shawula S, et al. Understanding job satisfaction and motivation among nurses in public health facilities of Ethiopia: a cross-sectional study. *BMC Nurs*. 2019;18:46. doi:10.1186/s12912-019-0373-8
- Almeida D, Figueiredo AR, Lucas P. Nurses' well-being at work in a hospital setting: a scoping review. *Healthcare*. 2024;12:173. doi:10.3390/healthcare12020173
- Haybron DM. *The Pursuit of Unhappiness: The Elusive Psychology of Well-Being*. Oxford, UK: Oxford University Press; 2008.
- Bakkeli NZ. Health, work, and contributing factors on life satisfaction: a study in Norway before and during the COVID-19 pandemic. *SSM Popul Health*. 2021;14:100804. doi:10.1016/j.ssmph.2021.100804
- Bąk-Sosnowska M, Skrzypulec-Plinta V. Health behaviors, health definitions, sense of coherence, and general practitioners' attitudes towards obesity and diagnosing obesity in patients. *Arch Med Sci*. 2017;13(2):433-440. doi:10.5114/aoms.2016.58145
- Short SE, Mollborn S. Social determinants and health behaviors: conceptual frames and empirical advances. *Curr Opin Psychol*. 2015;5:78-84. doi:10.1016/j.copsyc.2015.05.002
- Farhud DD. Impact of lifestyle on health. *Iran J Public Health*. 2015;44:1442-1444.
- Diener E, Oishi S, Tay L. Advances in subjective well-being research. *Nat Hum Behav*. 2018;2:253-260. doi:10.1038/s41562-018-0307-6
- Guo S, Fu H, Guo K. Effects of physical activity on subjective well-being: the mediating role of social support and self-efficacy. *Front Sports Act Living*. 2024;6:1362816. doi:10.3389/fspor.2024.1362816
- Organisation for Economic Co-operation and Development. *How's Life? 2023: Measuring Well-Being*. Paris, France: OECD Publishing; 2023.
- Ngamaba KH. Determinants of subjective well-being in representative samples of nations. *Eur J Public Health*. 2018;28:392-398. doi:10.1093/eurpub/ckw103
- Li X, Jiang T, Sun J, et al. The relationship between occupational stress, job burnout and quality of life among surgical nurses in Xinjiang, China. *BMC Nurs*. 2021;20:181. doi:10.1186/s12912-021-00703-2
- Shah MK, Gandrakota N, Cimiotti JP, et al. Prevalence of and factors associated with nurse burnout in the US. *JAMA Netw Open*. 2021;4:e2036469. doi:10.1001/jamanetworkopen.2020.36469
- Wills J, Hancock C, Nuttall M. The health of the nursing workforce: a survey of national nurse associations. *Int Nurs Rev*. 2020;67:294-299. doi:10.1111/inr.12586
- Kelly M, Wills J, Sykes S. Do nurses' personal health behaviours impact on their health promotion practice? A systematic review. *Int J Nurs Stud*. 2017;76:62-77. doi:10.1016/j.ijnurstu.2017.08.008
- Juczyński Z. *Narzędzia pomiaru w promocii i psychologii zdrowia*. Warszawa, Poland: Pracownia Testów Psychologicznych PTP; 2012.

25. Marczak P, Milecka D. Professional burnout of nurses and the level of rationing of nursing care: an observational preliminary study. *BMC Nurs.* 2024;23:269. doi:10.1186/s12912-024-01940-x
26. Ross A, Bevans M, Brooks AT, et al. Nurses and health-promoting behaviors: knowledge may not translate into self-care. *AORN J.* 2017;105:267-275. doi:10.1016/j.aorn.2016.12.018
27. Tong W, Chui PL, Chong MC, et al. Health-promoting behaviours and perceived lifestyle cancer risk factors among nurses. *Collegian.* 2023;30:32-38. doi:10.1016/j.colegn.2022.06.011
28. Kilar R, Harpula K, Nagórska M. Health behaviors in professionally active nurses—preliminary research. *Eur J Clin Exp Med.* 2019;17:222-228. doi:10.15584/ej-cem.2019.3.3
29. Gieniusz-Wojczyk L, Dąbek J, Kulik H. Type A Behaviour Pattern and Health Behaviour of Polish Nurses. *Int J Environ Res Public Health.* 2022;19(11):6358. doi:10.3390/ijerph19116358
30. Trojanowska A, Trojanowska P, Piasecka K, et al. Health behavior of pediatric nurses. *Fides Ratio.* 2020;43:177-194. doi:10.34766/fetr.v43i3.314
31. Zeng W, Shang S, Fang Q, et al. Health-promoting lifestyle behaviors and associated predictors among clinical nurses in China: a cross-sectional study. *BMC Nurs.* 2021;20:230. doi:10.1186/s12912-021-00752-7.
32. Baral P, Tamrakar N. Health promoting lifestyle among nurses of a tertiary level hospital: a descriptive cross-sectional study. *J Karnali Acad Health Sci.* 2020;3:1-10.
33. Razzakh SS, Singh R, Khan BU, Hassan N. The health-promoting lifestyle behaviors of healthcare employees in Qatar-a cross-sectional comparative study. *Qatar Med J.* 2024;2024:74. doi:10.5339/qmj.2024.74
34. Mustafaei Najaf-Abadi H, Rezaei B. Health-promoting behaviours of Iranian nurses and its relationship with occupational factors: a cross-sectional study. *J Nurs Manag.* 2018;26:717-725. doi:10.1111/jonm.12610
35. Cho H, Han K. Associations among nursing work environment, health-promoting behaviors and nursing performance quality: a multilevel modelling approach. *J Nurs Scholarsh.* 2018;50:403-410. doi:10.1111/jnu.12390
36. Ważna A, Dziechciaż M, Wieczorowska-Tobis K, et al. Sociodemographic and occupational predictors of health behaviors among nurses: a cross-sectional study in Poland. *BMC Nurs.* 2024;24(1):1135. doi:10.1186/s12912-025-03786-3
37. Hussein AHM, Abou Hashish EA, Younes BM. The relationship between nurses' psychological Well-Being and their work productivity loss: A descriptive correlational study. *SAGE Open Nurs.* 2024;10:23779608241285400. doi:10.1177/23779608241285400
38. Alharbi MS, Albughuli MB, Aljohani SS, et al. Nurses' well-being and its relationship with quality of nursing work life at alsalam and al Haram Hospitals, Saudi Arabia. *Front Public Health.* 2025;13:1678358. doi:10.3389/fpubh.2025.1678358
39. Zhang Y, Punnett L, Nannini A. Work-family conflict, sleep, and mental health of nursing assistants working in nursing homes. *Workpl Health & Saf.* 2017;65(7):295-303. doi:10.1177/2165079916665
40. Sternal D, Studencka A, Bąk E. Health behaviors of nursing staff in preventive programs, coping with stress and additional workload. *J Educ Health Sport.* 2023;31:94-113. doi:10.12775/JEHS.2023.31.01.009



ORIGINAL PAPER

α -Klotho, hypoxia-inducible factor-2 α , and oxidative stress in hemodialysis – interactions with anemia, secondary hyperparathyroidism, and intradialytic hypotension

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ABSTRACT

Introduction and aim. End-stage renal disease (ESRD) is associated with oxidative stress (OS) and hormonal disturbances linked to anemia, secondary hyperparathyroidism (SHPT), and intradialytic hypotension (IDH). This study evaluated the expression of α -klotho (α -KL) and hypoxia-inducible factor-2 α (HIF-2 α) together with OS markers in hemodialysis (HD) patients and explored their interrelationships and associations with anemia, SHPT, and IDH.

Material and methods. Fifty HD patients and 50 healthy controls were evaluated for clinical and biochemical parameters, malondialdehyde (MDA), catalase (CAT) activity, and α -KL and HIF-2 α expression. Correlation and network analyses were used to evaluate molecular interactions.

Results. Anemia, SHPT, and IDH were identified in 72%, 44%, and 18% of HD patients, respectively. Compared with controls, HD patients demonstrated significantly higher MDA levels and lower α -KL and HIF-2 α expression, and CAT activity (all $p < 0.001$). MDA positively correlated with parathyroid hormone (PTH) ($r = 0.26$, $p < 0.01$) and IDH ($r = 0.37$, $p < 0.001$), but inversely with hemoglobin (Hb) ($r = -0.24$, $p < 0.02$) and CAT activity ($r = -0.36$, $p < 0.001$). Lower α -KL and HIF-2 α expression levels, together with reduced CAT activity, correlated positively with Hb ($r = 0.27$ – 0.71) and inversely with PTH ($r = -0.27$ to -0.62) and IDH ($r = -0.40$ to -0.92) (all $p < 0.01$). Positive intercorrelations were additionally found among α -KL, HIF-2 α , and CAT ($r = 0.21$ – 0.38 , all $p < 0.05$).

Conclusion. Reduced α -KL and HIF-2 α expression together with altered OS markers were associated with anemia, SHPT, and IDH in HD patients. The close interrelationships observed among α -KL, HIF-2 α , and OS markers may reflect an integrated molecular pattern linked to dialysis-related complications. Additional prospective studies are warranted to further elucidate these relationships.

Keywords. α -klotho, anemia, hemodialysis, hypoxia-inducible factor-2 α , oxidative stress

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Introduction

End-stage renal disease (ESRD) represents the terminal stage of chronic kidney disease (CKD) and is associated with a substantial clinical and healthcare burden worldwide. Hemodialysis (HD) remains the most widely used form of renal replacement therapy (RRT).¹ In Egypt, the prevalence of ESRD patients receiving HD was reported as 442 per million population in Sharkia Governorate in 2017.²

Patients with ESRD undergoing dialysis frequently develop a broad spectrum of complications, such as anemia, altered mineral balance, cardiovascular instability, and intradialytic hypotension (IDH), which collectively may worsen clinical status and impair quality of life.^{3,4} Anemia in ESRD is multifactorial, involving impaired erythropoietin (EPO) production, disrupted oxygen-sensing mechanisms, inflammation, altered iron handling, and the effects of uremia.⁵ IDH is another important complication during HD and has been associated with impaired organ perfusion and increased cardiovascular risk.⁶

Growing evidence suggests that oxidative stress (OS) is closely linked to the development and progression of many ESRD-related complications. Excessive oxidative activity may contribute to cellular dysfunction, vascular injury, and chronic inflammation, particularly in patients undergoing HD.^{7,8} Catalase (CAT) is a key component of the antioxidant defense system,⁹ whereas malondialdehyde (MDA) is a widely recognized indicator of lipid peroxidation and oxidative damage.¹⁰

The α -klotho (α -KL) protein is involved in mineral metabolism regulation and exerts cytoprotective effects within the kidney and other tissues.¹¹ Hypoxia-inducible factor-2 α (HIF-2 α) plays an essential role in oxygen sensing and erythropoietic regulation.¹² Increasing evidence suggests that alterations in α -KL, HIF-2 α , and oxidative stress pathways may contribute to several complications encountered in HD patients. However, these factors are often investigated separately, and their interrelationships in the context of anemia, secondary hyperparathyroidism (SHPT), and IDH remain insufficiently characterized. Clarifying these associations may provide a broader understanding of the biological processes accompanying ESRD and HD-related complications.

Aim

This study was undertaken to evaluate α -KL and HIF-2 α gene expression and oxidative stress status in ESRD patients undergoing HD. It further investigated the associations between these molecular markers and major HD-related complications, including anemia, SHPT, and IDH. In addition, a correlation network was constructed to explore the interrelationships among α -KL, HIF-2 α , and oxidative stress markers.

Material and methods

Study design and participants

A case-control design was applied involving 50 Egyptian patients with ESRD undergoing maintenance HD and 50 apparently healthy individuals who served as controls for biomarker and gene expression analyses. Patients were enrolled from the Nephrology Department, Faculty of Medicine, Suez Canal University Hospital, Ismailia. Biochemical and molecular investigations were carried out at the Medical Biochemistry and Molecular Biology Department, Oncology Diagnostic Unit, and Clinical Pathology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.

Patients were considered eligible if they had undergone regular HD sessions three times weekly for at least three months. Control participants were matched for age and sex, exhibited normal renal function based on serum creatinine and urea measurements, and had no evidence of chronic systemic illness.

Ethical approval was granted by the Institutional Ethics Committee of Suez Canal University (Approval No. 4724). Written informed consent was secured from all participants prior to study inclusion.

Hemodialysis treatment characteristics

All patients underwent maintenance hemodialysis three times weekly for at least three months, with a four-hour duration per session. Ultrafiltration volume and interdialytic weight gain were documented. Standard management protocols for anemia and CKD-MBD were applied according to institutional protocols and Kidney Disease Improving Global Outcomes (KDIGO) recommendations.^{13,14} SHPT was assessed primarily according to parathyroid hormone (PTH) concentrations. Additional biochemical markers associated with bone and mineral metabolism, including calcium (Ca²⁺), phosphorus (P), and alkaline phosphatase (ALP) were also evaluated to provide indirect evidence of bone-related abnormalities. Direct evaluation of bone pathology, such as imaging studies or bone mineral density assessment, was not included.

For the purpose of this study, anemia was defined as hemoglobin (Hb) <110 g/L according to KDIGO guidelines.¹⁵ IDH was identified by a reduction in systolic blood pressure of ≥ 20 mmHg or a decline in mean arterial pressure of ≥ 10 mmHg during dialysis together with clinical symptoms or the need for therapeutic intervention, according to National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI) criteria.¹⁶ SHPT was defined as intact PTH levels exceeding 61.7 pmol/L, corresponding to >9-fold above the upper reference limit. KDIGO guidelines recommend maintaining PTH concentrations within approximately 2–9 times the upper normal range in stage 5 CKD patients; therefore, values beyond this range were considered indicative of SHPT in the present analysis.¹⁴ Interdialytic

weight gain (IDWG) was determined from the difference between post-dialysis body weight and pre-dialysis body weight at the subsequent dialysis session.

Blood samples collection and laboratory investigation

Peripheral venous blood samples (9 mL) were obtained from all participants. In HD patients, sample collection was performed immediately before dialysis initiation. Samples were processed as follows:

- Serum samples: 5 mL Blood was collected into plain vacutainer tubes, maintained at room temperature until clot formation, then centrifuged at 3,000 rpm for 10 minutes. Serum was subsequently separated into two aliquots:
 - One aliquot was utilized for biochemical investigations, including measurement of serum creatinine, urea, sodium (Na⁺), potassium (K⁺), Ca²⁺, P, ALP, iron, and ferritin using the Cobas c 6000 autoanalyzer (Roche Diagnostics, Mannheim, Germany). Serum PTH concentrations were determined by chemiluminescent immunoassay using the Access 2 Immunoassay System (Beckman Coulter, Brea, CA, USA).
 - The second aliquot was stored at –20°C for subsequent assessment of MDA and CAT.
- EDTA samples (4 mL): Divided into two portions:
 - 2 mL was used for complete blood count (CBC) using a 5-part differential hematology analyzer (Siemens AG, Erlangen, Germany).
 - The remaining 2 mL was used for molecular analysis to assess mRNA expression of α -KL and HIF-2 α .

Oxidative stress assessment

MDA represents an indicator of lipid peroxidation and membrane oxidative injury, reflecting enhanced oxidative stress and reactive oxygen species generation in CKD.¹⁷ CAT functions as an antioxidant enzyme responsible for converting hydrogen peroxide into water and oxygen. Simultaneous evaluation of MDA and CAT activity provides an integrated overview of oxidative injury and antioxidant defense status.

Serum levels of both markers were analyzed using a UV–VIS spectrophotometer (Unico S2100 Series, Wixom, MI, USA). Quantification of MDA levels and CAT activity was performed using commercially available Bio-diagnostic assay kits (Giza, Egypt) according to the manufacturer's recommendations and previously published methodologies.^{18,19}

Gene expression analysis

RNA extraction was performed from EDTA-treated blood samples using the QIAamp RNA Blood Mini Kit (Qiagen, Germany) in accordance with the manufacturer's protocol. RNA quantity and purity were evaluated using a Nano

Drop ND-1000 spectrophotometer V3.1.0 (Nano Drop Technologies Inc., Wilmington, DE, USA) through assessment of A260/A280 and A260/A230 absorbance ratios. Samples demonstrating A260/A280 ratios between 1.8 and 2.1 were considered acceptable for downstream analysis.

Equivalent quantities of RNA were reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen, USA) on a Biometra T Advanced thermocycler (Analytik Jena AG, Jena, Germany) according to the supplied protocol.

Quantitative real-time PCR (qRT-PCR) amplification was conducted using SYBR Green chemistry and Maxima SYBR Green qPCR Master Mix (ThermoFisher Scientific, USA, cat. no. K0251) following the manufacturer's recommendations. Thermal cycling conditions are summarized in Supplementary Table S1. Amplification reactions were performed using the StepOne™ Real-Time PCR System (Applied Biosystems, USA) for quantification of α -KL and HIF-2 α expression levels. GAPDH served as the endogenous reference gene, and all samples were analyzed in duplicate. Melting curve analysis was used to confirm amplification specificity and exclude nonspecific amplification or primer-dimer artifacts. Cycle threshold (Ct) obtained using a uniform fluorescence threshold applied across all experimental runs.

Gene-specific primers (Willowfort, UK) were designed and validated using multiple bioinformatics tools to ensure specificity and efficiency. The nucleotide sequences of all primers used in the present analysis are summarized in Supplementary Table S2. The following platforms were used: Integrated DNA Technologies (IDT) Custom Primer Design,²⁰ NCBI Primer-BLAST,²¹ Sequence Manipulation Suite: PCR Primer Stats,²² In silico PCR amplification²³ and UCSC In silico PCR.²⁴

Relative quantification of gene expression was determined using the $2^{-\Delta\Delta CT}$ method.²⁵⁻²⁷ Experimental procedures complied with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.²⁸

Statistical analysis

All statistical evaluations were carried out using SPSS software version 25 (IBM Corp., Armonk, NY, USA). Data normality was examined with the Shapiro-Wilk test, and continuous data were summarized as mean $\pm$ SD (SD). Group comparisons were conducted using the independent samples t-test or Mann–Whitney U test according to variable distribution characteristics.

Relationships between variables were examined using Spearman's rank correlation analysis, whereas Pearson correlation was applied exclusively for graphical network visualization. Multivariate logistic regression models were constructed to determine independent predictors of anemia and hyperparathyroidism. Predictor selection was based on biological relevance together with

significant associations observed in univariate analyses. To minimize the possibility of overfitting considering the relatively limited sample size, the number of predictors entered into each model was restricted. Findings are presented as ORs with 95% CIs. Statistical significance was considered at a two-sided p-value <0.05.

Results

Demographic and clinical characteristics of the study population

The study cohort comprised 50 HD patients and 50 healthy controls with generally comparable baseline characteristics. No statistically significant differences were identified between the groups regarding age, sex distribution, or body weight (Table 1).

Table 1. Baseline demographic profile of the HD and control groups*

Parameter	HD patients (n=50)	Controls (n=50)	p
Age (years), mean±SD	35.1±17.8	34.7±16.9	0.71
Male, n (%)	25 (50%)	24 (48%)	0.84
Female, n (%)	25 (50%)	26 (52%)	0.84
Weight (kg), mean±SD	55.5±17.1	56.9±15.8	0.63

* Values are expressed as mean±SD or number (%). Group comparisons were conducted using the independent samples t-test for continuous data and the chi-square test for categorical variables, SD – standard deviation, HD – hemodialysis

Table 2. Clinical characteristics, causes of ESRD and HD-related complications among HD patients*

Parameter	HD patients (n=50)
Duration of HD (years), mean±SD	8.5±5.97
IDWG (kg), mean±SD	2.05±0.73
HCV +ve (%)	28%
Patients' co-morbidities causing ESRD	
HTN (%)	30%
DM (%)	6%
SLE (%)	6%
Unknown cause (%)	58%
ESRD and HD-related complications	
Anemia (%)	72%
Hyperparathyroidism (%)	44%
IDH (%)	18%

* Data are expressed as mean±SD or percentage (%), HD – hemodialysis, ESRD – end-stage renal disease, IDWG – interdialytic weight gain, HCV – hepatitis C virus, HTN – hypertension, DM – diabetes mellitus, SLE – systemic lupus erythematosus, IDH – intradialytic hypotension, SD – standard deviation, +ve – positive

Among HD patients, the mean HD duration was 8.5±5.97 years, whereas the mean IDWG was 2.05±0.73 kg. HCV seropositivity was detected in 28% of patients. Hypertension represented the leading identified cause of

ESRD (30%), followed by diabetes mellitus and systemic lupus erythematosus (6% each), while the underlying etiology remained unidentified in 58% of cases. Anemia was the most frequent clinical complication (72%), followed by SHPT (44%) and IDH (18%) (Table 2).

Hematological and biochemical profiles of the study groups

HD patients demonstrated significantly lower Hb, serum Ca²⁺, and Na⁺ levels than controls. Conversely, serum iron, ferritin, P, PTH, ALP, creatinine, urea, and K⁺ concentrations were significantly elevated in the HD group (Table 3).

Table 3. Hematological and biochemical findings in HD patients and healthy controls *

Parameter (mean±SD)	Control (n=50)	Patients (n=50)	p
Hematological parameters			
Hb (g/L)	130.2±13.4	97±16.3	<0.001***
MCV (fL)	88.91±5.02	84.97±7.3	0.002**
MCH (pg)	29.99±1.84	27.86±3.16	<0.001***
Iron status			
Iron (µmol/L)	7.20±0.37	10.41±3.02	<0.001***
Ferritin (µg/L)	58.92±22.27	445.8±481.84	<0.001***
Mineral metabolism			
Ca ²⁺ (mmol/L)	2.29±0.14	2.01±0.24	<0.001***
P (mmol/L)	1.11±0.19	1.51±0.41	<0.001***
PTH (pmol/L)	3.58±0.96	63.8±50.3	<0.001***
ALP (U/L)	80.2±23.36	309.75±316.79	<0.001***
Renal function			
Creatinine (µmol/L)	89.3±14.1	925.4±235.1	<0.001***
Urea (mmol/L)	2.22±0.69	19.25±6.69	<0.001***
Electrolytes			
Na ⁺ (mmol/L)	137.86±3.04	134.67±3.02	<0.001***
K ⁺ (mmol/L)	4.03±0.48	5.18±0.87	<0.001***

* Results are presented as mean±SD. Statistical comparisons were performed using the independent samples t-test or Mann–Whitney U test according to data distribution., SD – standard deviation, Hb – hemoglobin, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, PTH – parathyroid hormone, ALP – alkaline phosphatase, Na⁺ – sodium, K⁺ – potassium, Ca²⁺ – calcium, P – phosphorus

Gene expression and oxidative status

HD patients exhibited significantly lower expression levels of α-KL, HIF-2α, and CAT activity compared with controls (all p < 0.001). In contrast, MDA concentrations were significantly increased in the patient group (p < 0.001) (Fig. 1).

Associations between biomarkers and clinical parameters (Hb, PTH, and IDH)

Hb levels showed inverse associations with P, ALP, PTH, creatinine, urea, MDA, HD duration, IDWG, and IDH, whereas positive associations were observed with iron, ferritin, CAT, α-KL, and HIF-2α.

An opposite trend was observed for PTH, which demonstrated negative correlations with Hb, Ca²⁺, CAT, α-KL, and HIF-2α, while positive correlations were noted with P, ALP, creatinine, urea, MDA, HD duration, IDWG, IDH, iron, and ferritin.

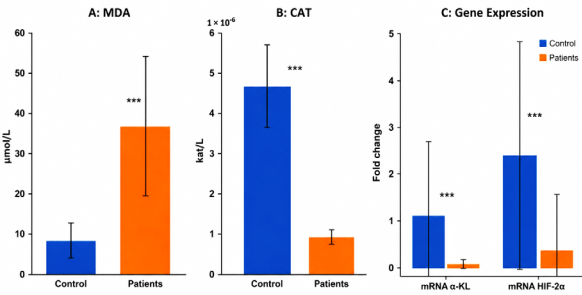


Fig. 1. Gene expression of α-KL and HIF-2α, and oxidative status in patients versus controls, A: Plasma MDA (μmol/L), B: CAT activity (kat/L), C: Relative mRNA expression of α-KL and HIF-2α in peripheral blood samples, bars represent mean±SD for control and patient groups, Group differences were evaluated using the independent samples t-test or Mann-Whitney U test, as appropriate, *** – p<0.001 indicates statistical significance, MDA – malondialdehyde, CAT – catalase, α-KL – α-Klotho, HIF-2α – hypoxia-inducible factor-2 alpha

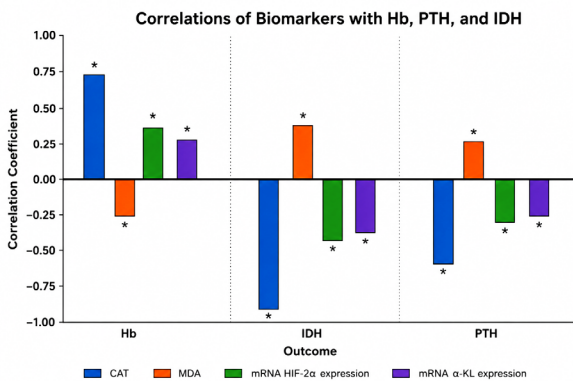


Fig. 2. Correlations between oxidative stress markers, gene expression parameters, and clinical outcomes, grouped bar chart showing correlation coefficients between MDA, CAT, mRNA α-KL expression, and mRNA HIF-2α expression with Hb, PTH, and IDH, positive and negative correlation values are shown on the y-axis, distinct colors represent different biomarkers, Correlations were analyzed using Spearman's rank correlation test, * – statistically significant correlations (p<0.05), MDA – malondialdehyde, CAT – catalase, α-KL – α-Klotho, HIF-2α – hypoxia-inducible factor-2 alpha, Hb – hemoglobin, PTH – parathyroid hormone, IDH – intradialytic hypotension

Likewise, IDH was associated with lower Hb, Ca²⁺, Na⁺, CAT, α-KL, and HIF-2α levels, whereas higher P, ALP, PTH, K⁺, creatinine, urea, MDA, dialysis duration,

and IDWG values were observed in affected patients (Figure 2, Table 4, Supplementary Table S3).

Table 4. Correlations of MDA, CAT, α-KL, and HIF-2α with clinical and biochemical parameters*

Variable		MDA	CAT	mRNA α-KL expression	mRNA HIF-2α expression
Hb	Correlation	-0.24	0.71	0.27	0.35
	p	0.016	<0.001	0.007	<0.001
Ferritin	correlation	0.078	-0.472	-0.209	-0.133
	p	0.442	<0.001	0.037	0.187
Ca ²⁺	Correlation	-0.11	0.56	0.29	0.22
	p	0.298	<0.001	0.004	0.031
P	Correlation	0.26	-0.52	-0.25	-0.11
	p	0.01	<0.001	0.014	0.296
PTH	Correlation	0.26	-0.62	-0.27	-0.3
	p	0.01	<0.001	0.006	0.002
ALP	Correlation	0.14	-0.43	-0.19	-0.23
	p	0.162	<0.001	0.061	0.021
Creatinine	Correlation	0.4	-0.89	-0.4	-0.41
	p	<0.001	<0.001	<0.001	<0.001
MDA	Correlation	1	-0.36	-0.15	-0.19
	p		<0.001	0.148	0.063
CAT	Correlation	-0.36	1	0.38	0.38
	p	<0.001		<0.001	<0.001
mRNA α-KL expression	Correlation	-0.15	0.38	1	0.21
	p	0.148	<0.001		0.032
mRNA HIF-2α expression	Correlation	-0.19	0.38	0.21	1
	p	0.063	<0.001	0.032	
IDH	Correlation	0.373	-0.916	-0.402	-0.436
	p	<0.001	<0.001	<0.001	<0.001

* Correlations involving MDA (μmol/L), CAT (kat/L), mRNA α-KL expression, and mRNA HIF-2α expression with clinical and biochemical parameters, Spearman's rank correlation analysis was applied to examine relationships between variables, and the results are expressed as correlation coefficients (r) and corresponding p-values, Hb – hemoglobin, Ca²⁺ – calcium, P – phosphorus, PTH – parathyroid hormone, ALP – alkaline phosphatase, MDA – malondialdehyde, CAT – catalase, α-KL – Klotho, HIF-2α – hypoxia-inducible factor, IDH – intradialytic hypotension

Correlation analysis

Oxidative stress markers demonstrated significant relationships with several clinical and biochemical parameters. MDA showed positive correlations with PTH (r=0.26, p=0.01), creatinine, HD duration, and IDH (r=0.37, p<0.001), while inverse correlations were observed with Hb (r=-0.24, p=0.016) and CAT (r=-0.36, p<0.001). CAT was positively associated with Hb (r=0.71, p<0.001), α-KL, and HIF-2α expression (r=0.38, p<0.001 for both), whereas negative associations were

identified with PTH ($r=-0.62$, $p<0.001$), MDA, HD duration, and IDH ($r=-0.916$, $p<0.001$).

HIF-2 α expression demonstrated positive correlations with Hb ($r=0.35$, $p<0.001$), Ca²⁺, CAT, and α -KL expression, while inverse correlations were observed with PTH ($r=-0.3$, $p=0.002$), creatinine, HD duration, IDH ($r=-0.44$, $p<0.001$), and IDWG. Similarly, α -KL expression was positively associated with Hb ($r=0.27$, $p=0.007$), CAT ($r=0.38$, $p<0.001$), and HIF-2 α , whereas negative correlations were identified with P, PTH ($r=-0.27$, $p=0.006$), creatinine, HD duration, IDH ($r=-0.4$, $p<0.001$), and IDWG. Detailed correlation coefficients and significance values are presented in Figure 2, Table 4 and Supplementary Table S3.

Correlation network

To further explore the relationships among OS and gene expression markers, a graphical correlation network was constructed (Figure 3). α -KL, HIF-2 α , and CAT formed a closely related cluster, whereas MDA demonstrated inverse relationships with CAT and HIF-2 α . In addition, α -KL and HIF-2 α exhibited a positive correlation pattern, suggesting potential interrelated regulatory mechanisms within oxidative stress pathways. These findings were generally consistent with the correlation matrix presented in Table 4 and Supplementary Table S3.

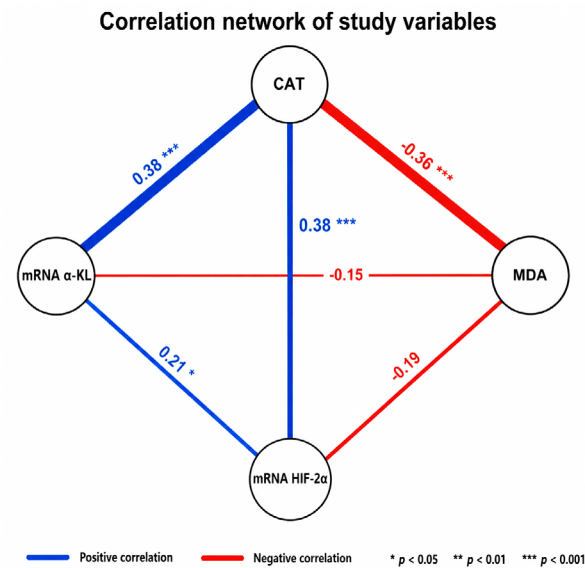


Fig. 3. Correlation network of study variables, graphical representation of the correlations among MDA, CAT, mRNA α -KL, and mRNA HIF-2 α , Pearson correlation coefficients were used for network visualization, Line colors indicate correlation direction (blue, positive correlation; red, negative correlation), while edge thickness reflects correlation strength ($|r|$), numbers indicate correlation coefficients, and statistical significance is indicated by asterisks (* – $p<0.05$, *** – $p<0.001$), MDA – malondialdehyde, CAT – catalase, α -KL – α -Klotho, HIF-2 α – hypoxia-inducible factor-2 alpha

Multivariate logistic regression analysis identified reduced α -KL, HIF-2 α , and CAT levels as independent predictors of anemia. In addition, SHPT was independently associated with lower α -KL, HIF-2 α , and CAT levels, together with higher MDA concentrations (Supplementary Table S4).

Discussion

ESRD patients undergoing HD commonly present with anemia, SHPT, and IDH, accompanied by disturbances in oxidative and hormonal homeostasis. In the present study, anemia was identified in 72% of patients, SHPT in 44%, and IDH in 18%, findings that are generally consistent with previous reports among HD populations.²⁹⁻³¹ These observations confirm the persistent burden of these complications in ESRD and provide the clinical context for interpreting the molecular and oxidative alterations observed in this study.

α -Klotho in ESRD: correlations and clinical significance

α -KL mRNA expression was markedly reduced in HD patients, consistent with previous reports demonstrating progressive KL downregulation across CKD stages.³² α -KL showed positive correlations with Hb, Ca²⁺, Na⁺, CAT, and HIF-2 α , and negative correlations with iron, ferritin, P, PTH, ALP, K⁺, creatinine, urea, HD duration, IDH, and IDWG.

These associations are consistent with the established role of α -KL in mineral metabolism and its relationship with erythropoiesis-related pathways. The inverse correlations with phosphorus and PTH agree with its recognized involvement in phosphate homeostasis and regulation of mineral balance.^{33,34} The observed associations of α -KL with Na⁺ and K⁺ are also consistent with previous reports suggesting links between α -KL expression and pathways involved in electrolyte regulation.^{35,36}

Furthermore, the inverse relationship with IDH is in line with reports associating reduced α -KL levels with vascular dysfunction and impaired cardiovascular health.³⁷ The positive association with CAT also supports a link between α -KL and antioxidant defense mechanisms.³⁸ Collectively, these findings suggest that reduced α -KL expression is associated with disturbances in mineral metabolism, oxidative balance, and dialysis-related complications and may represent part of a broader network involving HIF-2 α and OS markers.

HIF-2 α and its role in erythropoiesis and mineral disorders

HIF-2 α expression was significantly reduced in HD patients and showed positive correlations with Hb, Ca²⁺, Na⁺, CAT, and α -KL, while negative correlations were observed with PTH, ALP, K⁺, creatinine, urea, dialysis duration, IDH, and IDWG.

The positive association between HIF-2 α and Hb is consistent with its established role in hypoxia-responsive erythropoiesis and iron utilization.¹² Its correlation with α -KL is consistent with experimental findings suggesting a link between α -KL and HIF-2 α pathways.³³ Associations with electrolyte parameters and IDH may reflect links with adaptive responses to metabolic and hemodynamic stress, although the underlying mechanisms remain incompletely understood. In addition, previous studies have implicated HIF-2 α in bone remodeling processes, which may explain its association with markers of mineral metabolism.³⁹

Overall, the observed associations support a potential link between HIF-2 α , α -KL, and oxidative stress pathways in ESRD, highlighting their possible involvement in oxygen sensing, redox balance, and mineral metabolism.

Therapeutic perspective: hypoxia-inducible factor–prolyl hydroxylase inhibitors (HIF-PHIs)

HIF-PHIs have emerged as a promising therapeutic option for renal anemia through activation of HIF signaling, stimulation of endogenous erythropoietin production, and improvement of iron utilization. Their potential effects on inflammation and oxidative stress remain under investigation.⁴⁰

Oxidative stress: antioxidant defense

A marked oxidative imbalance was evident in HD patients. CAT activity was significantly reduced, whereas MDA levels were significantly elevated. CAT was positively correlated with Hb, Ca²⁺, Na⁺, α -KL, and HIF-2 α and negatively correlated with several markers of disease burden. In contrast, MDA showed the opposite pattern of associations.

These findings indicate a close association between OS, anemia, mineral metabolism abnormalities, and hemodynamic instability. The inverse relationship between CAT and MDA reflects impaired antioxidant defense and increased oxidative burden in ESRD, consistent with previous reports.⁴¹

Inflammation and OS interplay

The observed increase in MDA together with reduced CAT activity may also reflect an underlying pro-inflammatory state. Inflammation and OS are closely interconnected and have been implicated in impaired erythropoiesis and CKD progression.^{42,43} Although inflammatory markers were not directly measured, the observed biochemical profile is compatible with enhanced inflammatory activity. Reduced α -KL expression has also been associated with increased inflammatory signaling.⁴⁴

Previous studies have demonstrated a bidirectional interaction between OS and inflammation during CKD progression.^{42,43,45,46} Experimental evidence showing at-

tenuation of oxidative injury and inflammation following KL supplementation further supports the biological relevance of the associations observed in the present study.⁴⁷

Applicability to peritoneal dialysis (PD)

Although the present study focused on HD patients, some of the observed molecular associations may also be relevant in PD. However, differences between dialysis modalities may influence these relationships, and direct evaluation in PD populations is required.^{48,49}

Overall, a key finding of this study was the concurrent reduction of α -KL, HIF-2 α , and antioxidant activity together with increased OS, accompanied by significant interrelationships among these markers. The observed associations suggest the involvement of interconnected pathways related to oxidative balance, erythropoiesis, mineral metabolism, and dialysis-related complications in ESRD. However, given the cross-sectional design, these findings should be interpreted as associations rather than evidence of causality, and further longitudinal studies are required to clarify the nature of these interactions.

Study limitations

This study is limited by its cross-sectional design, single-center recruitment, and relatively modest sample size. In addition, α -KL and HIF-2 α were evaluated at the mRNA level, while inflammatory and treatment-related variables were not specifically analyzed. Nevertheless, the findings provide novel insights into the associations among α -KL, HIF-2 α , OS markers, and HD-related complications. Further studies are warranted to validate these observations.

Conclusion

This study identified significant associations among α -KL, HIF-2 α , and OS markers in ESRD patients undergoing HD. Reduced α -KL and HIF-2 α expression, together with decreased CAT activity and elevated MDA levels, were associated with anemia, SHPT, and IDH. The observed interrelationships among these biomarkers highlight potential links between oxidative balance, erythropoiesis, mineral metabolism, and dialysis-related complications. Further large-scale prospective studies are needed to confirm these associations and clarify their clinical significance.

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Supplementary material

Supplementary Tables associated with this article are provided in a single PDF file.

- **Supplementary Table S1** describes the thermal cycling protocol applied using the StepOne™ Real-Time PCR System.
- **Supplementary Table S2** presents the nucleotide sequences of primers used for real-time PCR analysis.
- **Supplementary Table S3** shows the correlation matrix between all independent variables, with statistically significant correlations defined at $p < 0.05$.
- **Supplementary Table S4** presents Multivariate logistic regression analysis identifying independent predictors of anemia and hyperparathyroidism in hemodialysis patients.

Declarations

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Author contributions

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

Conflicts of interest

The authors declare that they have no conflicts of interest relevant to this article.

Data availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

Ethics approval

This study was approved by the Research Ethics Committee of the Faculty of Medicine, Suez Canal University (Approval No. 4724).

Use of AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, QuillBot was used by H.M.Z. for language refinement and paraphrasing of selected text sections. All content was carefully reviewed and edited by the authors, who take full responsibility for the final version of the manuscript.

References

1. Lv JC, Zhang LX. Prevalence and disease burden of chronic kidney disease. *Adv Exp Med Biol.* 2019;1165:3-15. doi:10.1007/978-981-13-8871-2_1
2. Zahran A, Ahmed H, Issawi R. Prevalence and etiology of end-stage renal disease patients on maintenance hemodialysis. *Menoufia Med J.* 2020;33(3):766-771. doi:10.4103/mmj.mmj_395_18
3. Hashmi MF, Benjamin O, Lappin SL. End-stage renal disease. In: StatPearls [Internet]. StatPearls Publishing; 2023. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK499861/>
4. Agarwal A, Anjum F. Hemodialysis. In: StatPearls [Internet]. StatPearls Publishing; 2026. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK563296/>
5. Wang Y, Yu X. Stabilizing hypoxia-inducible factor to manage anemia in chronic kidney disease: from basic theory to clinical study. *Kidney Dis (Basel).* 2024;10(2):132-142. doi:10.1159/000536039
6. Hamrahian SM, Vilayet S, Herberth J, Fülöp T. Prevention of intradialytic hypotension in hemodialysis patients: current challenges and future prospects. *Int J Nephrol Renovasc Dis.* 2023;16:173-181. doi:10.2147/IJNRD.S245621
7. Liakopoulos V, Roumeliotis S, Zarogiannis S, Eleftheriadis T, Mertens PR. Oxidative stress in hemodialysis: Causative mechanisms, clinical implications, and possible therapeutic interventions. *Semin Dial.* 2019;32(1):58-71. doi:10.1111/sdi.12745
8. Rysz J, Franczyk B, Ławiński J, Gluba-Brzózka A. Oxidative stress in ESRD patients on dialysis and the risk of cardiovascular diseases. *Antioxidants.* 2020;9(11):1079. doi:10.3390/antiox9111079
9. Hong YA, Park CW. Catalytic antioxidants in the kidney. *Antioxidants.* 2021;10(1):130. doi:10.3390/antiox10010130
10. Piko N, Bevc S, Hojs R, Ekart R. The role of oxidative stress in kidney injury. *Antioxidants.* 2023;12(9):1772. doi:10.3390/antiox12091772
11. Fan Z, Wei X, Zhu X, et al. Correlation between soluble klotho and chronic kidney disease–mineral and bone disorder in chronic kidney disease: a meta-analysis. *Sci Rep.* 2024;14:4477. doi:10.1038/s41598-024-54812-4
12. Foresto-Neto O, da Silva ARPA, Cipelli M, Santana-Novelli FPR, Camara NOS. The impact of hypoxia-inducible factors in the pathogenesis of kidney diseases: a link through cell metabolism. *Kidney Res Clin Pract.* 2023;42(5):561-578. doi:10.23876/j.krcp.23.012
13. Kidney disease: Improving Global Outcomes (KDIGO) anemia work group. KDIGO 2026 Clinical practice guideline for the management of anemia in chronic kidney disease (CKD). *Kidney Int.* 2026;109(1S):S1-S99. doi:10.1016/j.kint.2025.06.006
14. Kidney disease: Improving Global Outcomes (KDIGO) CKD-MBD Update Work Group. KDIGO 2017 clinical practice guideline update for the diagnosis, evaluation, prevention, and treatment of chronic kidney disease-mi-

- neral and bone disorder (CKD-MBD). *Kidney Int Suppl* (2011). 2017;7(1):1-59. doi:10.1016/j.kisu.2017.04.001
15. Ye Y, Liu H, Chen Y, et al. Hemoglobin targets for the anemia in patients with dialysis-dependent chronic kidney disease: a meta-analysis of randomized, controlled trials. *Ren Fail.* 2018;40(1):671-679. doi:10.1080/0886022X.2018.1532909
 16. Kidney Disease Outcomes Quality Initiative (KDOQI). Clinical practice guidelines for cardiovascular disease in dialysis patients. *Am J Kidney Dis.* 2005;45(3 Pt 2):1-153. doi:10.1053/j.ajkd.2005.01.019
 17. Valentini J, Grotto D, Paniz C, Roehrs M, Burg G, Garcia SC. The influence of the hemodialysis treatment time under oxidative stress biomarkers in chronic renal failure patients. *Biomed Pharmacother.* 2008;62(6):378-382. doi:10.1016/j.biopha.2007.10.017
 18. Abogresha NM, Mohammed SS, Hosny MM, Abdallah HY, Gadallah AM, Greish SM. Diosmin mitigates cyclophosphamide induced premature ovarian insufficiency in rat model. *Int J Mol Sci.* 2021;22(6):3044. doi:10.3390/ijms22063044
 19. Hadwan MH, Hussein MJ, Mohammed RM, et al. An improved method for measuring catalase activity in biological samples. *Biol Methods Protoc.* 2024;9(1):bpae015. doi:10.1093/biomethods/bpae015
 20. Integrated DNA Technologies (IDT). Custom Primer Design. 2024. Available from: <https://eu.idtdna.com/page/products/qpcr-and-pcr/custom-primers>
 21. National Center for Biotechnology Information (NCBI). Primer-BLAST. 2024. Available from: <https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>
 22. Stothard P. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques.* 2000;28(6):1102-1104. doi:10.2144/00286ir01
 23. San Millán RM, Martínez-Ballesteros I, Rementeria A, Garaizar J, Bikandi J. Online exercise for the design and simulation of PCR and PCR-RFLP experiments. *BMC Res Notes.* 2013;6:513. doi:10.1186/1756-0500-6-513
 24. Casper J SM, Raney BJ, Perez G, Nassar LR, Lee CM, Hinrichs AS, et al. The UCSC Genome Browser database: 2026 update. *Nucleic Acids Res.* 2026;54(D1):D1331-D1335. doi:10.1093/nar/gkaf1250
 25. Zalewski D, Bogucka-Kocka A. RQdeltaCT: an open-source R package for relative quantification of gene expression using delta Ct methods. *Sci Rep.* 2025;15(1):29762. doi:10.1038/s41598-025-11822-0
 26. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc.* 2008;3(6):1101-1108. doi:10.1038/nprot.2008.73
 27. Edmunds RC, McIntyre JK, Luckenbach JA, Baldwin DH, Incardona JP. Toward enhanced MIQE compliance: reference residual normalization of qPCR gene expression data. *J Biomol Tech.* 2014;25(2):54-60. doi:10.7171/jbt.14-2502-003
 28. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 2009;55(4):611-622. doi:10.1373/clinchem.2008.112797
 29. Farag YMK, El-Sayed E. Global dialysis perspective: Egypt. *Kidney360.* 2022;3(7):1263-1268. doi:10.34067/kid.0007482021
 30. Wang Y, Liu J, Fang Y, Zhou S, Liu X, Li Z. Estimating the global prevalence of secondary hyperparathyroidism in patients with chronic kidney disease: a systematic review. *Front Endocrinol (Lausanne).* 2024;15:1400891. doi:10.3389/fendo.2024.1400891
 31. Arcentales-Vera K, Vera-Mendoza MF, Cevallos-Salas C, García-Aguilera MF, Fuenmayor-González L. Prevalence of cardiovascular instability during hemodialysis therapy in hospitalized patients: a systematic review and meta-analysis. *Sci Prog.* 2024;107(4):368504241308982. doi:10.1177/00368504241308982
 32. Li SS, Sheng MJ, Sun ZY, Liang Y, Yu LX, Liu QF. Upstream and downstream regulators of Klotho expression in chronic kidney disease. *Metabolism.* 2023;142:155530. doi:10.1016/j.metabol.2023.155530
 33. Li F, Ye X, Yang G, et al. Relationships between blood bone metabolic biomarkers and anemia in patients with chronic kidney disease. *Ren Fail.* 2023;45:2210227. doi:10.1080/0886022X.2023.2210227.
 34. Razzaque MS. Interactions between FGF23 and vitamin D. *Endocr Connect.* 2022;11(10):e220239. doi:10.1530/EC-22-0239
 35. Hu JW, Chu C, Shi T, Yan Y, Mu JJ. Effects of salt intervention on serum levels of Klotho influenced by salt sensitivity. *J Clin Hypertens.* 2020;22(11):2051-2058. doi:10.1111/jch.14044
 36. Jung HJ, Pham TD, Su XT, et al. Klotho is highly expressed in the chief sites of regulated potassium secretion, and it is stimulated by potassium intake. *Sci Rep.* 2024;14:10740. doi:10.1038/s41598-024-61481-w
 37. Liu L, Jia J, Cheng X, et al. The optimal cut-off values of Klotho for predicting all-cause and cardiovascular mortality among chronic kidney disease patients: results from NHANES. *Sci Rep.* 2024;14:4647. doi:10.1038/s41598-024-52701-4
 38. Typiak M, Piwkowska A. Antiinflammatory actions of klotho: implications for therapy of diabetic nephropathy. *Int J Mol Sci.* 2021;22(2):956. doi:10.3390/ijms22020956
 39. Lee SY, Park KH, Yu HG, et al. Controlling hypoxia-inducible factor-2α is critical for maintaining bone homeostasis in mice. *Bone Res.* 2019;7:14. doi:10.1038/s41413-019-0050-4
 40. Mima A. Hypoxia-inducible factor-prolyl hydroxylase inhibitors for renal anemia in chronic kidney disease: advantages and disadvantages. *Eur J Pharmacol.* 2021;912:174583. doi:10.1016/j.ejphar.2021.174583
 41. Navarro-García JA, Rodríguez-Sánchez E, Aceves-Ripoll J, et al. Oxidative status before and after renal replace-

- ment therapy: differences between conventional high-flux hemodialysis and online hemodiafiltration. *Nutrients*. 2019;11(11):2809. doi:10.3390/nu11112809
42. Stenvinkel P. Inflammation in end-stage renal disease: the hidden enemy. *Nephrology (Carlton)*. 2006;11(1):36-41. doi:10.1111/j.1440-1797.2006.00541.x
43. Ebert T, Neytchev O, Witasp A, Kublickiene K, Stenvinkel P, Shiels PG. Inflammation and oxidative stress in chronic kidney disease and dialysis patients. *Antioxid Redox Signal*. 2021;35(17):1426-1448. doi:10.1089/ars.2020.8184
44. Kuro-o M. Klotho and aging. *Biochim Biophys Acta*. 2009;1790(10):1049-1058. doi:10.1016/j.bbagen.2009.02.005
45. Mima A. Inflammation and oxidative stress in diabetic nephropathy: new insights on its inhibition as new therapeutic targets. *J Diabetes Res*. 2013;2013:248563. doi:10.1155/2013/248563
46. Mima A, Yasuzawa T, King GL, Ueshima S. Obesity-associated glomerular inflammation increases albuminuria without renal histological changes. *FEBS Open Bio*. 2018;8(4):664-670. doi:10.1002/2211-5463.12400
47. Fu Y, Cao J, Wei X, Ge Y, Su Z, Yu D. Klotho alleviates contrast-induced acute kidney injury by suppressing oxidative stress, inflammation, and NF-κB/NLRP3-mediated pyroptosis. *Int Immunopharmacol*. 2023;118:110105. doi:10.1016/j.intimp.2023.110105
48. Liakopoulos V, Roumeliotis S, Gorny X, Eleftheriadis T, Mertens PR. Oxidative stress in patients undergoing peritoneal dialysis: a current review of the literature. *Oxid Med Cell Longev*. 2017;2017:3494867. doi:10.1155/2017/3494867
49. Lu J, Shi D, Zhao X, Xi M, Qi H, He Q. Comparative analysis of efficacy and prognosis of hemodialysis and peritoneal dialysis for end-stage renal disease: a meta-analysis. *Comput Math Methods Med*. 2022;2022:6007698. doi:10.1155/2022/6007698



ORIGINAL PAPER

Vitamin C mitigates oxidative stress and preserves platelet efficacy during prolonged storage – an in vitro study

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ABSTRACT

Introduction and aim. Platelets stored at 22–24°C beyond 3–5 days undergo oxidative changes leading to storage lesions (PSL). Platelet additive solutions (PAS) improve platelet shelf-life, and antioxidant additives in PAS can mitigate PSL. This in vitro study evaluated the impact of Vitamin C (VC), a potent free radical scavenger, as an antioxidant additive in PAS-SSP+ on the redox status and functional integrity of rat platelets during prolonged storage.

Material and methods. Platelets from male *Wistar* rats (n=5) were grouped into controls (SSP+) and 10-VC (10mM VC in SSP+) and stored at 22–24°C for 11 days. Markers of antioxidant and oxidative status, platelet function and metabolism were investigated on days 1, 4, 7 and 11.

Results. Superoxide radicals, thiobarbituric acid reactive substances, and advanced oxidation protein products were significantly lower in 10-VC than controls. Superoxide dismutase and catalase were maintained whereas, glutathione was higher by 27% (p<0.01) and protein sulfhydryls by 65% (p<0.01) on day 7 than controls. Total antioxidant capacity was significantly higher than controls throughout storage in 10-VC. P-selectin lowered by 58% (p<0.001) on day 7 than controls while aggregation and cell viability were preserved in 10-VC.

Conclusion. 10-VC reduced oxidative stress by scavenging reactive species, inhibiting lipid and protein oxidation, augmenting antioxidant capacity, and preserving platelet functions and viability. Thus, VC in SSP+ maintained redox status and improved platelet efficacy, highlighting its potential as an additive in PAS for preserving platelet integrity during prolonged storage.

Keywords. antioxidants, blood platelets, oxidative stress, platelet banking, vitamin C

Introduction

Platelet transfusion is vital for treating thrombocytopenia. The development of platelet storage lesions (PSL) in plasma at 22–24°C limits platelet shelf-life to 3–5 days thereby, rendering them unfit for transfusion.^{1,2} Replacement of plasma with platelet additive solutions (PAS) such as SSP+ have been proven to mitigate PSL and prolong shelf-life for up to 9 days.^{3–6}

Platelet metabolism generates reactive oxygen species (ROS) leading to oxidative stress (OS) and

suppressing endogenous antioxidant defenses.⁷ OS promotes platelet activation, lipid and protein oxidation thereby, reducing platelet efficacy during storage.⁸ It also contributes to shedding of platelet receptors such as glycoprotein VI (GPVI) affecting platelet adhesion to collagen while altered integrin function caused by OS, impairs platelet aggregation.^{9–11} Studies have proven that antioxidant additives have mitigated OS and enhanced the quality of stored platelets.^{12–20} Antioxidants/ROS scavengers have the potential to preserve platelet func-

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tions such as GPVI-mediated adhesion and platelet response to agonists during storage.^{21,22} These studies have predominantly focused on the effects of antioxidants on metabolic stability, functional integrity, mitochondrial damage and apoptosis rather than maintaining redox homeostasis during platelet storage. Our previous studies have reported oxidative modulations occurring in platelets stored in PAS with antioxidant additives such as N-acetyl cysteine and caffeic acid.^{23,24}

Vitamin C (VC) is a potent ROS scavenger which inhibits lipid peroxidation and regenerates vitamin E and glutathione.²⁵⁻²⁷ VC is known to inhibit ROS-mediated platelet activation by specifically inhibiting CD40L ligand on platelets without affecting agonist-induced aggregation.²⁸ Mohammed et al. studied the impact of VC on functions of saline-stored platelets.²⁹ However, the influence of VC on oxidative modulations in stored platelets remains unclear. Continuous variations in platelet efficacy have not been studied in terms of redox status and antioxidant capacity to mitigate PSL at different time periods during storage. Previous studies have not assessed VC as an additive in SSP+ with continuous evaluation of redox status and platelet functions during extended storage. Thus, this study investigates the influence of VC on the continuous oxidative modulations and platelet efficacy during extended storage in PAS, SSP+. This is the first report on the responses of platelets to VC as an additive in SSP+ during extended storage period.

Wistar rats were studied as an exploratory model to gain insights into the platelet responses. Rat and human platelets share similar physiology although minor variations in receptor distribution may influence functional responses.

Aim

This study aimed to continuously monitor the progression of PSL, with the primary objective of evaluating the effects of VC on oxidative stress and redox status during extended storage, and the secondary objective of assessing its influence on platelet function, viability and metabolic status. To the best of authors' knowledge, this is the first in vitro study to evaluate VC as an additive in SSP+ for stored rat platelets during an extended storage period with continuous assessment of redox and functional parameters.

Material and methods

All experimental procedures were conducted in compliance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA) provided by the Institutional Animal Ethics Committee (IAEC), New Delhi, India. Whole blood samples were collected by cardiac puncture method under anesthesia in accordance with the approved animal care protocols of CCSEA. Animals were procured from Vertebrates (2138/PO/RcBiBt/S/21/CPCSEA) dated 1st Au-

gust 2024. Blood sampling was performed at Nargund College of Pharmacy according to the institutional ethics regulations (1051/PO/Re/S/07/CPCSEA).

Chemicals

2'-dichlorodihydrofluorescein diacetate (DCFH-DA), cytochrome c, thiobarbituric acid and adenine triphosphate (ATP) standard were purchased from Sisco Research Laboratories Pvt Ltd, India. Epinephrine, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), bathocuproinedisulfonic acid disodium salt (BCS), nicotinamide adenine dinucleotide (NADH), Griess reagent and collagen were purchased from Sigma Aldrich chemicals, Mumbai, India. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reagent (MTT), bovine serum albumin (BSA) and Vitamin C were purchased from HiMedia Laboratories Pvt Ltd, Mumbai, India. All other chemicals and solvents were reagent and spectral grade, respectively.

Autospan Liquid Gold Glucose kit, Arkray, Mumbai, India (Catalog no. 93LS100-75), Rat P-selectin GENLISA™ ELISA kit, KRISHGEN Biosystems, Mumbai, India (Ref: KLR2228), Rat Nicotinamide Adenine Dinucleotide Phosphate Oxidase 2 (NOX2) GENLISA™ ELISA kit, KRISHGEN Biosystems, Mumbai, India (Ref: KLR5069).

Blood sampling

Male *Wistar* rats (4 months old) were anesthetized with ketamine hydrochloride and approximately 4-5mL of whole blood was obtained by cardiac puncture and collected into tubes containing CPDA-1 (citrate phosphate dextrose adenine-1) anticoagulant.³⁰

Platelet isolation

Whole blood was subjected to centrifugation at 190g for 15min at room temperature to obtain platelet-rich plasma (PRP). The PRP was subsequently centrifuged at 2500g for 15min at 22°C.³⁰ The platelet pellet obtained was resuspended in SSP+ (pH 7.4) with 30% residual plasma. Platelet concentrates were prepared by resuspending the platelet pellet in SSP+ (1:60).

Experimental design

VC was optimized using ROS assay using DCFH-DA method with different concentrations – 3, 5 and 10mM. VC at 10mM (10-VC) exhibited maximum inhibition of ROS (Fig. S1) and was selected for further analysis. Platelet preparations obtained from individual animals served as experimental units (n=5 in each group). Platelets were grouped into i) Control: SSP+ without VC and ii) Experimental: 10-VC in SSP+. The samples were stored in polypropylene tubes under mild agitation at 22°C for 11 days. Oxidative stress, antioxidant status, platelet function, viability and metabolism were analyzed on storage days 1, 4, 7 and 11.

ROS DCFH-DA assay

A stock solution, 20 mM DCFH-DA, was prepared in methanol and diluted in Tyrode's buffer to obtain a 75 μ M working solution. Platelets were incubated at 37°C with 25 μ M DCFH-DA for 30 min and the fluorescence was read at 485 nm (excitation) and 520 nm (emission).³¹

Antioxidant status

The antioxidant status of stored platelets was evaluated by measuring superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and total antioxidant capacity (TAC).

SOD activity was determined by monitoring the inhibition of epinephrine oxidation in carbonate buffer (0.05 M, pH 10.2) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA) at 480nm and expressed as the amount of enzyme required to achieve 50% inhibition of epinephrine oxidation.³²

CAT activity was determined by treating the samples with absolute ethanol followed by incubation in an ice bath for 30 min and addition of phosphate buffer and 0.066 M hydrogen peroxide (H_2O_2). The absorbance was read at 240 nm using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.³³

GSH levels were estimated after treating the samples with 4% sulfosalicylic acid and centrifugation at 2500 g for 15 min; the resulting supernatant was treated with 10 mM DTNB, and absorbance was recorded at 412 nm.³⁴

TAC was assessed using BCS (0.25 mM), copper sulphate (0.5 mM) and EDTA (0.01 M). The absorbance was read at 490 nm, using uric acid as standard.³⁵

Oxidative stress

Oxidative status was evaluated by measuring superoxide and nitrite radicals, thiobarbituric acid reactive substances (TBARS), advanced oxidation protein products (AOPP) and protein sulfhydryls (SH).

Superoxide radicals were determined by monitoring cytochrome C reduction following incubation of stored platelets with cytochrome C (160 μ M) at 37°C, with absorbance measured at 550 nm.²⁶

Nitrite levels were estimated using Griess reagent after incubation in the dark at room temperature for 30 min. The absorbance was recorded at 548 nm using sodium nitrite as the standard.³⁶

Lipid peroxidation was measured by assessing TBARS by treating platelets with 20% cold trichloroacetic acid in 0.6 M hydrochloric acid, followed by reaction with 0.12 M thiobarbituric acid. After incubation in a boiling water bath for 15min, absorbance was measured at 532 nm.³⁷

Protein oxidation was evaluated by determining AOPP and SH levels.

AOPP was estimated by treating the samples with isotonic phosphate buffer, 1.16 M potassium iodide and glacial acetic acid. The absorbance was read at 340nm with an extinction coefficient of 26 $mM^{-1}cm^{-1}$.³⁸

SH content was determined using the DTNB method where platelets were treated with 0.08 M sodium phosphate buffer containing sodium dodecyl sulphate (2%) and sodium EDTA (0.5 mg/mL). After addition of DTNB, the color developed was recorded at 412 nm and SH content was estimated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$.³⁹

Platelet function

Platelet functions were evaluated by measuring NOX2 levels, P-selectin release (platelet activation), platelet aggregation and ATP secretion.

NOX2 and P-selectin were quantified using sandwich-ELISA kits specific for rat NOX2 (KRISHGEN Biosystems; Ref: KLR5069) and rat P-selectin (KRISHGEN Biosystems; Ref: KLR2228), respectively following the manufacturer's instructions. The absorbance was recorded at 450 nm.²³

Platelet aggregation was determined by incubating stored platelets with and without collagen (2 μ g/mL) at 37°C, followed by measurement of absorbance at 405 nm in shaking mode for 1.5–2 min.⁴⁰ % aggregation was calculated according to Tamang et al.⁴¹

ATP secretion was assessed after incubation of stored platelets with collagen (2 μ g/mL) at 37°C, followed by treatment with 1.2 M perchloric acid. Absorbance was measured at 260 nm and ATP levels were quantified using an ATP standard.⁴²

Platelet viability

Samples were incubated with MTT reagent (0.5 mg/mL) for 4 h at 37°C, followed by the addition of dimethyl sulfoxide and overnight incubation at 37°C. Absorbance was recorded at 570 nm.⁴³

Protein estimation

Protein was estimated according to Lowry et al. to determine the protein content of the samples. Stored platelets were treated with alkaline copper sulphate and Folin's reagent. The absorbance was measured at 660 nm.⁴⁴

Platelet metabolism

Platelet metabolism during storage was evaluated by measuring pH, levels of extracellular glucose and lactate dehydrogenase (LDH).

The pH of the samples was assessed using Fisher Scientific pH indicator strips.⁴⁵

Extracellular glucose present in the storage medium was quantified by the glucose oxidase-peroxidase (GOD-POD) method according to the manufacturer's instructions provided in the Autospan Gold kit, and absorbance was read at 546 nm.⁴⁶

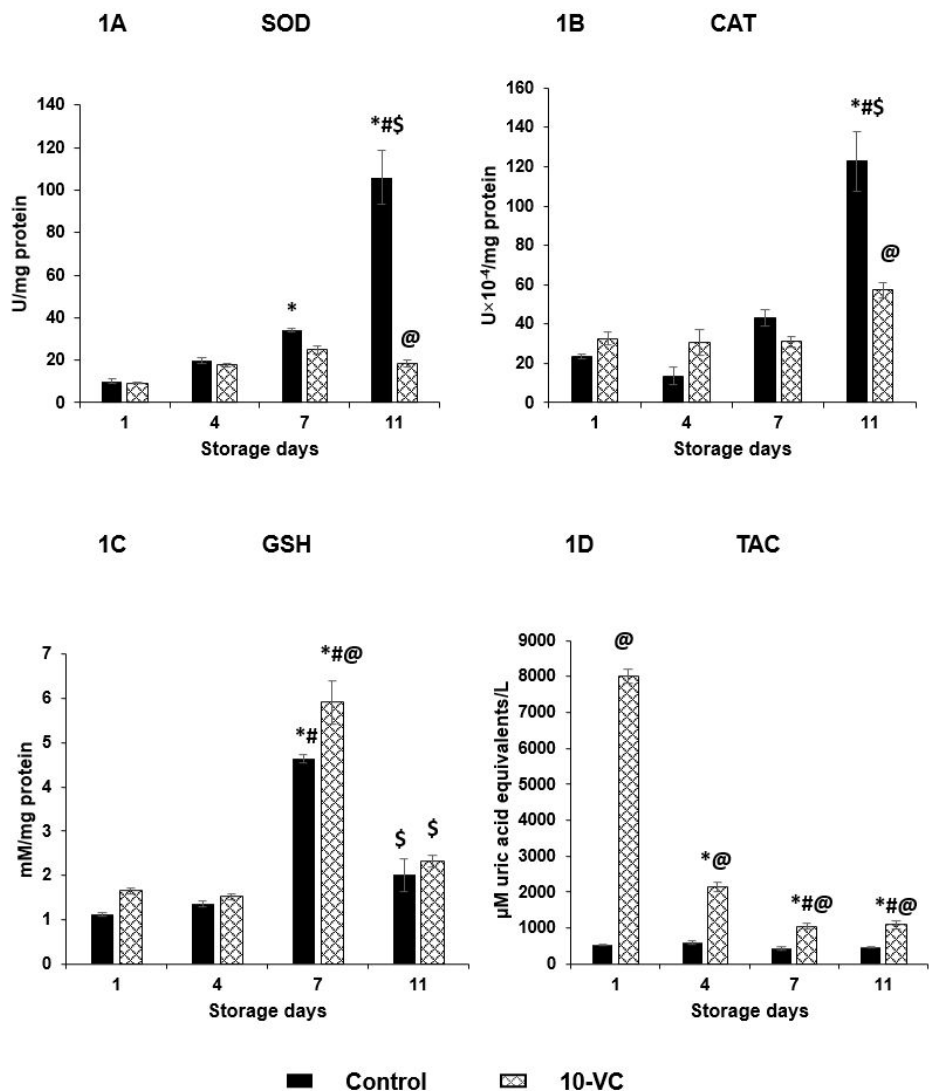


Fig. 1. Antioxidant status of platelets stored with VC, A: SOD, B: CAT, C: GSH, D: TAC (* – significant with day 1, # – significant with day 4, \$ – significant with day 7, @ – significant in 10-VC against controls)

LDH was determined following incubation of stored platelets with a reaction mixture containing Reagent 1 (80 mM Tris, 200 mM sodium chloride and 1.6 mM pyruvate) and Reagent 2 (0.2 mM NADH) at 37°C. Absorbance was recorded at 340 nm.⁴⁷

Statistical analyses

The data was assessed for normality (Shapiro-Wilk test) and passed the normality test. Results are presented as Mean with standard error from five independent biological samples (n=5). GraphPad Prism 8 software was used to perform two-way ANOVA (repeated-measures) and Bonferroni *post hoc* test to evaluate the effects between the storage days and the experimentals. Differences were considered statistically significant at $p < 0.05$. Pearson's correlation analysis was also performed to determine the relationships between superoxide radicals and SOD, CAT and SOD, and GSH and SH. Correlation coefficients (r) and corresponding p -values were calculated.

Results

Results are presented as i) changes between the storage days and ii) changes between the experimentals on a particular day of storage.

Antioxidant defenses

SOD

SOD was maintained in 10-VC whereas, it increased by twofold ($p < 0.05$) and ninefold ($p < 0.0001$) on day 7 and day 11 with respect to day 1 in controls.

SOD was lower by 82% ($p < 0.0001$) on day 11 in 10-VC compared to controls (Fig. 1A).

CAT

CAT was maintained throughout storage in 10-VC whereas it increased by fourfold ($p < 0.0001$) on day 11 with day 1 in controls. CAT was lower by 50% ($p < 0.0001$) on day 11 in 10-VC compared to controls (Fig. 1B).

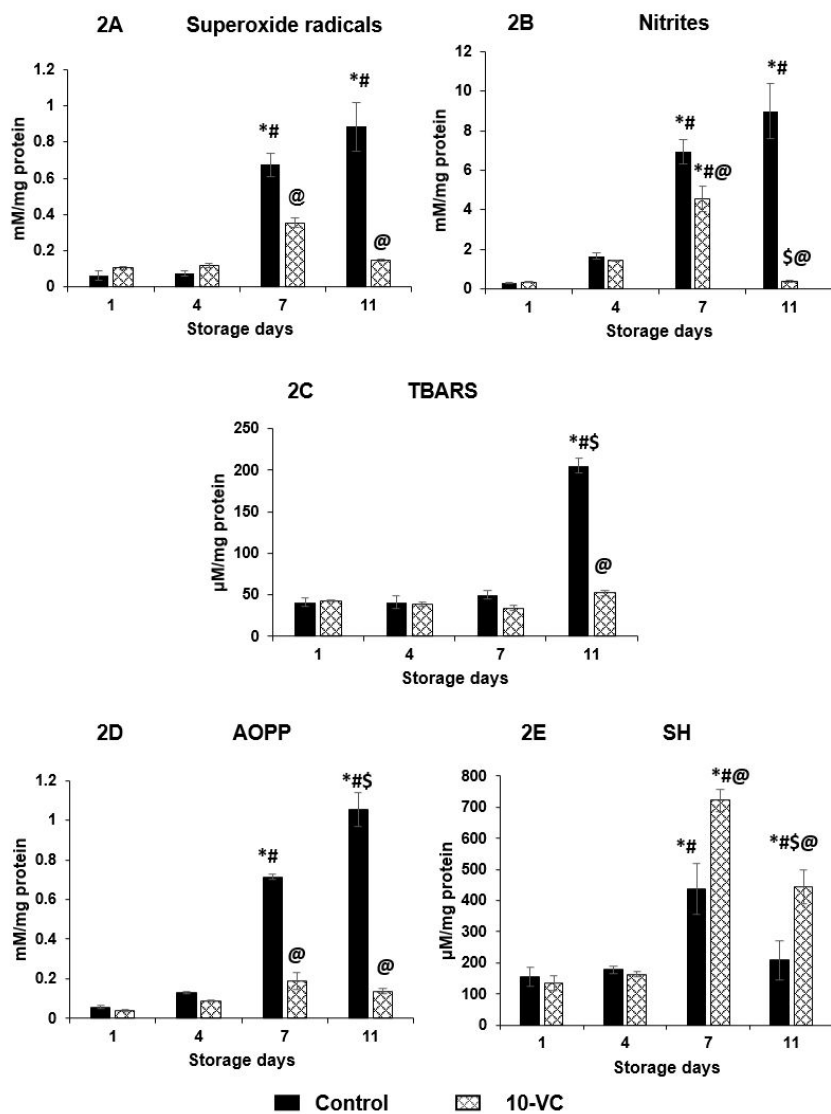


Fig. 2. Oxidative stress, lipid peroxidation and protein oxidation in platelets stored with VC, A: Superoxide radicals, B: Nitrites, C: TBARS, D: AOPP, E: SH (* – significant with day 1, # – significant with day 4, \$ – significant with day 7, @ – significant in 10-VC against controls)

GSH

GSH increased in 10-VC by 260% ($p<0.0001$) on day 7 with respect to day 1 and decreased by 60% ($p<0.0001$) on day 11 with day 7. GSH peaked 310% ($p<0.0001$) on day 7 with day 1 and decreased by 56% ($p<0.0001$) on day 11 with day 7 in controls.

GSH was higher by 27% ($p<0.01$) on day 7 in 10-VC compared to controls (Fig. 1C).

TAC

TAC decreased by 73% ($p<0.0001$) on day 4 with day 1 and decreased by 50% ($p<0.0001$) on day 7 and day 11 with day 4 in 10-VC whereas, it was maintained in controls.

TAC was higher by tenfold ($p<0.0001$) on day 1, twofold ($p<0.0001$) on day 4 and onefold ($p<0.01$) on days 7 and 11 in 10-VC compared to their respective controls (Fig. 1D).

Oxidative stress

Superoxide radicals

Superoxide radicals remained constant in 10-VC whereas it increased by ~ninefold ($p<0.0001$) on days 7 and 11 in controls.

Superoxide radicals were lower by 47% ($p<0.05$) and 83% ($p<0.0001$) on days 7 and 11 respectively, in 10-VC against controls (Fig. 2A).

Nitrites

Nitrites increased in 10-VC by tenfold ($p<0.0001$) on day 7 which decreased by 91% ($p<0.0001$) on day 11 with day 7. Nitrites increased by twentyfold ($p<0.0001$) on day 7 and day 11 in controls. Nitrites were lower by 34% ($p<0.05$) and 95% ($p<0.0001$) on days 7 and 11 respectively, in 10-VC against controls (Fig. 2B).

TBARS

TBARS was maintained throughout storage in 10-VC whereas it increased by fourfold ($p<0.0001$) on day 11 in controls.

TBARS was lower by 74% ($p<0.0001$) on day 11 in 10-VC against controls (Fig. 2C).

AOPP

AOPP remained constant in 10-VC whereas controls exhibited increments of tenfold ($p<0.0001$) on day 7 and day 11.

AOPP was lower by ~80% ($p<0.0001$) on days 7 and 11 in 10-VC against controls (Fig. 2D)

SH

SH increased in 10-VC by 435% ($p<0.0001$) on day 7 and decreased by 38% ($p<0.01$) on day 11 with day 7. SH increased by 180% ($p<0.01$) on day 7 and normalized to day 1 levels on day 11 in controls.

SH was higher by 65% ($p<0.01$) and 110% ($p<0.05$) on days 7 and 11 respectively, in 10-VC against controls (Fig. 2E).

Platelet function, viability and metabolism

NADPH Oxidase-2 (NOX2)

10-VC exhibited increments of 75% ($p<0.001$) on day 7 whereas, it increased by 73% ($p<0.0001$) and 41% ($p<0.05$) on days 7 and 11 respectively, in controls. NOX2 was lower by 25% ($p<0.01$) on day 7 in 10-VC against controls (Table 1).

Table 1. Platelet function during storage with VC^a

Storage days	Sub-groups	NOX2 (ng/mL)	P-selectin (pg/mL)	ATP secretion (µg/mL)
1	Control	35.10±2.37	2528.25±548.44	28760.00±2337.69
	10-VC	25.94±2.93	1214.69±312.89	34553.33±1359.35
4	Control	29.76±2.57	2570.62±754.86	30620.00±2427.03
	10-VC	26.48±1.28	1320.62±604.75	27960.00±857.54
7	Control	60.88±2.91**	18008.47±1986.82**	4153.33±132.33**
	10-VC	45.34±1.74**@	7531.78±222.46@	17593.33±2021.40**@
11	Control	49.56±2.51**	6709.04±1507.28\$	4660.00±316.65**
	10-VC	38.55±2.31	3820.62±908.49	19826.66±678.60**@

^a* – significant with day 1, # – significant with day 4, \$ – significant with day 7, @ – significant in 10-VC against controls

P-selectin

P-selectin remained constant in 10-VC whereas, it increased by 600% ($p<0.0001$) on day 7 and decreased by 62% ($p<0.0001$) on day 11 with day 7 in controls.

P-selectin was lower by 58% ($p<0.001$) on day 7 in 10-VC against controls (Table 1).

Aggregation

Aggregation levels in response to collagen were similar to day 1 throughout the storage period. (day 1: 85% in 10-VC and 53% in controls).

ATP secretion

ATP secretion declined by ~45% ($p<0.0001$) on day 7 and day 11 in 10-VC whereas, it decreased by ~85% ($p<0.0001$) on day 7 and day 11 in controls.

ATP levels were threefold higher ($p<0.0001$) on day 7 and day 11 in 10-VC against controls (Table 1).

Table 2. Platelet viability and metabolism during storage with VC^a

Storage days	Subgroups	MTT (570 nm)	pH	Glucose (mmol/L)	LDH (U×10-6/mg protein)
1	Control	0.45±0.03	7.0±0.0	2.32±0.26	10.73±1.20
	10-VC	0.42±0.04	7.0±0.0	1.80±0.17	9.35±0.29
4	Control	0.302±0.02*	7.0±0.0	0.67±0.08*	9.14±1.13
	10-VC	0.31±0.01	7.2±0.2	0.63±0.11	6.52±0.79
7	Control	0.18±0.01**	7.5±0.0	0.45±0.13*	7.67±1.39
	10-VC	0.25±0.02*	7.2±0.2	0.53±0.09*	5.68±0.78
11	Control	0.11±0.02**	7.6±0.16	0.59±0.14*	25.49±6.22**\$
	10-VC	0.17±0.005**	7.25±0.32	0.53±0.21*	9.41±1.16@

^a* – significant with day 1, # – significant with day 4, \$ – significant with day 7, @ – significant in 10-VC against controls

Cell viability

Viable cells decreased by 40% ($p<0.001$) and 58% ($p<0.0001$) on day 7 and day 11 in 10-VC. Controls exhibited decrements of 33% ($p<0.01$) on day 4 and 40% ($p<0.01$) and 60% ($p<0.0001$) on day 7 and day 11 respectively, with day 4.

Changes in platelet viability were similar between controls and 10-VC on each storage day (Table 2).

pH

pH was maintained in both controls and 10-VC. Variations in pH were similar between controls and 10-VC on each day of storage (Table 2).

Glucose

10-VC showed decrements of ~68% on day 4 ($p<0.001$), day 7 ($p<0.0001$) and day 11 ($p<0.001$). Glucose decreased by ~75% ($p<0.0001$) on days 4, 7 and 11 in controls.

Variations in glucose were similar between controls and 10-VC on each storage day (Table 2).

LDH

LDH remained constant in 10-VC whereas, it increased by twofold ($p<0.01$) on day 11 in controls. LDH was lower by 63% ($p<0.01$) on day 11 in 10-VC against controls (Table 2).

Correlation analysis

Pearson correlation analysis exhibited positive correlation between superoxide radicals and SOD ($r=0.83$, $p<0.05$) and GSH and SH ($r=0.93$, $p<0.05$) in 10-VC. Controls also showed positive correlation between superoxide radicals and SOD ($r=0.85$, $p<0.05$), SOD and CAT ($r=0.98$, $p<0.05$), GSH and SH ($r=0.99$, $p<0.01$).

Discussion

Storage of platelets in SSP+ has mitigated OS-induced storage lesions by augmenting antioxidant defenses and improving platelet viability and function during 7-day storage period.³⁰ Antioxidants as additive in PAS can attenuate OS. Hence, VC was used as an additive in SSP+ to investigate its effects on platelet quality, function and OS during storage.

10 mM VC was chosen as an antioxidant additive in SSP+ for platelet storage based on the ROS assay. Previous studies with VC at 10 mM concentration as an additive in erythrocyte storage solutions have mitigated OS by reducing superoxide radicals and elevating endogenous antioxidant defenses.^{48,49} VC at 10 mM concentration has preserved erythrocyte efficacy during storage. Therefore, 10 mM VC was used in platelet storage solution as an additive in this study.

Platelets are equipped with endogenous antioxidant defenses, namely SOD and CAT, that are activated by high ROS levels i.e., superoxide radicals and H_2O_2 respectively.²⁷ SOD and CAT are proportional as the dismutation product of SOD (H_2O_2 at high concentrations) activate CAT. Superoxide radicals and SOD were maintained throughout storage ($r=0.83$) in 10-VC indicating the ROS scavenging property of VC. VC directly scavenges ROS by donating electrons/hydrogen atoms from its two ionizable -OH groups.²⁷ It is reported that VC reduced ROS levels induced by cisplatin in pig platelets and inhibited superoxide production by 90% compared to resveratrol.^{25,26} VC being an efficient ROS scavenger limited ROS generation which resulted in lower SOD and CAT levels thereby, maintaining the redox status of the cells. SOD and CAT were proportional to the superoxide radicals in controls on days 7 and 11 (Superoxide radicals vs SOD, $r=0.85$; SOD vs CAT, $r=0.98$).

GSH is an endogenous antioxidant that directly scavenges ROS. The pentose phosphate pathway (PPP) contributes to GSH recycling by providing NADPH required for glutathione reductase (GR) activity.²⁷ The higher levels of GSH in 10-VC compared to controls on day 7 indicate that VC could maintain GSH in reduced state. This may be associated with improved GSH recycling thereby, maintaining redox balance. Similar observations have been reported by Puskas et al. who demonstrated that pretreatment with dehydroascorbate to T-lymphocytes increased GSH levels, potentially through the activation of PPP.⁵⁰ Antioxidant additives

have been reported to improve platelet viability during storage.¹⁷ This was evidenced in low LDH levels on day 11 in 10-VC than controls, suggesting reduced platelet damage. Variations of ~50% in 10-VC was observed compared to controls although the changes were insignificant. Thus, VC could lower storage lesions and preserve platelet integrity by reducing ROS levels during storage. TAC was higher than controls throughout storage in 10-VC which revealed the antioxidant potential of VC. However, it is reported that PPP is activated during platelet storage as a stress response in order to maintain the redox status.⁵¹ This was evidenced in the elevated GSH levels on day 7 in controls which could be associated with enhanced GSH recycling. However, GSH decreased on day 11 in both 10-VC and controls due to high ROS. The GSH levels were also in line with SH content in controls (GSH vs SH, $r=0.99$). High metabolic rate evidenced by lower glucose levels throughout storage in controls and 10-VC may be indicative of an active glycolytic pathway/PPP. These results suggest that VC has worked in synergy with the endogenous antioxidant defenses as the SOD and CAT levels were maintained with augmentation of GSH thereby, resulting in mitigation of OS.

Cellular lipids and proteins are prone to ROS attack during OS. Oxidation of cellular lipids and proteins disrupt membrane structure and function eventually leading to apoptosis. VC in synergy with VE inhibits lipid peroxidation.⁵² This was evident in the constant levels of TBARS throughout storage in 10-VC whereas, it increased on day 11 in controls due to accumulation of ROS causing lipid peroxidation. Olas et al. reported that VC reduced lipid peroxidation in pig platelets during OS conditions.^{25,26} Proteins form dityrosine cross-linkages called AOPPs during OS which disrupt several cellular signaling cascades.⁵³ AOPPs were lower than controls in 10-VC which can be attributed to the ROS scavenging and antioxidant regenerating ability of VC. This was also evidenced in the levels of protein sulfhydryls. The disulfides (S-S) formed during oxidation can be reversed back to SH when ROS lowers or due to the interventions of antioxidant defenses. SH was higher than controls on days 7 and 11 due to the antioxidant potential of VC in maintaining thiols in reduced state which was also in line with GSH ($r=0.93$).

NOX2 is a major enzymatic source of ROS in platelets and is closely associated with platelet activation and functional responses.^{54,55} NOX2 contributes to high ROS generation during storage which promotes undesirable platelet activation and impair responses to agonists.^{8,10,56} The effects of VC was evidenced in low P-selectin levels in 10-VC group compared to controls although ATP secretion (granule release) was higher and aggregation response to collagen was maintained. VC has been reported to modulate platelet redox status and inhibit

CD40 ligand expression (found on activated platelets) without affecting agonist-induced platelet aggregation.^{28,29} This is in accordance with the results of platelet function markers. This suggests that VC could modulate redox balance and attenuate ROS-mediated activation pathways thereby, preserving platelet responses. Thus, VC could selectively inhibit platelet activation while maintaining functional integrity during storage.

Platelets primarily use glucose as their metabolic fuel. Glucose consumption increases lactate production which decrease the pH of the storage medium.⁵⁷ Glucose levels lowered in both controls and 10-VC throughout storage indicating its higher consumption although pH was maintained. Storage of platelets in an artificial medium or PAS with minimal residual plasma, preserves metabolic integrity.⁵⁷ The salts in SSP+ provide a buffering effect as well as the acetate component can act as a secondary metabolic fuel as evidenced in glucose and pH levels.³⁰ Platelet metabolic integrity was maintained by the components of SSP+ although, metabolic stress was observed (lower glucose levels) during storage. These results are in line with the findings of Rajanand et al. where pH was maintained despite lower glucose levels when stored in PAS-SPP+.²³ This was attributed to the salts present in SSP+ which preserved the metabolic status.

Study limitations

The present study used animal models as they are bred in controlled conditions. Thus, the variations in age, diet, genetics and microenvironment are minimized, allowing the observed effects to be more directly associated with the experimental interventions. However, the study was conducted using a limited number of biological samples (n=5) from male *Wistar* rats, which were selected to minimise potential influences of estrogen-associated antioxidant effects. Therefore, these findings require validation in human platelets.

Although the concentration of VC was initially optimized using ROS assay, only 10mM concentration was evaluated during extended storage. In addition, in vivo platelet recovery, post-transfusion survival and clinical transfusion efficacy were not assessed. Therefore, the present findings should be interpreted within the context of an in vitro platelet storage model. Further studies using human platelets and in vivo validation are required to confirm the translational relevance of these findings.

Conclusion

10-VC reduced oxidative stress by scavenging superoxide and nitrite radicals, inhibiting lipid peroxidation, protein oxidation, augmenting antioxidant capacity and preserving platelet functions and viability during storage. Thus, VC (10mM) in SSP+ was able to maintain

the redox status and improve selected markers of platelet efficacy during extended storage. This in vitro study highlights the prospects of employing vitamin C as an additive in PASs to preserve platelet efficacy during prolonged storage. However, further studies using human platelets are required to confirm the translational relevance of these findings.

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Supplementary material

Fig. S1. Dose optimization of Vitamin C (VC) in stored platelets.

Declarations

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Author contributions

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

Conflicts of interest

The authors declare no conflicts of interest.

Competing interests

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Ethics approval

All experimental procedures were conducted in compliance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA)

provided by the Institutional Animal Ethics Committee (IAEC), New Delhi, India. Whole blood samples were collected by cardiac puncture method under anesthesia in accordance with the approved animal care protocols of CCSEA. Animals were procured from Vertebrates (2138/PO/RcBiBt/S/21/CPCSEA) dated 1st August, 2024. Blood sampling was performed at Nargund College of Pharmacy according to the institutional ethics regulations (1051/PO/Re/S/07/CPCSEA).

References

1. Rajashekaraiiah V, Rajanand MC. Platelet storage: Progress so far. *J Thromb Thrombolysis*. 2023;55(1):9-17. doi:10.1007/s11239-022-02716-3
2. Shrivastava M. The platelet storage lesion. *Transfus Apher Sci*. 2009;41(2):105-113. doi:10.1016/j.transci.2009.07.002
3. Gulliksson H. Additive solutions for the storage of platelets for transfusion. *Transfus Med*. 2000;10(4):257-264. doi:10.1046/j.1365-3148.2000.00262.x
4. Ringwald J, Zimmermann R, Eckstein R. The new generation of platelet additive solution for storage at 22 C: development and current experience. *Transfus Med Rev*. 2006;20(2):158-164. doi:10.1016/j.tmr.2005.11.003
5. Hornsey VS, McColl K, Drummond O, et al. Extended storage of platelets in SSP+ platelet additive solution. *Vox Sang*. 2006;91(1):41-46. doi:10.1111/j.1423-0410.2006.00771.x
6. Saunders C, Rowe G, Wilkins K, Holme S, Collins P. In vitro storage characteristics of platelet concentrates suspended in 70% SSP+ TM additive solution versus plasma over a 14-day storage period. *Vox Sang*. 2011;101(2):112-121. doi:10.1111/j.1423-0410.2011.01468.x
7. Alexandru N, Popov D, Georgescu A. Intraplatelet oxidative/nitrative stress: inductors, consequences, and control. *Trends Cardiovas Med*. 2010;20(7):232-238. doi:10.1016/j.tcm.2011.11.007
8. Rinalducci S, Zolla L. Biochemistry of storage lesions of red cell and platelet concentrates: A continuous fight implying oxidative/nitrosative/phosphorylative stress and signaling. *Transfus Apher Sci*. 2015;52(3):262-269. doi:10.1016/j.transci.2015.04.005
9. Arthur JF, Shen Y, Kahn ML, Berndt MC, Andrews RK, Gardiner EE. Ligand binding rapidly induces disulfide-dependent dimerization of glycoprotein VI on the platelet plasma membrane. *J Biol Chem*. 2007;282(42):30434-30441. doi:10.1074/jbc.M701330200
10. Qiao J, Arthur JF, Gardiner EE, Andrews RK, Zeng L, Xu K. Regulation of platelet activation and thrombus formation by reactive oxygen species. *Redox Biol*. 2018;14:126-130. doi:10.1016/j.redox.2017.08.021
11. Ray PD, Huang BW, Tsuiji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal*. 2012;24(5):981-990. doi:10.1016/j.cellsig.2012.01.008
12. Zhuang Y, Ren G, Li H, et al. In vitro properties of apheresis platelet during extended storage in plasma treated with anandamide. *Transfus Apher Sci*. 2014;51(1):58-64. doi:10.1016/j.transci.2014.03.009
13. Wang X, Fan Y, Shi R, Li J, Zhao S. Quality assessment of platelets stored in a modified platelet additive solution with trehalose at low temperature (10C) and in vivo effects on rabbit model of thrombocytopenia. *Platelets*. 2015;26(1):72-79. doi:10.3109/09537104.2013.872772
14. Handigund M, Bae TW, Lee J, Cho YG. Evaluation of in vitro storage characteristics of cold stored platelet concentrates with N acetylcysteine (NAC). *Transfus Apher Sci*. 2016;54(1):127-138. doi:10.1016/j.transci.2016.01.006
15. Mithun M, Rajashekaraiiah. L-Carnitine as an additive in Tyrode's buffer during platelet storage. *Blood Coagul Fibrinolysis*. 2018;29(7):613-621. doi:10.1097/MBC.0000000000000760
16. Ekaney ML, Gray GG, McKillop IH, Evans SL. Enhanced platelet function in cold stored whole blood supplemented with resveratrol or cytochrome C. *J Trauma Acute Care Surg*. 2018;85(1):92-97. doi:10.1097/TA.0000000000001887
17. Hosseini E, Ghasemzadeh M, Atashibarg M, Haghshenas M. ROS scavenger, N-acetyl-L-cysteine and NOX specific inhibitor, VAS2870 reduce platelets apoptosis while enhancing their viability during storage. *Transfus*. 2019;59(4):1333-1343. doi:10.1111/trf.15114
18. Wang L, Xie R, Fan Z, et al. The contribution of oxidative stress to platelet senescence during storage. *Transfus*. 2019;59(7):2389-2402. doi:10.1111/trf.15291
19. Amiri F, Dahaj MM, Siasi NH, Deyhim MR. Treatment of platelet concentrates with the L-carnitine modulates platelets oxidative stress and platelet apoptosis due to mitochondrial reactive oxygen species reduction and reducing cytochrome C release during storage. *J Thromb Thrombolysis*. 2021;51(2):277-285. doi:10.1007/s11239-020-02241-1
20. Hegde S, Wellendorf AM, Zheng Y, Cancelas JA. Antioxidant prevents clearance of hemostatically competent platelets after long-term cold storage. *Transfus*. 2021;61(2):557-567. doi:10.1111/trf.16200
21. Zhang P, Du J, Zhao L, et al. The role of intraplatelet reactive oxygen species in the regulation of platelet glycoprotein Iba ectodomain shedding. *Thromb Res*. 2013;132(6):696-701. doi:10.1016/j.thromres.2013.09.034
22. Stef G, Csiszar A, Xiangmin Z, Ferdinandy P, Ungvari Z, Veress G. Inhibition of NAD (P) H oxidase attenuates aggregation of platelets from high-risk cardiac patients with aspirin resistance. *Pharmacol Rep*. 2007;59(4):428. PMID: 17901572
23. Rajanand MC, Ananthakrishna AB, Rajashekaraiiah V. A laboratory study on N-acetyl cysteine in SSP+ modulating oxidative stress and delaying the progres-

- sion of storage lesion in platelets. *Biomed Res Ther.* 2024;11(11):6950-6959. doi:0.15419/bmrat.v11i11.941
24. Rajanand MC, Ananthakrishna AB, Rajashekaraiah V. Caffeic Acid Alleviates Oxidative Stress and Prolongs the Shelf-Life of Rat Platelets. *Biopreserv Biobank.* 2026;24(1):41-51. doi:10.1089/bio.2025.0039
 25. Olas B, Wachowicz B, Buczyn A. Vitamin C suppresses the cisplatin toxicity on blood platelets. *Anti-cancer drugs.* 2000;11(6):487-493. doi:10.1097/00001813-200007000-00010
 26. Olas B, Wachowicz B. Resveratrol and vitamin C as antioxidants in blood platelets. *Thromb Res.* 2002;106(2):143-148. doi:10.1016/S0049-3848(02)00101-9
 27. Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*, 5th ed. New York: Oxford University Press; 2015.
 28. Pignatelli P, Sanguigni V, Paola SG, Coco EL, Lenti L, Violi F. Vitamin C inhibits platelet expression of CD40 ligand. *Free Rad Biol Med.* 2005;38(12):1662-1666. doi:10.1016/j.freeradbiomed.2005.02.032
 29. Mohammed BM, Sanford KW, Fisher BJ, et al. Impact of high dose vitamin C on platelet function. *World J Crit Care Med.* 2017;6(1):37. doi:10.5492/wjccm.v6.i1.37
 30. Rajanand MC, Ananthakrishna AB, Rajashekaraiah V. Oxidative modulations in platelets stored in SSP+, PAS-G and Tyrode's buffer: a comparative analysis. *Hematol Transfus Cell Ther.* 2024;46:80-89. doi:10.1016/j.htct.2024.04.121
 31. Blasa M, Angelino D, Gennari L, Ninfali P. The cellular antioxidant activity in red blood cells (CAA-RBC): a new approach to bioavailability and synergy of phytochemicals and botanical extracts. *Food Chem.* 2011;125(2):685-691. doi:10.1016/j.foodchem.2010.09.065
 32. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem.* 1972;247(10):3170-3175. doi:10.1016/S0021-9258(19)45228-9
 33. Aebi H. Catalase in vitro. *Methods Enzymol.* 1984;105:121-126. doi:10.1016/s0076-6879(84)05016-3
 34. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. *J Lab Clin Med.* 1963;61:882-888. PMID: 13967893.
 35. Da Cruz G. Use of bathocuproine for the evaluation of the antioxidant power in liquids and solutions. 2003;US patent 6613577.
 36. Chen LY, Mehta P, Mehta JL. Oxidized LDL decreases L-arginine uptake and nitric oxide synthase protein expression in human platelets: relevance of the effect of oxidized LDL on platelet function. *Circ.* 1996;93(9):1740-1746. doi:10.1161/01.CIR.93.9.1740
 37. Olas B, Nowak P, Kolodziejczyk J, Ponczek M, Wachowicz B. Protective effects of resveratrol against oxidative/nitrative modifications of plasma proteins and lipids exposed to peroxynitrite. *J Nutr Biochem.* 2006;17(2):96-102. doi:10.1016/j.jnutbio.2005.05.010
 38. Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int.* 1996;49(5):1304-1313. doi:10.1038/ki.1996.186
 39. Habeeb AFSA. Reaction of protein sulfhydryl groups with Ellman's reagent. *Methods Enzymol.* 1972;25(25):457-464. doi:10.1016/S0076-6879(72)25041-8
 40. Born GVR, Cross MOJ. The aggregation of blood platelets. *J Physiol.* 1963;168(1):178. doi:10.1113/jphysiol.1963.sp007185
 41. Tamang HK, Stringham EN, Tourdot BE. Platelet functional testing via high-throughput microtiter plate-based assays. *Curr Protoc.* 2023;3(2):668. doi:10.1002/cpz1.668
 42. Wachowicz B, Olas B, Zbikowska HM, Buczyński A. Generation of reactive oxygen species in blood platelets. *Platelets.* 2002;13(3):175-182. doi:10.1080/09533710022149395
 43. Shiri R, Yari F, Ahmadinejad M, Vaeli S, Tabatabaei MR. The caspase-3 inhibitor (peptide Z-DEVD-FMK) affects the survival and function of platelets in platelet concentrate during storage. *Blood Res.* 2014;49(1):49. doi:10.5045/br.2014.49.1.49
 44. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193(1):265-275. doi:10.1016/S0021-9258(19)52451-6
 45. Amorini AM, Tuttobene M, Lazzarino G, Denti G. Evaluation of biochemical parameters in platelet concentrates stored in glucose solution. *Blood Transfus.* 2007;5(1):24. doi:10.2450/2007.0019-06
 46. Basak A. Development of a rapid and inexpensive plasma glucose estimation by two-point kinetic method based on glucose oxidase-peroxidase enzymes. *Indian J Clin Biochem.* 2007;22(1):156-160. doi:10.1007/BF02912902
 47. Buhl SN, Jackson KY. Optimal conditions and comparison of lactate dehydrogenase catalysis of the lactate-to-pyruvate and pyruvate-to-lactate reactions in human serum at 25, 30, and 37 degrees C. *Clin Chem.* 1978;24(5):828-831. doi:10.1093/clinchem/24.5.828
 48. Soumya R, Vani R. Vitamin C as a modulator of oxidative stress in erythrocytes of stored blood. *Acta Haematol Pol.* 2017;48(4):350-356. doi:10.1016/j.achaem.2017.08.005
 49. Pallavi M, Rajashekaraiah V. Vitamin C with N-acetylcysteine ameliorates the antioxidant defenses in erythrocytes during storage. *J Exp Clin Med.* 2024;41(2):302-310. doi:10.52142/omujecm.41.2.14
 50. Puskas F, Gergely Jr P, Banki K, Perl A. Stimulation of the pentose phosphate pathway and glutathione levels by dehydroascorbate, the oxidized form of vitamin C. *FASEB J.* 2000;14(10):1352-1361. doi:10.1096/fasebj.14.10.1352
 51. Guo L, Qian C, Gao C, et al. Activation of the pentose phosphate pathway mitigates platelet storage lesions and improves platelet preservation quality. *Thromb Res.* 2025;252:109377. doi:10.1016/j.thromres.2025.109377

52. Çimen MB. Free radical metabolism in human erythrocytes. *Clin Chim Acta*. 2008;390(1-2):1-1. doi:10.1016/j.cca.2007.12.025
53. Ou H, Huang Z, Mo Z, Xiao J. The characteristics and roles of advanced oxidation protein products in atherosclerosis. *Cardiovasc Toxicol*. 2017;17(1):1-12. doi:10.1007/s12012-016-9377-8
54. Masselli E, Pozzi G, Vaccarezza M, et al. ROS in platelet biology: functional aspects and methodological insights. *Int J Mol Sci*. 2020;21(14):4866. doi:10.3390/ijms21144866
55. Dionisio LM, Zheng Y, Cancelas JA. Redox Control in Platelet Activity and Therapy. *Antioxidants*. 2025;14(11):1286. doi:10.3390/antiox14111286
56. Freedman JE. Oxidative stress and platelets. *Arterioscler Thromb Vasc Biol*. 2008;28(3):11-16. doi:10.1161/ATVBAHA.107.15917
57. Gulliksson H. Defining the optimal storage conditions for the long-term storage of platelets. *Transfus Med Rev*. 2003;17(3):209-215. doi:10.1016/S0887-7963(03)00020-8



ORIGINAL PAPER

Expression of the miR-155, miR-326 and IL-17/IL-23 axis in patients with essential hypertension – a case-control study

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ABSTRACT

Introduction and aim. Essential hypertension (EH) is a major cardiovascular risk factor worldwide. The interleukin (IL)-17 and IL-23 axis is a key driver of Th17-mediated immune response; however, the involvement of microRNAs in modulating this axis in patients with essential hypertension remains poorly understood. This study aimed to evaluate expression profiles of miR-155 and miR-326 together with the serum of IL-17A and IL-23 in patients with EH.

Material and methods. This case-control study enrolled 100 participants (50 patients with essential hypertension and 50 healthy controls). The expression of miR-155 and miR-326 was determined by (RT-qPCR), whereas serum IL-17A and IL-23 concentrations were measured using (ELISA).

Results. IL-17A and IL-23 levels were significantly elevated in patients with essential hypertension compared to healthy controls ($p < 0.001$). Additionally, miR-155 was significantly overexpressed, whereas miR-326 was significantly downregulated in patients with essential hypertension ($P < 0.001$ for both). Exploratory receiver operating characteristic analysis showed preliminary discriminatory performance for miR-155, miR-326, IL-17A and IL-23 with AUC values of 0.988, 0.927, 0.974 and 0.946, respectively. No direct correlation between miRNA expression and cytokine levels was identified.

Conclusion. Patients with essential hypertension exhibit dysregulation of miR-155 and miR-326, IL-17A, and IL-23. Lack of significant correlations suggests that these biomarkers may reflect distinct aspects of hypertension-associated inflammation. Further prospective and longitudinal investigations are needed to validate these findings and clarify underlying mechanisms.

Keywords. hypertension, interleukin-17, interleukin-23, microRNAs, miR-155, miR-326

Introduction

Essential hypertension is a chronic elevation of blood pressure and is considered both a disease itself and a major risk factor for other diseases leading to global public health challenge. While hemodynamic, renal and neurohormonal mechanisms have traditionally defined the pathophysiological framework of essential hypertension, accumulating evidence highlights low-grade chronic inflammation as a critical driver of vascular dysfunction and sustained blood pressure elevation.¹ Patients with hypertension exhibit elevated circulat-

ing inflammatory markers, oxidative stress, and altered immune cell phenotypes.^{2,3} The renin-angiotensin-aldosterone system interacts closely with immunity via angiotensin II, which not only constricts blood vessels but also serves as an immunomodulator, stimulating dendritic cell activation and promoting inflammatory cell recruitment.⁴

Among the immune pathways implicated in essential hypertension, the Interleukin-17 and Interleukin-23 (IL-17/IL-23) axis has been recognized as an important contributor to inflammation in essential hy-

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pertension.⁵ Within the axis, IL-23 stabilizing the Th17 phenotype and sustaining IL-17A production.⁶ IL-17A promotes endothelial dysfunction by impairing nitric oxide (NO) bioavailability, promoting oxidative stress and increased renal sodium reabsorption, collectively leading to elevated blood pressure.^{7–9} Although most mechanistic evidence arises from preclinical models, human studies support the relevance of these inflammatory cytokines in the pathogenesis of essential hypertension.^{5,10–12}

MicroRNAs (miRNAs) are small conserved non-coding RNA molecules that regulate gene expression at the post-transcriptional level. They are increasingly recognized as major regulators of immune cell differentiation, cytokine production, and vascular homeostasis. Among miRNAs with established roles in immune regulation, miR-155 has been reported to enhance Th17 differentiation by inhibiting suppressor of cytokine signaling 1 (SOCS1), a key inhibitor of cytokine signaling, thereby enhancing IL-17 production and perpetuating inflammatory cascades.¹³ Conversely, miR-326 has been reported to regulate Th17 differentiation by targeting negative regulator (Ets-1), thereby promoting the expansion of Th17 cells and potentially augmenting IL-17A-driven immune responses.¹⁴ In particular, these two miRNAs are thought to play distinct regulatory roles within the same Th17 pathway.

Moreover, assessing miR-155 and miR-326 together with IL-17A and IL-23 may help clarify potential interactions between epigenetic regulation and inflammatory responses in essential hypertension. Most previous investigations have focused exclusively on studying IL-17/IL-23 axis components or miR-155 alone.^{3,12,15–17} To our knowledge, only a few studies have comprehensively analyzed the combined expression pattern of circulating miR-326 and miR-155, IL-17A and IL-23 in patients with essential hypertension. Addressing this gap may improve our understanding of how specific miRNAs and the IL-17/IL-23 axis are associated with immune dysregulation in essential hypertension.

Aim

This study aimed to perform an exploratory analysis of the combined expression profiles of miR-155 and miR-326 together with circulating IL-17A and IL-23 levels in patients with essential hypertension. Secondary analyses examined the correlations among these biomarkers and explored their discriminatory performance using receiver operating characteristic analysis.

Material and methods

Study design and population

This study was designed to evaluate the expression profiles of specific microRNAs and the IL-17/IL-23 inflammatory axis in patients with essential hyper-

tension. The study cohort was comprised of 100 participants: 50 patients with a confirmed diagnosis of essential hypertension (patients group; 26 men, 24 women; mean age, 48.74 ± 7.83 years) and 50 healthy individuals (healthy control; 28 men, 22 women; mean age, 46.16 ± 8.03 years).

Participants were consecutively enrolled from the outpatient clinics and physical examination unit of Al-Diwaniyah Teaching Hospital (Iraq, Al-Diwaniyah Governorate) between October 2025 and January 2026. The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki (Number 41, Date 20/10/2025). The institutional ethics committee specifically approved the use of verbal informed consent as part of the approved study protocol because the study was observational and involved routine venous blood collection without any additional intervention.

Inclusion and exclusion criteria

Rigorous exclusion criteria were applied to minimize confounding variables. Participants were excluded if they fulfilled any of the following criteria: immunocompromised status, active malignancies, diabetes mellitus, active smoking, secondary hypertension of known etiology, autoimmune disorders or chronic inflammatory diseases. Healthy volunteers in the healthy control group were required to be strictly normotensive defined systolic blood pressure (SBP) <120 mmHg and diastolic blood pressure (DBP) <80 mmHg with no personal history of chronic inflammatory or cardiovascular diseases. Blood pressure was measured by physicians using a calibrated mercury sphygmomanometer with the participant in the seated position after at least 5 minutes of rest. Two consecutive measurements were obtained at 1–2-minute intervals, and the average of the two readings was recorded. Essential hypertension was confirmed as a (SBP) ≥ 140 mmHg and/or (DBP) ≥ 90 mmHg, confirmed on repeated measurements during separate clinical visits according to the current clinical guidelines.

Sample size calculation

The study enrolled 50 patients with essential hypertension and 50 healthy controls. Sample size was determined based on participant availability during the predefined study period. A formal a priori power analysis was not performed. Accordingly, this limitation should be taken into account when interpreting the study findings.

Blood collection, processing and storage

Each participant underwent peripheral venous blood collection (5 mL) after fasting overnight for 8–10 hours. A (2 mL) aliquot of whole blood was immediately transferred into RNase-free tubes to preserve RNA

integrity. The remaining (3 mL) was transferred into clean sterile tubes, at room temperature allowed to clot for 20–30 minutes, and to isolate serum blood centrifuged for 10 minutes at 3000 rpm. Both whole-blood aliquots with serum were immediately stored at -80°C until downstream molecular and biochemical analyses. Before serum analysis, frozen samples were thawed to room temperature, mixed gently by vortexed for 3 seconds, then centrifuged at 10,000×g for 5 minutes at 4°C to precipitate any cellular debris. Visually hemolysis, lipemic, or icteric samples were rigorously discarded.

Total RNA extraction

Total RNA was isolated from the processed whole blood samples using TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, USA) in strict accordance with the protocol of manufacturer. The concentration and purity of the extracted RNA were evaluated using a NanoDrop spectrophotometer (Thermo Scientific, USA), only samples with an absorbance (A260/280) between 1.8 to 2.0 were acceptable for downstream processing. RNA structure integrity was further evaluated by 1.5% agarose gel electrophoresis through visual assessment of 28S and 18S ribosomal RNA bands. Only samples showing intact ribosomal RNA bands with an approximate 28S:18S ratio of 2:1 and no evidence of degradation were included for subsequent analyses.

DNase treatment and cDNA synthesis

To remove residual genomic DNA contamination, total RNA was treated with DNase I (Promega, Madison, WI, USA) for 30 minutes at 37°C, followed by enzyme inactivation for 10 minutes at 65°C. For microRNA-specific complementary DNA (cDNA) synthesis, a stem-loop reverse transcription method was employed. A universal RT primer was utilized alongside the M-MLV Reverse Transcriptase kit (Bioneer, Republic of Korea) with 100 ng/μL of template RNA. The RNA-primer mix was denatured at 70°C for 10 minutes and instantly chilled on ice. Reverse transcription was conducted at 42°C for 60 minutes followed by enzyme inactivation for 5 minutes at 65°C. For the amplification of the housekeeping reference gene (GAPDH), cDNA synthesis was performed under analogous conditions using random hexamer primers, initiated with a denaturation step for 10 min at 65°C.

Quantitative real-time polymerase chain reaction (RT-qPCR)

The expression of the selected miRNAs was quantified by (RT-qPCR). Specific qPCR primers for hsa-miR-155 (MIMAT0000646) and hsa-miR-326 (MIMAT0000756) were designed using the Sanger Center miRNA database (miRBase) registry to select the appropriate mature miRNA sequences, with assistance from a specialized

miRNA primer design tool. Primers for the GAPDH reference gene (NM_001256799.3) were generated using the NCBI database and Primer3Plus online software.¹⁸ Quantitative PCR was performed using GoTaq® qPCR Master Mix (Promega, USA) on a MiniOpticon Real-Time PCR Detection system (Bio-Rad, USA) and each amplification was prepared in a final total volume of 20 μL, containing cDNA template, reverse primer, forward primer, GoTaq qPCR Master Mix, and DE-PC-treated water. Thermocycling parameters were: start denaturation for 5 min at 95°C, followed by 45 amplification cycles including denaturation at 95°C for 20 s and 60°C for 30 s. The sequences of all primers used in this study are presented in Table 1.

Table 1. The primer sequences used for RT-qPCR

Primer	Sequence
RT primer (universal)	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACTTTTITTTTTVN
hsa-miR-155 qPCR primer	F AACACGCTTAATGCTAATCGTGATA R GTCGTATCCAGTGCAGGGT
hsa-miR-326 qPCR primer	F AACAACTCCTCTGGGCCCTTC R GTCGTATCCAGTGCAGGGT
GAPDH qPCR primer	F TCACCAGGCTGCTTTTAAC R TGACGGTGCCATGGAATTG

RT-qPCR quality control

Relative gene expression levels were calculated via the comparative 2^{-ΔΔCt} method, using healthy normotensive group serving as the calibrator.¹⁹ GAPDH was used as the endogenous reference gene. Its stability was assessed by comparing Ct values between patients with essential hypertension and healthy controls. Amplification specificity was evaluated by melt curve analysis which conformed to a single distinct melting peak for each target, indicating specific amplification. Amplification efficiencies were determined using 10-fold serial cDNA dilutions, yielding acceptable ranges between 90% and 110% ((R²)>0.99). Intra-assay and inter-assay variability were assessed by running samples in triplicate within the same plate and across three independent runs, respectively, with coefficients of variation (CV) consistently <5%. Although small RNA reference genes like U6, miR-16, SNORD44, and SNORD48 are generally recommended for circulating miRNA normalization according to the MIQE guidelines, these controls were not incorporated during the original study design. Consequently, retrospective re-normalization was not feasible. GAPDH was retained as the endogenous control because it demonstrated highly stable Ct values across all study samples under the present experimental conditions. Nevertheless, the use of GAPDH represents a methodological limitation, and future studies should validate normalization using multiple endogenous reference controls.

In silico target prediction analysis

In silico miRNAs target interactions were analysis using the publicly available miRDB and TargetScan databases to identify potential down-stream inflammatory pathways regulated by miR-155 and miR-326 relevant to inflammatory signaling pathways.

Measurement of circulating cytokines

Commercially available sandwich ELISA kits were used to measure serum interleukin-17A (IL-17A) and interleukin-23 (IL-23) concentrations. Human IL-17A was quantified using an ELISA kit (Cat. No. E0047Hu, Bioassay Technology Laboratory, China) with detection range (5–1000 ng/L) and assay sensitivity (2.38 ng/L), and IL-23 was measured using ELISA kit (Cat. No. E0074Hu, Bioassay Technology Laboratory, China) with detection range (3–9000 ng/L) with sensitivity (1.52 ng/L). The inter-assay coefficient of variation (CV) was <10% for both assays according to the manufacturer. All laboratory procedures strictly adhered to the manufacturer’s protocols. Standard solutions were prepared to generate standard curves, and serum samples were analyzed in duplicate wells. After the required incubation and washing steps, the appropriate detection antibodies were added, then followed by substrate development. Absorbance was measured at the recommended wavelength using a microplate reader, and cytokine level were measured from the corresponding standard curves.

Sample quality and exclusion criteria

Samples were excluded based on predefined criteria: (1) visual evidence of hemolysis, lipemia, or icterus; (2) RNA purity outside the acceptable range ($A_{260/280}<1.8$ or >2.0) or RNA degradation observed on agarose gel; (3) failure of the internal control gene (GAPDH) amplification with a cycle threshold (Ct) value >35 ; and (4) coefficient of variation (CV) $>10\%$ among technical replicates in RT-qPCR or ELISA assays. Only samples that satisfied all predefined quality control criteria were retained for the final statistical analysis.

Statistical analysis

Statistical analyses were demonstrated using the IBM SPSS Statistics software (version 26.0 and IBM Corp., Armonk, NY, USA). For normally distributed continuous variables, descriptive statistics are shown as the mean $\pm$ SD accompanied by 95% confidence intervals (CIs), whereas categorical data are described using frequencies and percentages. To assess normality of data distribution, we used the Shapiro–Wilk test before applying parametric statistical analyses. To confirm homogeneity of variances among groups was assessed using Levene’s test prior to independent t test ($P>0.05$).

Between-group comparisons were performed using the independent samples t-test for continuous variables with normal distribution and the chi-square test for categorical variables. The strength of the associations between continuous variables was evaluated by Pearson’s correlation coefficient (r). Exploratory receiver operating characteristic (ROC) curve analysis was performed to evaluate the preliminary discriminatory value of IL-17A, IL-23, miR-155, and miR-326 in distinguishing patients with essential hypertension from healthy controls. Sensitivity, specificity, optimal cutoff values, and the area under the curve (AUC) were calculated. Multivariable binary logistic regression analysis was performed to for potentially confounding variables including BMI and age. A statistically significant difference was considered at $P<0.05$. Because the study was performed in a single-center cohort without external validation, the finding of the ROC analysis should be considered exploratory.

Results

The clinical and demographic profiles of the study population are shown in Table 2. Statistical analysis demonstrated that the groups were comparable with respect to age and sex distribution, with no significant differences observed ($p=0.155$ and $p=0.688$, respectively). In contrast, the essential hypertensive group had significantly higher BMI and obesity prevalence than the healthy controls group ($p=0.001$ and $p=0.002$, respectively). Additionally, 80% of patients with essential hypertension had a family history of hypertension.

Information regarding antihypertensive treatment status (treated or untreated) was collected from patient interviews. However, detailed information regarding the specific classes, doses, and duration of antihypertensive medications was not consistently available and therefore was not included in the analysis.

Table 2. Demographics and clinical profile of study groups*

Characteristic	EH patients (n=50)	Healthy controls (n=50)	Test	p
Age, Mean $\pm$ SD (years)	48.74 $\pm$ 7.83	46.16 $\pm$ 8.03		0.155
Male, n (%)	26 (52.0%)	28 (56.0%)	$\chi^2=0.160$	0.688
Female, n (%)	24 (48.0%)	22 (44.0%)		
SBP (mmHg), Mean $\pm$ SD	148.25 $\pm$ 15.99	112.25 $\pm$ 5.60	t=14.898	0.001
DBP (mmHg), Mean $\pm$ SD	96.84 $\pm$ 8.69	74.37 $\pm$ 5.12	t=15.719	0.001
BMI (kg/m ²), Mean $\pm$ SD	31.47 $\pm$ 6.72	26.75 $\pm$ 5.45	–	0.001
Obese (BMI ≥ 30), n (%)	29 (58.0%)	12 (24.0%)	–	0.002
Positive family history, n (%)	40 (80.0%)	–	–	–
Negative family history	10 (20.0%)	–	–	–
Duration of disease $\downarrow$ 5 years, n (%)	28 (56.0%)	–	–	–
Duration of disease $\uparrow$ 5 years, n (%)	22 (44.0%)	–	–	–
Treating, n (%)	16 (32.0%)	–	–	–
Non-treating, n (%)	34 (68.0%)	–	–	–

* Family history, disease duration, and treatment status were assessed only in patients with essential hypertension

Stability assessment of GAPDH

To assess the suitability of GAPDH as a reference gene, Ct values were compared between patients with essential hypertension and healthy controls. Gene reference expression demonstrated minimal variability across samples, with mean Ct values of 22.03 ± 0.45 in patients with essential hypertension and 22.08 ± 0.47 in healthy controls with ($p > 0.05$), supporting its suitability as an internal control under the experimental conditions of the present study (Table 3).

Table 3. Stability assessment of GAPDH Ct values in study groups

Groups	n	Mean CT $\pm$ SD	Minimum CT	Maximum CT
EH Patients	50	22.03 $\pm$ 0.45	21.29	22.78
Healthy controls	50	22.08 $\pm$ 0.47	21.26	22.68
p		0.710		

As presented in Table 4, miR-155 expression was significantly upregulated in patients with essential hypertension compared with healthy controls (9.26-fold upregulation, $p < 0.001$). In contrast, miR-326 expression was significantly downregulated in patients with essential hypertension, relative expression level (0.092-fold compared with healthy controls ($p < 0.001$)).

Table 4. miR-155 and miR-326 expression in patients with EH and healthy controls

miRNA	EH patients (n=50) Mean $\pm$ SD	Healthy controls (n=50) Mean $\pm$ SD	Fold change (EH)	p
miR-155	9.26 $\pm$ 2.71	1.0 $\pm$ 0.31	9.26 $\uparrow$	<0.001
miR-326	0.092 $\pm$ 0.031	1.0 $\pm$ 0.23	0.092 $\downarrow$	<0.001

* Values are mean $\pm$ SD, $\uparrow$ – upregulated. $\downarrow$ – downregulated

There were marked elevations in the concentrations of serum IL-17A (59.66 $\pm$ 6.75 pg/mL vs. 38.93 $\pm$ 6.41 pg/mL, $p < 0.001$) and IL-23 (107.82 $\pm$ 11.33 pg/mL vs. 77.21 $\pm$ 8.56 pg/mL, $p < 0.001$) in patients with essential hypertension relative to healthy subjects (Table 5).

Table 5. Serum IL-17A and IL-23 level in patients with EH and healthy controls*

Parameter	EH Patients (n=50)	Healthy Controls (n=50)	p
IL-17A (pg/mL)	59.66 $\pm$ 6.75	38.93 $\pm$ 6.41	<0.001
Range	45.96–74.12	23.69–56.79	
IL-23 (pg/mL)	107.82 $\pm$ 11.33	77.21 $\pm$ 8.56	<0.001
Range	86.87–131.12	54.31–102.40	

* Values are mean $\pm$ SD

Correlation analysis between miR-155 and miR-326

Correlation analysis showed a weak association between miR-155 and miR-326 expression levels ($r = -0.126$). No significant correlation was detected in this relationship ($p > 0.05$) as presented in Figure 1.

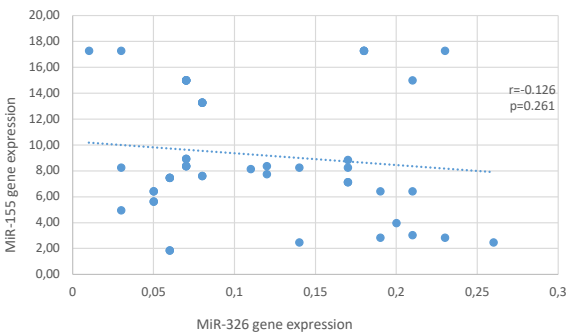


Fig. 1. The correlation scatter miR-155 and miR-326 levels in patients with EH

Correlations between immunological parameters in patients with EH

Correlation analysis demonstrated that the immunological parameters were significantly correlated, with IL-17A and IL-23 showing a positive correlation among patients with essential hypertension ($r = 0.618$, $p < 0.001$, Fig. 2).

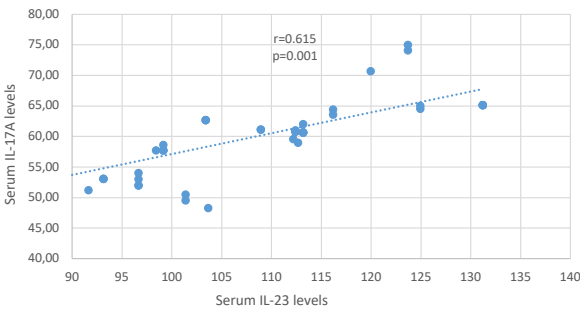


Fig. 2. The correlation scatter of IL-17A and IL-23 levels in patients with EH

Table 6. Correlation between miRNAs gene expression parameters and cytokine axis and other parameters in patients with EH*

Parameters	miRNAs gene expression parameters			
	miR-155 gene expression		miR-326 gene expression	
	r	p	r	p
IL-17A	0.181	0.209	-0.210	0.194
IL-23	0.171	0.234	-0.103	0.528
Age	0.383	0.006*	-0.113	0.435
BMI	0.502	0.001*	-0.323	0.042*

* r – correlation coefficient

Correlation between miRNAs expression parameters and other parameters in patients with EH

Among patients with EH, miR-155 expression demonstrated significant positive correlations with age ($r = 0.383$, $p = 0.006$) and BMI ($r = 0.502$, $p = 0.001$). Expression of miR-326 was significantly negatively correlated with BMI ($r = -0.323$, $p = 0.042$) but not with age ($r = -0.113$, $p = 0.435$). Neither miR-155 nor miR-326 expression correlated significantly with serum IL-17A or IL-23 concentrations (for all $p > 0.05$).

In silico target prediction analysis identified SOCS1 as a potential target of miR-155 and Ets-1 as a potential target of miR-326. Both genes have been implicated in cytokine signaling and regulation of Th17-cell differentiation.

ROC curve analysis was conducted to evaluate the preliminary discriminatory performance of both cytokines and miRNA in patients with essential hypertension compared with controls, as shown in Table 7 and Table 8. IL-17A, at a cutoff value of >50.37 pg/mL yielded an AUC of 0.974 (95% CI: 0.950–0.998), achieving a sensitivity of 96.0% and a specificity of 92.0%. IL-23 at a cutoff of >92.38 pg/mL yielded an AUC of 0.946 (95% CI: 0.908–0.985), with corresponding sensitivity and specificity values of 92.0% and 94.0%, respectively.

Table 7. Accuracy of IL-17A and IL-23 by ROC curve analysis*

Biomarker	Cutoff	AUC (95% CI)	Sensitivity %	Specificity %	PPV %	NPV %
IL-17A	>50.37 pg/mL	0.974 (0.950–0.998)	96.0	92.0	92.3	95.8
IL-23	>92.38 pg/mL	0.946 (0.908–0.985)	92.0	94.0	93.9	92.2

* CI – confidence interval, AUC – area under the curve, NPV – negative predictive value, PPV – positive predictive value

At a cutoff of >2.24-fold expression, miR-155 yielded an AUC of 0.988 (95% CI: 0.973–1.000), with sensitivity of 96.0% and specificity of 98.0%. miR-326 at a cutoff of <0.22-fold expression yielded an AUC of 0.927 (95% CI: 0.848–1.000), with 90.0% specificity and 92.0% sensitivity. MiR-155 achieved the highest AUC across all four biomarkers. These findings require external validation before any clinical application.

Table 8. Accuracy of miR-155 and miR-326 by ROC curve analysis*

Biomarker	Cutoff	AUC (95% CI)	Sensitivity %	Specificity %	PPV %	NPV %
miR-155	>2.24 fold	0.988 (0.973–1.000)	96.0	98.0	98.0	96.1
miR-326	<0.22 fold	0.927 (0.848–1.000)	92.0	90.0	90.2	91.8

* AUC – area under the curve CI – confidence interval, PPV – positive predictive value, NPV – negative predictive value

To account for potential confounding by age and BMI, multivariable binary logistic regression models were applied to examine whether the relationships of miR-155, miR-326, IL-17A, and IL-23 with essential hypertension remained statistically significant. The results demonstrated that all four biomarkers remained significantly associated with hypertension.

Table 9. Multivariable logistic regression model of related factors in patients with EH

Model	Variable	B	SE	χ^2 Wald	df	p	OR	95% CI for OR
miR-155	Age	0.057	0.101	0.325	1	0.569	1.059	0.869–1.291
	BMI	-0.511	0.255	4.000	1	0.045	0.600	0.364–0.990
	miR-155	-6.310	2.654	5.652	1	0.017	0.002	0.000–0.330
miR-326	Age	-0.005	0.030	0.027	1	0.870	0.995	0.939–1.055
	BMI	-0.069	0.044	2.490	1	0.115	0.933	0.856–1.017
	miR-326	2.412	0.612	15.542	1	0.000	11.153	3.363–36.993
IL-17	Age	-0.005	0.059	0.008	1	0.931	0.995	0.885–1.118
	BMI	-0.192	0.090	4.574	1	0.032	0.825	0.692–0.984
	IL17	-0.358	0.077	21.569	1	0.000	0.699	0.601–0.813
IL-23	Age	0.003	0.055	0.002	1	0.963	1.003	0.900–1.117
	BMI	-0.187	0.071	6.910	1	0.009	0.830	0.722–0.954
	IL23	-0.230	0.057	16.090	1	0.000	0.795	0.710–0.889

Discussion

This case-control study provides clinical evidence for the dysregulation of circulating miR-155 and miR-326, as well as the IL-17/IL-23 inflammatory axis in patients with essential hypertension. An experimental study demonstrated that IL-17A is not a secondary marker, but part of pathogenesis of hypertension.²⁰ Wang et al. demonstrated significantly elevated circulating Th17 cells and IL-23 levels in patients with hypertension, supporting the involvement of Th17-mediated inflammation in hypertension.¹⁵ Ahmed et al., demonstrated significant activation of the IL-23/Th17 in Egyptian patients with grade 2 hypertension, supporting the role of Th17-mediated inflammation in hypertension.¹² Furthermore, Yao et al. reported elevated serum IL-17 concentrations even among individuals with prehypertension, suggesting that activation of the IL-17 pathway may occur early during blood pressure dysregulation and persist throughout disease progression.²¹

These clinical observations are further supported by recent large-scale evidence highlighting the central role of immune and inflammatory pathways, particularly the IL-23/IL-17/Th17 axis, in the development and progression of hypertension. A systematic review and meta-analysis of circulating cytokines in hypertension underscored the contribution of immune mediators to development of hypertension, while contemporary reviews have emphasized the central role of inflammation disease progression.^{22,23}

The positive correlation between IL-17A and IL-23 levels identified in our study ($R^2=0.3816$) reinforces potential the interdependent nature of these cytokines and is consistent with prior reports demonstrating a synergistic relationship within the axis in patients with essential hypertension.¹⁵

The exploratory discriminatory performance of IL-17A (AUC=0.974) and IL-23 (AUC=0.946) in distinguishing patients with essential hypertension from

healthy controls underscores their potential as inflammatory biomarkers, although requiring further validation.

The concurrent upregulation of miR-155 and elevation of IL-17A in patients with essential hypertension may be associated with inflammatory marker. This finding is partly agreement with previous clinical studies suggesting a role for miR-155 in hypertension-associated inflammation. Huang et al. reported an association between increased circulating gene expression as well as miR-155 with elevated inflammatory markers in patients with white coat hypertension, supporting the involvement of miR-155 in early hypertensive inflammatory responses.³ While Kara et al. did not observe differences in miR-155 levels among patients newly diagnosed hypertension, resistant hypertension, and normotensive controls.²⁴ These discrepancies may reflect differences in patient populations, disease phenotypes and treatment status. Collectively, the available evidence suggests that the role of miR-155 may be dependent on and potentially influenced by both inflammatory and clinical characteristics of the studied population.

Conversely, the significant downregulation of miR-326 in patients with essential hypertension, occurring together with elevated IL-17A and IL-23 levels, presents a complex regulatory scenario. Similar alterations in circulating miRNA profiles have been reported in patients with essential hypertension. A study reported significant upregulation of miR-4516 and downregulation of miR-145 in patients with hypertension, supporting the concept that miRNA dysregulation may associated with the pathophysiology of hypertension.²⁵

Previous studies in autoimmune diseases, particularly multiple sclerosis, have reported increased miR-326 expression associated with enhanced Th17 differentiation through suppression of Ets-1.¹⁴ In contrast, the present study demonstrated a marked reduction in circulating miR-326 levels in patients with essential hypertension. Furthermore, the relationship between miR-326 and IL-17A does not appear to be straightforward; a study in celiac disease reported increased IL-17A levels despite downregulation of miR-326, suggesting that increased IL-17A production may occur independently of miR-326 expression under certain inflammatory conditions.²⁶ This apparent discrepancy suggests that the biological role of miR-326 may be disease-specific and associated with differences in the immune microenvironment, tissue origin, or metabolic factors. Furthermore, Th17 differentiation is controlled by a complex regulatory network involving multiple microRNAs and signaling pathways.²⁷ This discrepancy may indicate that the regulatory role of miR-326 is highly dependent and differs between autoimmune diseases and cardiovascular inflammatory conditions such as essential hypertension.

It can be hypothesized that IL-23-driven inflammatory mechanisms may become dominant in sustaining Th17 activity, potentially overriding or compensating for miR-326 regulatory role. However, this hypothesis was not directly investigated in the present study and requires experimental validation.

The significant positive correlations between miR-155 expression, age and BMI observed in this study are clinically relevant. Advancing age is associated with heightened oxidative stress and low-grade inflammation, while adipose tissue is a recognized source of circulating miR-155 that engages macrophage polarization and sustains pro-inflammatory signaling.²⁸

The downregulation of miR-326 expression and its negative correlation with BMI indicate that metabolic factors, particularly obesity-related immune dysregulation, may modulate miR-326 expression in a dependent manner. As knowing miRNA secretion from the growing adipose tissue which is one of the sources of circulating miRNAs and vary significantly as obesity develops.^{29,30}

There is no correlation between miRNA and the measured cytokine, despite their concurrent dysregulation in patients with essential hypertension. Therefore, the present findings do not provide direct evidence of a mechanistic interaction between these biomarkers. There is no evidence of a molecular cascade in this study. Further studies are required to clarify whether selected biomarkers interact mechanistically or reflect independent aspects of hypertensive inflammation.

The multivariable logistic regression analysis provided additional support for the observed associations. When adjustment for age and BMI, miR-155, miR-326, IL-17A, and IL-23 remained significantly associated with essential hypertension, suggesting that these associations may not fully explain the differences in age or BMI between the study groups. Although, obesity is recognized as a low-grade chronic inflammatory condition may contribute to the inflammatory milieu associated with hypertension, capable of promoting Th17-cell activation and improving the production of pro-inflammatory cytokines, including IL-17.^{31–33}

The simultaneous evaluation of circulating miRNAs and inflammatory cytokines offers a much more thorough insight into the nature of the inflammatory landscape in hypertension than the measurement of isolated markers. *In silico* target prediction was performed using publicly available databases (miRDB and TargetScan). The analysis identified SOCS1 as a potential target of miR-155 and Ets-1 as a potential target of both miR-155 and miR-326. The findings of present study generate hypotheses regarding possible regulatory pathways but do not provide experimental evidence.

Despite the high AUC value found through ROC analysis, the results should be interpreted cautiously

not conclusive for diagnosis because of the small number limits the generalizability of the results, and the observed exploratory discriminatory performance cannot be considered definitive evidence of diagnostic utility, although further studies on a larger scale are necessary.

Obesity and treatment status should also be considered when interpreting the present findings and may independently influence circulating cytokine concentrations and miRNA expression profiles.³⁴ The high prevalence of untreated disease (68.0%) in our cohort and detailed information regarding antihypertensive medication classes and treatment duration was unavailable, likely contributed to a persistently activated inflammatory milieu, further amplifying these cytokine levels. Likewise, antihypertensive medications have been reported to modulate inflammatory pathways and immune responses.³⁵ Therefore, the observed biomarker alterations may have been partially influenced by these factors, and future investigations with larger study populations and multivariable analyses are needed to further clarify their independent effects.

Study limitations

Several limitations in the present study. The sample size may insufficient to detect possible correlations between the expressions of miRNA and inflammatory cytokines levels. The single-center design generalizability to populations with different demographic and clinical characteristics. No formal adjustment for multiple comparisons was applied. In addition, multiple statistical comparisons across several biomarkers and correlation analyses may increase the risk of type I error. Findings of this study should be interpreted cautiously until confirmed in larger, independent investigation. Therefore, these findings suggest potential discriminatory value; however, validation in larger independent cohorts is required. It would be premature to consider diagnostic efficacy as per ROC analysis as definitive. Moreover, without any target gene validation for the specific miRNA, the study cannot prove its mechanism directly for example, SOCS1 is the target of miR-155. Future longitudinal and functional studies are needed to assess gene expression together with cytokine levels and miRNA profiling.

The use of a single housekeeping gene (GAPDH) for normalization, which may not fully comply with MIQE recommendations; however, its stable expression across study groups was confirmed by comparable Ct values. The multivariable regression models were adjusted only for age and BMI. Additional factors, including antihypertensive treatment characteristics and family history of hypertension, were not involved in the regression models and residual confounding cannot be completely excluded. Therefore, these factors should be considered in future studies.

Conclusion

Patients with essential hypertension showed significant upregulation of miR-155, IL-17A, and IL-23, along with downregulation of miR-326 compared with normotensive control. Although these biomarkers were significantly dysregulated, no direct correlation was identified between miRNA expression and cytokine concentrations. The present finding suggests that these biomarkers may perform distinct aspects of the hypertension-associated inflammation rather than a confirmed molecular pathway. The findings should be regarded preliminary, require validation in larger, independent, preferably prospective cohorts before their potential clinical relevance can be established.

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Declarations

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Author contributions

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

Conflicts of interest

The authors have no competing interests to declare in relation to this study.

Data availability

Data and material analyzed during the current study can be available by contacting the corresponding author upon reasonable requests.

Ethics approval

The research followed the ethical guidelines of the College of Medicine at the University of Al-Qadisiyah, in according with the Declaration of Helsinki (Approval Number 41, Date 20/10/2025).

Use of AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used AI to improve the language, grammar.

References

- Harrison DG, Guzik TJ, Lob HE, et al. Inflammation, immunity, and hypertension. *Hypertension*. 2011;57(2):132-140. doi:10.1161/HYPERTENSIONAHA.110.163576
- Zhang Z, Zhao L, Zhou X, Meng X, Zhou X. Role of inflammation, immunity, and oxidative stress in hypertension: New insights and potential therapeutic targets. *Front Immunol*. 2023;13:1098725. doi:10.3389/fimmu.2022.1098725
- Huang Y qing, Huang C, Zhang B, Feng Y. Association of circulating miR-155 expression level and inflammatory markers with white coat hypertension. *J Hum Hypertens*. 2020;34(5):397-403. doi:10.1038/s41371-019-0250-7
- Rudemiller NP, Crowley SD. Interactions Between the Immune and the Renin–Angiotensin Systems in Hypertension. *Hypertension*. 2016;68(2):289-296. doi:10.1161/HYPERTENSIONAHA.116.06591
- Higaki A, Mahmoud AUM, Paradis P, Schiffrin EL. Role of interleukin-23/interleukin-17 axis in T-cell-mediated actions in hypertension. *Cardiovasc Res*. 2021;117(5):1274-1283. doi:10.1093/cvr/cvaa257
- Bloch Y, Bouchareychas L, Merceron R, et al. Structural Activation of Pro-inflammatory Human Cytokine IL-23 by Cognate IL-23 Receptor Enables Recruitment of the Shared Receptor IL-12R β 1. *Immunity*. 2018;48(1):45-58. e6. doi:10.1016/j.immuni.2017.12.008
- Davis GK, Fehrenbach DJ, Madhur MS. Interleukin 17A: Key Player in the Pathogenesis of Hypertension and a Potential Therapeutic Target. *Curr Hypertens Rep*. 2021;23(3):13. doi:10.1007/s11906-021-01128-7
- Jung R, Lehmann A, Knopp T, et al. Antagonizing IL-17A Reduces Vascular Inflammation and Attenuates Oxidative Stress Formation but Does Not Significantly Improve Vascular Dysfunction Induced by One Week of Angiotensin II Treatment. *Antioxidants (Basel)*. 2026;15(2):229. doi:10.3390/antiox15020229
- Norlander AE, Saleh MA, Kamat NV, et al. Interleukin-17A Regulates Renal Sodium Transporters and Renal Injury in Angiotensin II-Induced Hypertension. *Hypertension*. 2016;68(1):167-174. doi:10.1161/HYPERTENSIONAHA.116.07493
- Madhur MS, Lob HE, McCann LA, et al. Interleukin 17 promotes angiotensin II-induced hypertension and vascular dysfunction. *Hypertension*. 2010;55(2):500-507. doi:10.1161/HYPERTENSIONAHA.109.145094
- Krebs CF, Lange S, Niemann G, et al. Deficiency of the Interleukin 17/23 Axis Accelerates Renal Injury in Mice With Deoxycorticosterone Acetate+Angiotensin II-Induced Hypertension. *Hypertension*. 2014;63(3):565-571. doi:10.1161/HYPERTENSIONAHA.113.02620
- Ahmed EES, Ali FHA, Basry AE, El-Deeb MA. Assessment of IL23/Th17 Axis in Egyptian Patients with Grade 2 Hypertension. *Egyptian Journal of Hospital Medicine*. 2022;87(1):2259-2264. doi:10.21608/ejhm.2022.234293
- Yao R, Ma YL, Liang W, et al. MicroRNA-155 Modulates Treg and Th17 Cells Differentiation and Th17 Cell Function by Targeting SOCS1. *PLoS ONE*. 2012;7(10):e46082. doi:10.1371/journal.pone.0046082
- Du C, Liu C, Kang J, et al. MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. *Nat Immunol*. 2009;10(12):1252-1259. doi:10.1038/ni.1798
- Wang J, Chen J, Chen C, Huang S, Rao X, Zhong J. Elevated Th17 and IL-23 in Hypertensive Patients with Acutely Increased Blood Pressure. *Am J Immunol*. 2012;8(2):27-32. doi:10.3844/ajisp.2012.27.32
- Abo-Ali F, Ahmed EE, El-deeb MA, Basry AEA. Elevated IL 23 in Hypertensive Patients with Acutely Increased Blood Pressure. *QJM*. 2020;113(1):hcaa052.059. doi:10.1093/qjmed/hcaa052.059
- Saleh MA, Norlander AE, Madhur MS. Inhibition of Interleukin-17A, But Not Interleukin-17F, Signaling Lowers Blood Pressure, and Reduces End-Organ Inflammation in Angiotensin II-Induced Hypertension. *JACC: Basic to Translational Science*. 2016;1(7):606-616. doi:10.1016/j.jacbts.2016.07.009
- Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res*. 2007;35(Web Server issue):W71-74. doi:10.1093/nar/gkm306
- Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262
- Itani HA, McMaster WG, Saleh MA, et al. Activation of human T cells in hypertension: Studies of Humanized Mice and Hypertensive Humans. *Hypertension*. 2016;68(1):123-132. doi:10.1161/HYPERTENSIONAHA.116.07237
- Yao W, Sun Y, Wang X, Niu K. Elevated Serum Level of Interleukin 17 in a Population With Prehypertension. *J Clin Hypertens (Greenwich)*. 2015;17(10):770-774. doi:10.1111/jch.12612
- Guzik TJ, Nosalski R, Maffia P, Drummond GR. Immune and inflammatory mechanisms in hypertension. *Nat Rev Cardiol*. 2024;21(6):396-416. doi:10.1038/s41569-023-00964-1
- Caiazzo E, Sharma M, Rezig AOM, et al. Circulating cytokines and risk of developing hypertension: A systematic review and meta-analysis. *Pharmacol Res*. 2024;200:107050. doi:10.1016/j.phrs.2023.107050
- Kara SP, Ozkan G, Yilmaz A, Bayrakçı N, Güzel S, Geyik E. MicroRNA 21 and microRNA 155 levels in resistant hypertension, and their relationships with aldosterone. *Ren Fail*. 2021;43(1):676-683. doi:10.1080/0886022X.2021.1915800
- Özkan G, Ulusoy Ş, Geyik E, Erdem Y. Down-regulation of miRNA 145 and up-regulation of miRNA 4516 may be associated with primary hypertension. *J Clin Hypertens (Greenwich)*. 2019;21(11):1724-1731. doi:10.1111/jch.13704

26. Nafari M, Asri N, Rostami-Nejad M, et al. Elevated interleukin-17A levels despite reduced microRNA-326 gene expression in celiac disease patients under gluten-free diet. *Rom J Intern Med.* 2022;60(3):166-172. doi:10.2478/rjim-2022-0011
27. Huang J, Xu X, Yang J. miRNAs Alter T Helper 17 Cell Fate in the Pathogenesis of Autoimmune Diseases. *Front Immunol.* 2021;12:593473. doi:10.3389/fimmu.2021.593473
28. Cerda A, Amaral AA, de Oliveira R, et al. Peripheral Blood miRome Identified miR-155 as Potential Biomarker of MetS and Cardiometabolic Risk in Obese Patients. *Int J Mol Sci.* 2021;22(3):1468. doi:10.3390/ijms22031468
29. Thomou T, Mori MA, Dreyfuss JM, et al. Adipose-derived circulating miRNAs regulate gene expression in other tissues. *Nature.* 2017;542(7642):450-455. doi:10.1038/nature21365
30. Vega-Cárdenas M, Uresti-Rivera EE, Cortés-García JD, et al. Increased levels of adipose tissue-resident Th17 cells in obesity associated with miR-326. *Immunology Letters.* 2019;211:60-67. doi:10.1016/j.imlet.2019.05.010
31. Chehimi M, Vidal H, Eljaafari A. Pathogenic Role of IL-17-Producing Immune Cells in Obesity, and Related Inflammatory Diseases. *J Clin Med.* 2017;6(7):68. doi:10.3390/jcm6070068
32. Zhang S, Gang X, Yang S, et al. The Alterations in and the Role of the Th17/Treg Balance in Metabolic Diseases. *Front Immunol.* 2021;12:678355. doi:10.3389/fimmu.2021.678355
33. Kang YS. Obesity Associated Hypertension: New Insights into Mechanism. *Electrolyte Blood Press.* 2013;11(2):46-52. doi:10.5049/EBP.2013.11.2.46
34. Rakib A, Kiran S, Mandal M, Singh UP. MicroRNAs: a crossroad that connects obesity to immunity and aging. *Immun Ageing.* 2022;19(1):64. doi:10.1186/s12979-022-00320-w
35. Bryniarski P, Nazimek K, Marcinkiewicz J. Immunomodulatory Activity of the Most Commonly Used Antihypertensive Drugs-Angiotensin Converting Enzyme Inhibitors and Angiotensin II Receptor Blockers. *Int J Mol Sci.* 2022;23(3):1772. doi:10.3390/ijms23031772



ORIGINAL PAPER

Nebulized budesonide vs. ipratropium as an add-on to salbutamol in preschool wheeze – a randomized controlled trial

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ABSTRACT

Introduction and aim. Acute wheeze is common cause of emergency visits and hospitalization in preschool children. This randomized controlled trial directly compared nebulized budesonide versus ipratropium bromide as add-on therapy to salbutamol in children aged 1–5 years with moderate-to-severe acute wheeze.

Material and methods. This hospital-based randomized controlled trial included 84 children equally assigned to two groups. Group A received nebulized budesonide with salbutamol, and Group B received nebulized ipratropium bromide with salbutamol. The co-primary outcomes were the change in Pediatric Respiratory Assessment Measure (PRAM) score from baseline to 60 minutes and the PRAM score trajectory during the first 60 minutes.

Results. Baseline PRAM scores were comparable between groups. Group A showed significantly lower PRAM scores at 20, 40, 60 minutes, with greater mean PRAM reduction at 60 minutes ($p < 0.05$). Mean time to achieve $\text{PRAM} \leq 4$ was shorter in Group A. Group A children had a significantly shorter hospital stay.

Conclusion. Nebulized budesonide as an add-on to salbutamol was associated with greater short-term improvement in PRAM scores and shorter hospital stay than ipratropium bromide; larger multicenter studies are needed.

Keywords. budesonide, child, ipratropium, preschool, salbutamol, wheezing

Introduction

Acute exacerbations of wheeze are among the most common reasons for emergency visits and hospital admissions among the children aged 1–5 years, contributing substantially to pediatric morbidity, health care utilization, and caregiver burden worldwide.¹ In early childhood, wheezing episodes are commonly triggered by viral respiratory infections and are associated with airway inflammation, mucosal edema, bronchoconstriction, and increased mucus secretion, leading to respiratory distress.² Because of smaller airway caliber and heightened inflammatory responses, young children are more vulnerable to severe manifestations and

may require prompt treatment to prevent clinical deterioration.³

The mainstay of acute wheeze management is rapid reversal of bronchospasm and improvement in oxygenation, with nebulized salbutamol forming the cornerstone of therapy.⁴ The bronchodilatory effect of salbutamol is through stimulation of β_2 adrenergic receptors present in the bronchial smooth muscle. Salbutamol has limited effect on the underlying inflammatory process, and some children show suboptimal responses when treated with salbutamol alone.⁵ For moderate-to-severe exacerbations, ipratropium bromide is frequently added because it blocks muscarinic recep-

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tors, reduces vagally mediated bronchoconstriction, and may improve clinical outcomes, especially in severe cases.^{6,7} However, its action is largely bronchodilation and does not directly target airway inflammation.

This has led to increasing interest in early use of inhaled corticosteroids during acute wheezing episodes. Budesonide, a potent inhaled corticosteroid with high topical anti-inflammatory activity and a favorable safety profile, has been proposed as an adjunct to bronchodilator therapy.^{8,9} Nebulized budesonide is particularly suitable for young children because it allows targeted pulmonary delivery with minimal systemic absorption and can be administered even in those unable to use inhaler devices effectively.¹⁰ Previous studies suggest that early administration of inhaled corticosteroids may hasten symptom resolution, improve respiratory scores, and reduce the need for systemic corticosteroids and prolonged hospitalization.¹¹ Nevertheless, evidence remains inconsistent, particularly in children under 5 years, because of variations in study design, patient characteristics, dosing regimens, and outcome measures across trials.¹²

At present, there is no universal consensus regarding the best add-on therapy to salbutamol in preschool children with acute wheeze. Although ipratropium bromide is widely used in emergency care, its comparative efficacy against nebulized budesonide has not been adequately established in well-designed randomized controlled trials among children aged 1–5 years.^{1,6} This question is clinically important because recurrent or inadequately treated wheeze in early childhood may increase the risk of recurrent hospitalizations, persistent airway hyperresponsiveness, and progression to chronic asthma.^{8,9}

The Pediatric Respiratory Assessment Measure (PRAM) score is a validated tool used to assess the severity of wheeze and monitor response to treatment in children with acute respiratory distress.²

Despite widespread clinical use of both therapies, direct randomized comparisons between nebulized budesonide and ipratropium bromide as add-on treatment to salbutamol in preschool wheeze remain limited. Along with PRAM improvement, duration of hospital stay is a clinically meaningful outcome that reflects both recovery and healthcare resource use.^{3,10–12}

Against this background, the present randomized controlled trial was designed to address the existing evidence gap by directly comparing nebulized budesonide plus salbutamol with ipratropium bromide plus salbutamol in children aged 1–5 years with moderate-to-severe acute exacerbations of wheeze. By directly comparing these two commonly used adjunctive regimens, the study aims to generate evidence on symptom resolution, PRAM score improvement, and hospital stay and thereby help guide evidence-based management of acute wheeze in young children. This study pro-

vides a direct head-to-head comparison of nebulized budesonide and ipratropium bromide as add-on therapies in preschool children, a population in which comparative evidence remains limited. Additionally, this study evaluates early response using serial PRAM scoring at short intervals, which has been inadequately explored in previous trials. Ipratropium bromide is widely used as an adjunct to salbutamol in moderate-to-severe acute wheeze; therefore, comparison with this commonly employed regimen was considered more clinically relevant than comparison with salbutamol alone.

Aim

The aim was to compare the efficacy of nebulized budesonide versus ipratropium bromide as add-on therapy to salbutamol in preschool children with moderate-to-severe acute wheeze

Co-primary objectives

1. To compare the change in Pediatric Respiratory Assessment Measure (PRAM) score from baseline to 60 minutes between treatment groups.
2. To compare the trajectory of PRAM scores during the first 60 minutes following treatment between treatment groups.

Secondary objectives

1. To compare the time required to achieve a PRAM score ≤ 4 between the two treatment groups.
2. To compare the duration of hospital stay between the two treatment groups.
3. To assess the need for additional bronchodilator or corticosteroid therapy following the initial treatment period.

Material and methods

Study design and setting

This hospital-based randomized controlled trial with a parallel-group design was carried out during the period from 2025 to 2026 in the Department of Pediatrics, SRM Medical College Hospital and Research Centre, a tertiary care teaching hospital. The participants were recruited from the pediatric emergency department and PICU, where children presenting with acute exacerbation of wheeze were routinely evaluated and managed. During the study period, participant recruitment, intervention administration, follow-up assessment, data collection and data analysis were carried out.

Study population

Children of age 1 to 5 years, presenting with acute exacerbation of wheeze, were screened for eligibility. The children were included if written informed consent was provided by a parent or legal guardian, children were aged 1 to 5 years, had clinically diagnosed wheeze, char-

acterized by audible high-pitched whistling sounds predominantly during expiration, had moderate to severe exacerbation, defined by a Pediatric Respiratory Assessment Measure (PRAM) score of >4 . The children presenting with life-threatening exacerbation of wheeze, had received systemic or inhaled corticosteroid therapy within the preceding 24 hours, had known cardiovascular disease, primary or secondary immunodeficiency, or chronic systemic illness, had mild exacerbation of wheeze, defined as a PRAM score of 0–4, were excluded.

Eligible participants were screened and enrolled at the time of presentation to the pediatric emergency department or PICU before initiation of the study intervention. Participants were recruited by consecutive sampling. All eligible children presenting to pediatric emergency department or PICU during the study period were assessed for eligibility and enrolled consecutively until the estimated sample size was attained. The sample size was estimated based on previously published studies evaluating nebulized budesonide in preschool wheeze. Assuming a two-sided α error of 0.05, a study power of 80%, and equal allocation between groups, the minimum required sample size was estimated as 42 participants per group (total $n=84$). The participants were equally allocated into treatment groups A and B, with 42 children in each treatment arm.

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of SRM Medical College Hospital and Research Centre (approval No.: SRMIEC-ST0724-1721). Written informed consent was obtained from parents or legal guardians. Parents were informed that participation was voluntary and they could withdraw their child from the study at any time without affecting the treatment. The study involved minimal risk and no invasive procedures beyond standard care. Patient confidentiality was strictly maintained throughout the study. The study was self-funded, and no external financial support or commercial sponsorship was involved.

Randomization and allocation

Randomization was performed using a computer-generated random sequence with a 1:1 allocation ratio. Allocation concealment was ensured using sequentially numbered, opaque, sealed envelopes prepared by an independent investigator. Due to the nature of the intervention, blinding was not feasible. All randomized participants were included in the final analysis. The study utilized an open-label design owing to the nature of the interventions; however, outcome assessment via PRAM scoring adhered to standardized criteria to mitigate observer bias. PRAM assessments were performed by pediatric residents and consultants familiar with the

PRAM scoring system and its standardized assessment criteria. All assessors received orientation regarding standardized PRAM assessment before study initiation. The same standardized PRAM assessment criteria were applied throughout the study to minimize inter-observer variability. However, formal certification using the online PRAM training module and assessment of inter-rater reliability were not performed.

Intervention

The enrolled participants were assigned to one of the following treatment groups:

Group A: Nebulized Salbutamol and Nebulized Budesonide

Group B: Nebulized Salbutamol and Nebulized Ipratropium Bromide

Dose:

1. Salbutamol: <20 kg – 2.5 mg; >20 kg – 5 mg
2. Ipratropium bromide: 250 μ g for children <20 kg and 500 μ g for children ≥ 20 kg, as per standard pediatric dosing guidelines
3. Budesonide: 0.5 mg

The study medications were administered according to standard pediatric dosing protocols. Each child received three consecutive nebulization cycles at 20-minute intervals during the first hour of treatment. Nebulization was administered using oxygen-driven jet nebulizers according to institutional protocol. Supportive care, including oxygen supplementation, was provided whenever required.

The study intervention consisted of three consecutive nebulization cycles administered during the first hour of treatment. Following completion of the study intervention, further management, including bronchodilator therapy, corticosteroid administration, oxygen supplementation, and discharge decisions, was provided according to the institutional treatment protocol and at the discretion of the treating pediatrician. No additional study-specific intervention was administered beyond the initial treatment period. Hospital admission and discharge decisions were made by the treating pediatric team according to institutional clinical protocols and physician judgment.

Outcome measures

The co-primary outcomes were (1) the change in Pediatric Respiratory Assessment Measure (PRAM) score from baseline to 60 minutes and (2) the PRAM score trajectory during the first 60 minutes following initiation of therapy. Secondary outcomes included time to achieve a PRAM score ≤ 4 and duration of hospital stay. Respiratory rate, heart rate, oxygen saturation, Glasgow Coma Scale, and need for additional therapy were monitored clinically but were not systematically retained for detailed secondary analysis.

Baseline demographic variables such as age and sex were also recorded. Laboratory parameters including complete blood count (CBC), eosinophil count, and C-reactive protein (CRP) were documented. Viral testing was not performed routinely and was undertaken only when clinically indicated using nasopharyngeal or throat swab RT-PCR testing. Therefore, viral identification was available only for a subset of participants. At presentation, all eligible children underwent detailed clinical evaluations, including history taking and physical examination. Baseline assessment included PRAM score, RR, HR, oxygen saturation, and GCS. After enrollment and randomization, the assigned nebulization regimen was administered. Participants were reassessed at 20, 40, and 60 minutes after nebulization using PRAM scores and vital parameters. Children were subsequently monitored until clinical stabilization and discharge. Data were collected using a standardized pro forma designed for the study.

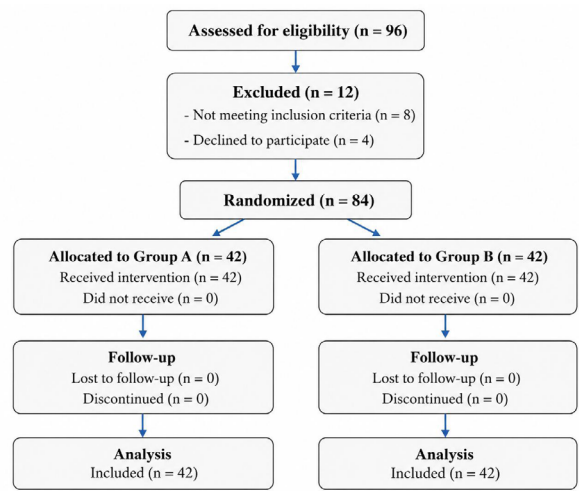


Fig. 1. CONSORT flow diagram showing participant recruitment, randomization, follow-up, and analysis

Statistical analysis

Data were coded and entered into Microsoft Excel and analyzed using SPSS software version 28 (IBM, Armonk, NY, USA). Frequencies and percentages were used to express categorical variables, while mean and standard deviation were used for continuous variables. Inferential statistics included the chi-square test and Fisher’s exact test for categorical variables. For the co-primary outcomes, the change in PRAM score from baseline to 60 minutes was compared between groups using the independent samples t-test, whereas the PRAM score trajectory during the first 60 minutes was analyzed using repeated-measures ANOVA. Post hoc pairwise comparisons with Bonferroni correction were performed where applicable. The p value of <0.05 was taken as statistical significance. No participants were lost to follow-up during the predefined observation period. Therefore, no imputation for missing outcome data was required.

Results

A total of 96 children were evaluated for eligibility, with 84 satisfying the inclusion criteria and subsequently randomized into two equal groups (n=42 each). No participants were lost to follow-up, and all randomized participants were included in the final analysis.

The baseline demographic and clinical characteristics, treatment response, and outcome measures were analyzed and compared between the nebulized budesonide plus salbutamol group and the nebulized ipratropium bromide plus salbutamol group.

Baseline demographic and clinical characteristics

The mean age was 2.93 years (SD 1.42) in Group A and 3.21 years (SD 1.50) in Group B. Although the distribution across age categories showed some imbalance between groups despite randomization, the mean ages were broadly similar. Sex distribution and weight were also comparable between groups.

All children in both groups had no cyanosis. Chest radiograph findings, viral profile, and mode of oxygen supplementation were broadly comparable between the two groups. Viral testing was available only for a subset of participants because testing was performed when clinically indicated.

Table 1. Baseline demographic and clinical characteristics of study participants*

S. No	Variable	Category	Group A	Group B
1	Age group	1 to 2 years	6 (14.3 %)	10 (23.8 %)
		2 to 3 years	22 (52.4 %)	10 (23.8 %)
		3 to 5 years	14 (33.3 %)	22 (52.4 %)
	In years	Mean (SD)	2.93 (1.42)	3.21 (1.50)
2	Sex	Male	16 (48.5%)	17 (51.5%)
		Female	26 (51%)	25 (49%)
3	Weight (kg)	Mean (SD)	12.3 (2.8)	13.6 (3.08)
4	Cyanosis	Absent	42 (100%)	42 (100%)
5	Chest x ray	Normal	29 (48.3%)	31 (51.7%)
		Hyperinflation	13 (54.2%)	11 (45.8%)
6	Throat swab	Positive	7 (46.7%)	8 (53.3%)
		Negative	35 (50.7%)	34 (49.3%)
		Influenza	5 (50%)	5 (50%)
7	Virus type	RSV	2 (40%)	3 (60%)
		NA	35 (50.7%)	34 (49.3%)
		HFNC	7 (46.7%)	8 (53.3%)
8	Oxygen mode	Simple O2	35 (50.7%)	34 (49.3%)

* Data are presented as number (%) or mean±SD, HFNC – high flow nasal cannula, viral testing was performed only when clinically indicated; percentages are based on available data

Comparison of PRAM scores at different time intervals

For the first co-primary outcome, baseline PRAM scores were similar between Group A (6.64±1.428) and Group B (6.71±1.312; p=0.291). At 20 minutes, PRAM scores were lower in Group A (5.05±1.67) compared to

Group B (6.14 ± 1.28 ; $p=0.048$). At 40 minutes, PRAM scores were lower in Group A (3.76 ± 1.68) compared to Group B (5.76 ± 1.36 ; $p=0.034$). At 60 minutes, PRAM scores were lower in Group A (2.50 ± 1.50) compared to Group B (5.26 ± 1.43 ; $p=0.038$).

PRAM scores decreased more rapidly in Group A compared to Group B at all post-treatment time points.

Table 2. Comparison of PRAM scores between groups at different time intervals*

Time point	Group A (n=42)	Group B (n=42)	p
Baseline	6.64±1.428	6.71±1.312	0.291
20 minutes	5.05±1.667	6.14±1.280	0.048
40 minutes	3.76±1.679	5.76±1.358	0.034
60 minutes	2.50±1.502	5.26±1.432	0.038
Change at 60 minutes	4.14±0.647	1.45±0.889	0.006

* Data are presented as mean±SD

For the second co-primary outcome, repeated measures ANOVA demonstrated a statistically significant time × group interaction ($p < 0.05$) (Fig. 2), indicating that the reduction in PRAM scores over time differed between groups.

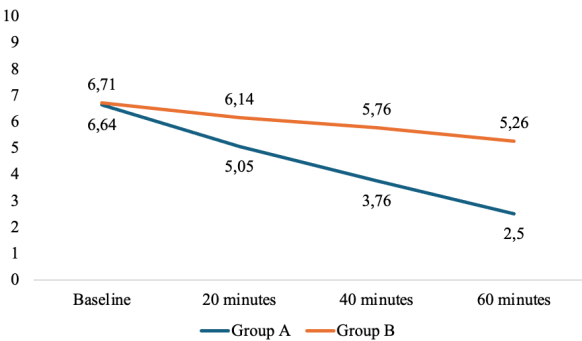


Fig. 2. Comparison of PRAM scores at different time intervals

Comparison of time to achieve PRAM≤4

The mean time to achieve a PRAM score ≤ 4 was shorter in group A compared to group B (38.45 ± 3.351 minutes vs. 52.40 ± 3.883 minutes; $p=0.011$).

Table 3. Comparison of time to achieve PRAM ≤ 4*

	Study group	N	Mean	SD	p
Time to PRAM≤ 4	Group A	42	38.45	3.351	0.011
	Group B	42	52.40	3.883	

* Data are presented as mean±SD

Comparison of duration of hospital stay

The hospital stay was found to be significantly lower in group A than in group B (2.414 ± 0.2581 vs 3.495 ± 0.2793 days; $p=0.007$).

Respiratory rate, heart rate, oxygen saturation, and Glasgow Coma Scale were monitored clinically during

patient management but were not systematically retained for detailed secondary analysis

Table 4. Comparison of duration of hospital stay*

	Study group	n	Mean	SD	p
Hospital stay days	Group A	42	2.414	0.2581	0.007
	Group B	42	3.495	0.2793	

* Data are presented as mean±SD

Adverse events

No adverse events were observed in either group during the study period.

Discussion

This randomized controlled trial compared two commonly used add-on nebulized regimens given with salbutamol in children aged 1–5 years presenting with moderate-to-severe acute exacerbation of wheeze. The principal finding was that nebulized budesonide plus salbutamol (group A) was superior to nebulized ipratropium bromide plus salbutamol (group B) for both co-primary outcomes, namely the change in PRAM score from baseline to 60 minutes and the PRAM score trajectory during the first 60 minutes. This was accompanied by a shorter time to achieve PRAM ≤4 and a reduced hospital stay.

Baseline comparability between the two groups was largely acceptable. Although age-group distribution differed between groups, mean age and baseline PRAM scores were comparable. Nevertheless, the possibility of residual confounding related to age cannot be completely excluded. This age profile is close to preschool wheeze cohorts studied by Razi et al.¹³ and Razi and Andiran et al.¹⁴ whereas studies by Upham et al.¹⁵ and Wyatt et al.¹⁶ included broader pediatric age groups and therefore greater phenotypic heterogeneity. Thus, the present study reflects a preschool-aged population presenting with acute wheezing, although wheezing phenotypes were not systematically characterized. Sex distribution was balanced, consistent with prior studies.^{13,15} Mean weight was also comparably similar to other studies.^{13,14} The absence of cyanosis in both groups further indicates that the cohort represented moderate-to-severe wheeze without overt cyanotic respiratory failure, and therefore the findings are most applicable to this clinical spectrum, comparable to other studies.^{13,15} Chest X-ray findings, throat swab results, virus type, and oxygen support mode were similar between groups.

Both co-primary outcomes, namely change in PRAM score from baseline to 60 minutes and PRAM score trajectory, favored nebulized budesonide. Baseline PRAM scores were similar, but group A (nebulized salbutamol and budesonide) showed significantly lower PRAM scores at 20, 40 and 60 minutes, with greater overall reduction at 60 minutes. This indicates a more

rapid and sustained clinical response with nebulized budesonide plus salbutamol. These findings are consistent with studies showing benefit from early nebulized budesonide in acute asthma or wheeze. Chen et al. demonstrated better early lung function recovery and higher remission rates with high-dose budesonide.¹⁷ Razi et al. also reported improved clinical scores and lower hospitalization rates in preschool children receiving nebulized budesonide.¹³ Murphy et al. similarly concluded that early nebulized budesonide may reduce symptom severity and hospitalization in children aged ≤ 5 years.⁹ However, not all studies have shown benefit. Upham et al. found no difference in early asthma score improvement or admission rates,¹⁵ and Razi and Andiran et al. reported no difference in length of stay in hospitalized wheezing children.¹⁴ These differences may be due to variations in severity, dosing, and concurrent systemic corticosteroid use.

The shorter time to achieve PRAM ≤ 4 in group A (nebulized salbutamol and budesonide) is clinically meaningful because it reflects earlier stabilization and may reduce repeated nebulization, prolonged monitoring, and escalation of care. This is comparable to findings from Razi et al., who reported shorter discharge time with budesonide,¹³ and Chen et al., who found earlier remission.¹⁷ Although ipratropium-containing regimens have shown benefit in some studies, particularly when compared with salbutamol alone,^{19,21} the present study suggests that budesonide was superior to ipratropium bromide as an add-on to salbutamol. Nevertheless, ipratropium bromide remains an effective adjunct bronchodilator in acute wheeze and has demonstrated clinical benefit when combined with salbutamol, particularly in moderate-to-severe exacerbations.

A further important finding was the significantly shorter hospital stay in group A. This suggests early improvement being translated into a downstream service benefit. Similar reductions in hospitalization burden with budesonide have been reported previously,^{13,18} although some studies did not find a significant length-of-stay advantage.^{14,16} These results should be interpreted with caution due to the limited sample size, single-center design, and baseline age distribution imbalance.

All enrolled children were admitted for observation according to institutional protocol because they presented with moderate or severe wheezing requiring repeated nebulization. Discharge decisions were based on clinical stability, oxygen independence, adequate oral intake, and caregiver readiness. Therefore, duration of hospitalization may have been influenced not only by clinical response but also by institutional discharge practices. Consequently, differences in hospital stay should be interpreted as exploratory findings rather than definitive measures of treatment efficacy.

Study strengths and limitations

Randomized equal allocation improved internal validity. The study focused on preschool children, a clinically important age group with limited comparative evidence. Serial PRAM scoring at fixed intervals captured early response objectively. Clinically relevant outcomes such as time to PRAM ≤ 4 and hospital stay increased practical applicability to emergency and inpatient pediatric care.

Despite randomization, age-group distribution differed between treatment groups at baseline. Since younger children may have different wheezing phenotypes and treatment responses compared with older preschool children, residual confounding cannot be excluded.

Larger studies with stratified randomization according to age are warranted. The study did not systematically characterize wheezing phenotypes, including recurrent wheeze history, atopic status, family history of asthma, or allergic sensitization. Information regarding previous hospitalization for wheeze, environmental tobacco smoke exposure, fever, and detailed atopic history was not systematically collected and therefore could not be analyzed. Therefore, subgroup analyses according to wheezing phenotype could not be performed, and differential treatment responses among specific phenotypic subgroups cannot be excluded. The sample size was modest, and the study was conducted in a single center, limiting generalizability. PRAM scoring may be subject to observer variation. Although PRAM scoring followed standardized criteria and assessors received orientation regarding PRAM assessment, formal assessor certification and inter-rater reliability testing were not conducted. Respiratory rate, heart rate, oxygen saturation, and Glasgow Coma Scale were monitored clinically but were not systematically retained for detailed secondary analysis. Systemic corticosteroid administration after the initial study intervention was not systematically recorded and may have influenced later outcomes, including duration of hospitalization. Viral categorization was limited, and the observed reduction in hospital stay may have reflected institutional discharge practices in addition to treatment response; therefore, hospital stay should not be interpreted as a measure of treatment efficacy alone. Outcome assessors were not blinded to treatment allocation because of the open-label design. Although PRAM scoring was performed using standardized criteria and assessors received orientation regarding PRAM assessment, the open-label design could have influenced subjective clinical assessment, and observer bias cannot be excluded. Criteria for treatment failure and initiation of rescue systemic corticosteroids were based on institutional clinical protocols and physician judgment rather than predefined study-specific criteria.

Although the study achieved the planned sample size, detailed assumptions underlying the original sample size calculation, including the expected treatment

effect and standard deviation, were unavailable from archived study records. Therefore, the adequacy of statistical power should be interpreted cautiously. Treatment failure was not predefined as a study outcome. Escalation of therapy was determined by the treating pediatrician according to institutional clinical protocols.

Clinical implications

The findings suggest that nebulized budesonide as an add-on to salbutamol may provide faster symptom relief, earlier stabilization, and shorter hospitalization in moderate-to-severe wheeze. This can improve bedside decision-making, reduce escalation of care, optimize bed utilization, and lessen caregiver burden. PRAM also proved useful as a practical monitoring tool in acute settings. The regimen associated with faster PRAM improvement and shorter hospital stay may be considered as an add-on therapy to salbutamol in similar clinical settings; however, these findings require confirmation in larger multicenter randomized trials before routine clinical implementation. PRAM should be monitored at regular short intervals. Non-responding by 40–60 minutes should be reassessed early. Standardized discharge criteria, oxygen weaning, and correct nebulization techniques should be ensured.

Future directions

Large multicenter randomized trials are needed to confirm these findings. Future studies should use stratified randomization by age and severity, standardize co-interventions, and assess additional outcomes such as recurrence of wheezing episodes, emergency department revisit rates, subsequent systemic corticosteroid therapy, oxygen weaning time, and cost-effectiveness. Subgroup analyses according to viral status, atopy, and recurrent wheeze may improve individualized treatment selection.

Conclusion

In this randomized direct comparison, nebulized budesonide as an add-on to salbutamol was associated with greater short-term improvement in PRAM scores and shorter hospital stay than nebulized ipratropium bromide as an add-on to salbutamol in children aged 1–5 years presenting with moderate or severe wheezing. Larger multicenter studies with detailed phenotypic characterization of preschool wheezing are needed to confirm these findings and determine the patient subgroups most likely to benefit. These findings may help optimize the acute management of preschool wheeze pending confirmation in larger multicenter randomized trials.

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Declarations

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Author contributions

Conceptualization, V.V.; Methodology, R.A.; Investigation, R.A.; Data curation, R.A.; Writing – original draft, R.A.; Writing – review & editing, V.V., A.R., S.S.; Supervision, S.S.

Conflicts of interest

The authors declare no competing interests.

Data availability

The datasets are available from the corresponding author on reasonable request.

Ethics approval

The study was approved by the Institutional Ethics Committee of SRM Medical College Hospital and Research Centre (Approval No: SRMIEC-ST0724-1721). Written informed consent was obtained from the parents or legal guardians of all participating children.

Use of AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT (OpenAI) to assist with language refinement and manuscript polishing. After using these tools, the authors reviewed and edited all content as needed and take full responsibility for the content of the published article.

Trial registry

CTRI/2025/04/085680 (registered on 25/04/2025).

References

1. Abood HA, Almusawi ZH, Mahdi AA, Hameed R. Effects of nebulized budesonide plus salbutamol and nebulized salbutamol monotherapy on mild to moderate acute exacerbation of asthma in children. *J Pak Med Assoc.* 2021;71:29–34.
2. Ducharme FM, Chalut D, Plotnick L, et al. The pediatric respiratory assessment measure: a valid clinical score for assessing acute asthma severity from toddlers to teenagers. *J Pediatr.* 2008;152(4):476–480. doi:10.1016/j.jpeds.2007.08.034
3. Global Initiative for Asthma. Global strategy for asthma management and prevention. Updated 2023.
4. British Thoracic Society, Scottish Intercollegiate Guidelines Network. British guideline on the management of asthma. *Thorax.* 2019;74(Suppl 3):1–214.

5. Rodrigo GJ, Castro-Rodriguez JA. Anticholinergics in the treatment of children and adults with acute asthma: a systematic review with meta-analysis. *Thorax*. 2005;60:740-746. doi:10.1136/thx.2005.040444
6. Xu H, Tong L, Gao P, et al. Combination of ipratropium bromide and salbutamol in children and adolescents with asthma: a meta-analysis. *PLoS One*. 2021;16(2):e0237620. doi:10.1371/journal.pone.0237620
7. Chen AH, Chen RC, Zhan JY, et al. The efficacy of nebulized budesonide in acute moderate to severe exacerbations of asthma in children. *Zhonghua Jie He He Hu Xi Za Zhi*. 2012;35(4):269-274.
8. Milani GK, Rosário Filho NA, Riedi CA, Figueiredo BC. Nebulized budesonide to treat acute asthma in children. *J Pediatr (Rio J)*. 2004;80(2):106-112.
9. Murphy KR, Hong JG, Wandalsen G, et al. Nebulized inhaled corticosteroids in asthma treatment in children 5 years or younger: a systematic review. *J Allergy Clin Immunol Pract*. 2020;8(6):1815-1827. doi:10.1016/j.jaip.2020.01.042
10. Cyril J, Baburaj S, Nair P, Kuruvilla T, Bobby C, Das L. Comparative study of nebulized budesonide with levosalbutamol versus ipratropium bromide with levosalbutamol in acute asthma in children. *Int J Contemp Pediatr*. 2020;8:65-70. doi:10.18203/2349-3291.ijcp20205507
11. Li CY, Liu Z. Effect of budesonide on hospitalization rates among children with acute asthma attending pediatric emergency department: a systematic review and meta-analysis. *World J Pediatr*. 2021;17(2):152-163. doi:10.1007/s12519-020-00403-y
12. Afshar AA, Rahmati M, Maleki A, Kamali K. Effectiveness of budesonide in the treatment of asthma attacks in children: a systematic review and meta-analysis. *Prev Care Nurs Midwifery J*. 2023;13(3):9-18.
13. Razi C, Cörüt N, Andiran N. Budesonide reduces hospital admission rates in preschool children with acute wheezing. *Pediatr Pulmonol*. 2017;52:720-728. doi:10.1002/ppul.23667
14. Akbaş ED, Razi C, Andiran N. Effects of using montelukast during acute wheezing attack in hospitalized preschool children on the discharge rate and the clinical asthma score. *Pediatr Pulmonol*. 2021;56:1931-1937. doi:10.1002/ppul.25394
15. Upham BD, Mollen CJ, Scarfone RJ, Seiden JA, Chew A, Zorc JJ. Nebulized budesonide added to standard pediatric emergency department treatment of acute asthma: a randomized, double-blind trial. *Acad Emerg Med*. 2011;18(7):665-673. doi:10.1111/j.1553-2712.2011.01114.x
16. Wyatt EL, Borland ML, Doyle SK, Geelhoed GC. Metered-dose inhaler ipratropium bromide in moderate acute asthma in children: a single-blinded randomized controlled trial. *J Paediatr Child Health*. 2015;51:1033-1039. doi:10.1111/jpc.12692
17. Chen A, Zeng G, Chen R, et al. Effects of nebulized high-dose budesonide on moderate-to-severe acute exacerbation of asthma in children: a randomized, double-blind, placebo-controlled study. *Respirology*. 2013;18:122-128. doi:10.1111/resp.12168
18. Alangari AA, Malhis N, Mubasher M, et al. Budesonide nebulization added to systemic prednisolone in the treatment of acute asthma in children: a double-blind, randomized, controlled trial. *Chest*. 2014;145(4):772-778. doi:10.1378/chest.13-2298
19. Kartiningsih L, Setiawati L. Comparison of clinical efficacy and safety between salbutamol-ipratropium bromide nebulization and salbutamol alone in children with asthmatic attack. *Paediatr Indones*. 2016;46(6):241-245. doi:10.14238/pi46.6.2006.241-5
20. Iramain R, Castro-Rodriguez JA, Jara A, et al. Salbutamol and ipratropium by inhaler is superior to nebulizer in children with severe acute asthma exacerbation: a randomized clinical trial. *Pediatr Pulmonol*. 2019;54:372-377. doi:10.1002/ppul.24244
21. Özdemir M. Efficacy of ipratropium bromide in moderate and severe asthma attack in children. *Haydarpaşa Numune Med J*. 2019;59:358-366. doi:10.14744/hnhj.2019.99705
22. Joiya SJ, Khan MKA, Haq F, Ahmad ZP. Comparison of outcome of salbutamol alone and salbutamol in combination with ipratropium bromide in acute asthma in children. *Prof Med J*. 2020;27(6):1175-1181. doi:10.29309/TPMJ/2020.27.06.3218



ORIGINAL PAPER

Evaluation of serum CCN6, VEGF-A, and NF- κ B p65 and their clinicopathological correlations in breast cancer

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ABSTRACT

Introduction and aim. CCN6, a transcriptional regulator within the CCN family, influences breast tumorigenesis by interacting with key angiogenic (VEGF-A) and inflammatory (NF- κ B p65) pathways. This study establishes simultaneous evaluation of these serum biomarkers and comparison with CA 15-3 across untreated and treated breast cancer cohorts. This study aimed to evaluate their serum level and clinicopathological associations to understand their role in tumor progression and potential diagnostic utility.

Material and methods. Serum biomarkers were measured via ELISA in 130 breast cancer patients (56 untreated and 74 treated) and 50 controls. Demographic and clinical data were collected through questionnaire-based interviews and clinicopathological data from medical records.

Results. CCN6 had the greatest effect size ($F=1.10$), followed by NF- κ B p65 ($F=0.51$) and VEGF-A ($F=0.22$). The diagnostic efficacy ($AUC=0.995$; $p=0.0001$), sensitivity (96%), and specificity (100%) of CCN6 were superior to those of CA15-3 ($AUC=0.855$) and NF- κ B p65 ($AUC=0.813$). Logistic regression demonstrated that downregulated CCN6 was inversely associated with disease outcome ($OR=0.440$; $p=0.0001$), whereas elevated NF- κ B p65 ($OR=1.737$) levels increased breast cancer risk.

Conclusion. CCN6 showed promising diagnostic performance and exhibits an inverse association with breast cancer, outperforming NF- κ B p65 and CA 15-3; these findings underscore CCN6's potential as a preliminary non-invasive diagnostic biomarker.

Keywords. breast cancer, CA 15-3, CCN6, NF- κ B p65, VEGF-A

Introduction

Cellular communication network factor 6 (CCN6) is a matricellular protein member of the CCN family, which comprises six proteins: CCN1 (CYR61), CCN2 (CTGF), CCN3 (NOV), CCN4 (WISP1), CCN5 (WISP2), and CCN6 (WISP3).¹ These proteins regulate various biological processes, including cell proliferation, differentiation, adhesion, migration, angiogenesis, and tissue repair.^{1,2} Particularly, it has been shown that CCN6 maintains the integrity of epithelial cells by preventing epithelial-mesenchymal transition (EMT) and restraining the invasive phenotype.³ Since breast cancer is a highly prevalent ma-

lignancy,⁴ recent studies have investigated how CCN6 might participate in its development and progression.^{5,6} By downregulating the insulin growth factor-1 (IGF-1)/insulin growth factor-1 receptor (IGF-1R), Wnt/ β -catenin signaling pathway, and phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) pathways, CCN6 preserves the epithelial phenotype and thereby inhibits EMT, which is one of the main mechanisms through which tumors invade and metastasize.⁵ resulting in reduced proliferation, differentiation, and motility of cancer cells.⁷ Consequently, CCN6 downregulation correlates with invasive tumor behavior and poor prognosis.³

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Concurrent research has investigated vascular endothelial growth factor A (VEGF-A) and nuclear factor kappa B (NF-κB), two pivotal regulators of angiogenesis, inflammation, and cancer cell survival.^{8,9} VEGF-A stimulates endothelial proliferation and vascularization by VEGFR-2-activated mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, and its expression is associated with advanced tumor stage and reduced patient survival.¹⁰ NF-κB, particularly its p65 subunit, regulates the expression of pro-inflammatory, proliferation, and apoptosis-resistant genes. Persistent NF-κB activation has been observed in invasive forms of breast cancer, where NF-κB stimulates the expression of VEGF-A, B-cell lymphoma-2 (Bcl-2), and matrix metalloproteinase-9 (MMP-9) to facilitate angiogenesis and invasion.¹¹ Conversely, the inhibition of NF-κB activity leads to a significant decrease in VEGF-A mRNA levels.¹²

Clinically, CA15-3 is a widely utilized serum tumor marker in the management of breast cancer due to its role in diagnosis and prognostication.¹³ It is secreted from mucin 1 (MUC1) glycoprotein and serves as a measure of tumor burden and is useful for disease monitoring and recurrence detection. However, its decreased sensitivity in early-stage disease has prompted studies into its use in combination with other molecular markers to provide a more effective and comprehensive diagnostic profile.¹⁴

Despite advances in conventional breast cancer treatments, the molecular heterogeneity of malignancy necessitates the continuous evolution of therapeutic strategies. While the individual roles of these biomarkers are recognized, it remains unknown how their serum levels interact across different breast cancer clinical states or how a combinatorial panel performs relative to the standard marker. The single markers fail to capture the multi-faceted tumor microenvironment; therefore, the simultaneous evaluation of these four biomarkers represents a vital advance.

Aim

This study aims to determine the diagnostic potential of CCN6, VEGF-A, NF-κB p65, and CA 15-3 by comparing their levels across untreated and treated breast cancer patients with healthy controls and assess their correlation with critical clinicopathological factors, which may contribute to a better understanding of the underlying molecular mechanism and improve early disease detection.

Material and methods

Study design

A cross-sectional study was conducted between October 2024 and June 2025, enrolling 180 women aged 25–80, as illustrated in Fig. 1. Of these, 130 were patients with breast cancer diagnosed by an oncologist at the Oncology Center of Al-Sadr Teaching Hospital in Basrah City, Southern Iraq.

Participants and eligibility

This study involved 180 subjects classified into patient and control groups. Patients were subdivided based on their treatment status into two cohorts:

- Untreated group: 56 newly diagnosed patients with breast cancer aged 25–80 years, 1–12 months post-diagnosis, who had yet to receive any treatment modality.
- Treated group: 74 patients with breast cancer, aged 30–75 years, either currently receiving treatment or 2–12 years post-treatment. Treatment protocols varied according to the cancer stage, lymph node involvement, organ metastasis, and tumor grade, encompassing radiotherapy; chemotherapy (with doxorubicin, cyclophosphamide, docetaxel, paclitaxel, and carboplatin); hormonal therapy (used for hormone-positive breast cancer with tamoxifen, letrozole, or anastrozole depending on menopausal status); and HER2-targeted therapy with trastuzumab if HER2 was positive, given with chemotherapy if indicated; the heterogeneity of these treatment modalities acknowledged a potential source of biological variability in biomarkers' expression. The AJCC Tumor-Node-Metastasis (TNM) classification is used for breast cancer staging.¹⁵ This system standardizes breast cancer treatment and prognosis via stage.
- Control group: 50 healthy female volunteers (hospital staff, community) matched to the patients' cohort by age and geographical distributions, with no history of autoimmune, chronic, or genetic diseases or sickle cell anemia.

Inclusion criteria

- female patients, either newly diagnosed before treatment (untreated group) or previously/actively receiving treatment (treated group),
- patients with complete histological and laboratory data,
- patients providing written informed consent.

Exclusion criteria

- pregnant women,
- individuals with psychiatric or cognitive disorders,
- patients aged <25 years,
- patients refusing to provide written informed consent,
- patients with incomplete medical records or missing clinicopathological data.

Sample and effect size calculation

The total sample size required to conduct this study was calculated a priori using G*Power version 3.1.9.7 for a three-group comparison with a medium effect size ($f=0.25$), two-tailed testing ($\alpha=0.05$), and power ($1-\beta$) of 0.80, resulting in a minimum requirement of approx-

imately 159 total samples. To ensure robust statistical power, a total of 180 samples were collected.

Data collection

Demographics and clinical data (age, weight, Body Mass Index [BMI], family history, and menopausal status) were collected via questionnaire-based interviews. Clinicopathological data (disease stage, histological type, molecular subtype, tumor grade, lymphovascular invasion [LVI], KI-67% proliferation index, CA 15-3, ER, PR, and HER-2 status) were collected from the oncology center's medical records and electronic laboratory system. The health assessments were conducted according to STORBE guidelines. BMI was computed as the subject's weight in kilograms (kg) divided by height in square meters (m^2) and grouped according to the WHO standards into four groups: underweight (under $18.5 \text{ kg}/m^2$), normal weight ($18.5\text{--}25 \text{ kg}/m^2$), overweight ($25\text{--}30 \text{ kg}/m^2$), and obese (above $30 \text{ kg}/m^2$).¹⁶ In the statistical analysis, these categories of BMI were used to establish any potential association between BMI and the serum concentrations of the biomarkers under investigation (CCN6, VEGF-A, NF- κ B p65, and CA 15-3) among the study participants.

Sample collection

Five milliliters of whole blood were collected via venipuncture from breast cancer patients during their scheduled morning visits to the oncology center following an overnight fast, either pre-treatment or before chemotherapy/hormonal therapy cycles, and from healthy controls. Two milliliters of blood were drawn into purple ethylenediaminetetraacetic acid (EDTA) tubes for complete blood count analysis of hematological parameters. The remaining 3 mL, collected in yellow-colored gel separator tubes, was permitted to coagulate at ambient temperature and then centrifuged at 4100 rpm for 4 minutes and stored in a deep freezer (-80°C). The samples underwent only a single freeze-thaw cycle before biomarker quantification and routine laboratory tests assessment.

Routine laboratory tests assessment

Complete blood count test

Hematological parameters were quantified using 2 milliliters of blood collected in EDTA-anticoagulant tubes. A Sysmex automated hematology analyzer (Sysmex, Kobe, Japan) was utilized to execute complete blood counts and quantify white blood cells (WBCs), neutrophils, monocytes, lymphocytes, red blood cell (RBC) counts, and hemoglobin levels.

Enzyme-linked immunosorbent assay (ELISA)

Serum concentrations of CCN6, VEGF-A, and NF- κ B p65 were quantified using commercially available ELISA kits, following the manufacturer's instructions. Spe-

cifically, CCN6 was measured using the BT Lab Human CCN6 ELISA kit (Cat. No. E6409Hu; range: $0.05\text{--}30 \text{ ng}/\text{mL}$, sensitivity: $0.018 \text{ ng}/\text{mL}$). VEGF-A levels were measured using an Elabscience Human VEGF-A ELISA kit (Cat. No. E-EL-H0111; range: $31.25\text{--}2000 \text{ pg}/\text{mL}$, sensitivity: $18.75 \text{ pg}/\text{mL}$). NF- κ B p65 levels were measured using an Elabscience Human NF- κ B p65 ELISA kit (Cat. No. E-EL-H1388; range: $0.16\text{--}10 \text{ ng}/\text{mL}$; sensitivity: $0.09 \text{ ng}/\text{mL}$). For quality controls, all standards and samples were tested in duplicates. An ELISA washer (Thermo Scientific, USA) was employed for washing, and absorbance was recorded at 450 nm using an ELISA microplate reader (Thermo Scientific, USA). The concentrations were calculated from the standard curves. The inter- and intra-assay coefficient of variation (CV) for CCN6 ELISA were $<8\%$ and $<10\%$, respectively; for NF- κ B p65, ($6.09\text{--}8.5\%$) and ($6.55\text{--}6.83\%$), respectively; and for VEGF-A, ($6.61\text{--}8.27\%$) and ($4.92\text{--}6.95\%$), respectively. Laboratory blinding was enforced during the biomarker assessment to alleviate the information bias.

Statistical analysis

IBM SPSS Statistics, version 31 (SPSS Inc., Chicago, IL, USA), was used for data analysis, while graphs were plotted using GraphPad Prism, version 8.0.1 (GraphPad Software, San Diego, CA, USA). Continuous variables are presented as mean $\pm$ standard deviation (SD). Categorical variables were presented as frequencies and percentages extracted from the chi-square and Fisher's exact tests. Shapiro-Wilk and Kolmogorov-Smirnov tests were used for the normality test. The Kruskal-Wallis test with Dunn's post-hoc test was used for the three-group comparison. Spearman's correlation analysis was performed to assess associations. Bonferroni correction was applied following multiple comparisons and correlations to adjust the significance threshold and minimize the risk of type 1 errors. Diagnostic performance of the serum biomarkers was evaluated by ROC curve analysis, and the AUC, optimal cut-offs, sensitivity, specificity, and accuracy were calculated. The cutoff points were assessed by the Youden index. Univariate binary logistic regression was used to identify independent risk factors of breast cancer, followed by multivariate binary logistic regression. To avoid the confounding influence of the therapeutic interventions, the previously mentioned analyses were purposefully restricted to the untreated group versus healthy controls. Statistical significance was set at $p < 0.05$.

Results

Patients' demographic and histopathologic characteristics

The study cohort comprised 180 participants, categorized into three groups: untreated patients ($n=56$), treated patients ($n=74$), and healthy controls ($n=50$). Patients' demographic and histopathologic characteris-

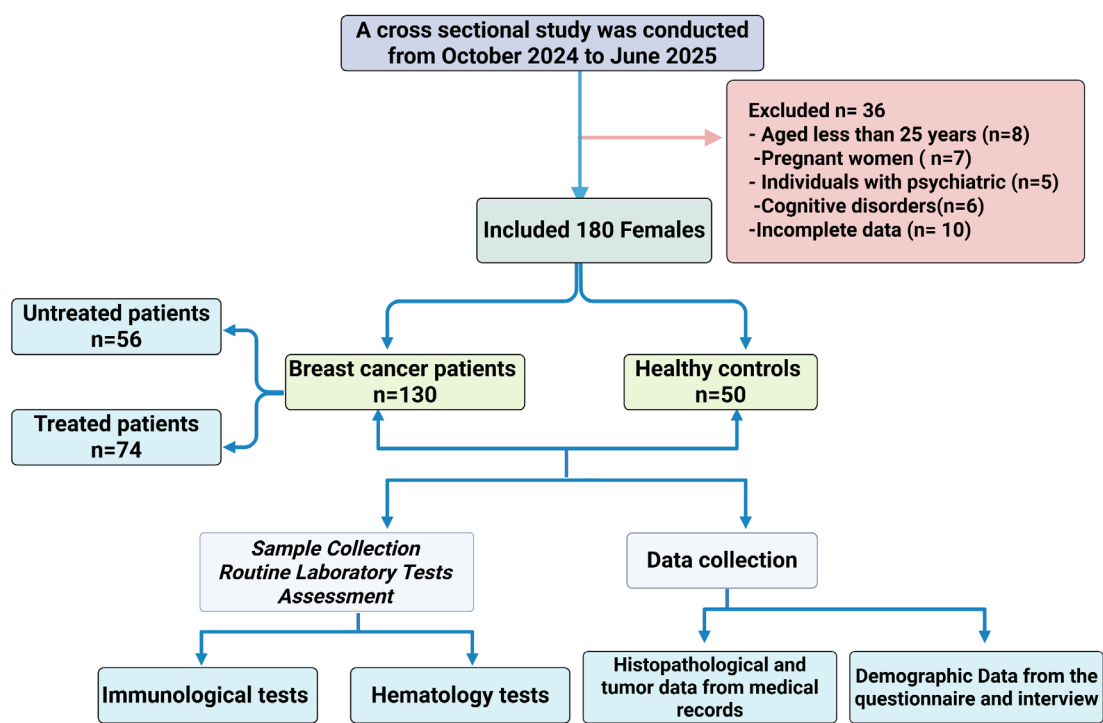


Fig. 1. Flow diagram of participant selection and enrollment in the study

tics are detailed in Table 1. The analysis confirmed that age, BMI, and family history were statistically balanced across the patient groups ($p>0.05$). The majority of patients in both groups were aged between 40–55 years, overweight ($\text{BMI}>25\text{ kg/m}^2$), and had a negative family history. However, a significant disparity was observed in the menopausal status ($p=0.0001$), with peri-menopausal status being the predominant status in the treated group (55.4%), whereas patients were more evenly distributed between pre- (39.3%) and post-menopausal (35.7%) statuses in the untreated group.

The treated patients presented with more aggressive disease features characterized by a higher frequency of grade III tumors (63.5%), while grade II was more prevalent in the untreated group (57.1%), $p=0.010$. Furthermore, tumor invasiveness varied significantly ($p=0.032$), though invasive disease was predominant in both groups (100% of treated and 92.9% of untreated patients). Histological subtypes showed significant variation ($p=0.008$), with invasive ductal carcinoma, no specific type (IDC NST), being the prevalent subtype across both groups. Other clinicopathological characteristics, including LVI, disease stage, hormone receptor status (ER, PR), HER-2 status, and molecular subtypes, did not differ significantly between the untreated and treated cohorts ($p>0.05$). Stage II accounted for the highest proportion of cases in both groups. Immunohistochemical profiling indicated that estrogen and progesterone receptor positivity was predominant in both groups, whereas HER-2 receptor negativity was more frequent.

Consequently, luminal B was the most prevalent subtype in the untreated group (39.3%), compared to the treated group, which represents equal proportions of both luminal A and B (36.5% each).

Surgical interventions differed significantly between the two groups ($p=0.0001$), with most untreated patients enrolled before the surgery. Nearly all the treated patients underwent surgical resection, accounting for 97.3% of the cases. Similarly, the treatment protocol distributions were highly significant ($p=0.0001$), as all the untreated patients were enrolled prior to the initiation of therapeutic interventions, while the majority of treated patients (79.7%) had received a combination therapy consisting of chemotherapy, radiation, and hormonal therapy; alternative combination types were presented less frequently within the group.

Table 1. Demographic, anthropometric, and histopathological characteristics of breast cancer patients*

Age, n (%)			
<40	13 (23.2)	9 (12.2)	0.130
40–55	23 (41.1)	42 (56.8)	
>55	20 (35.7)	23 (31.1)	
Mean ± SD	49.8±11.6	52±9.69	0.347
Body mass index, n (%)			
Under weight	10 (17.9)	16 (21.6)	0.235
Normal weight	15 (26.8)	28 (37.8)	
Overweight	31 (55.4)	30 (40.5)	
Obese	10 (17.9)	16 (21.6)	
Family history, n (%)			
Positive	10 (17.9)	13 (17.6)	0.966
Negative	46 (82.1)	61 (82.4)	

Menopausal status, n (%)			
Pre-menopausal	22 (39.3)	9 (12.2)	0.0001
Perimenopausal	14 (25)	41 (55.4)	
Post-menopausal	20 (35.7)	24 (32.4)	
Disease stage, n (%)			
Stage 0 (DCIS)	2 (3.6)	0	0.313
Stage I	8 (14.3)	10 (14.9)	
Stage II	26 (46.4)	27 (36.5)	
Stage III	12 (21.4)	20 (27)	
Stage IV	8 (14.3)	16 (21.6)	
Tumor grade, n (%)			
I	2 (3.6)	0	0.010
II	32 (57.1)	27 (36.5)	
III	22 (39.3)	47 (63.5)	
Lymphovascular invasion, n (%)			
Present	18 (32.1)	13 (17.6)	0.063
Not identified	38 (67.9)	61 (82.4)	
Tumor invasiveness, n (%)			
Noninvasive	4 (7.1)	0	0.032
Invasive	52 (92.9)	74 (100)	
Histological type, n (%)			
Ductal carcinoma in situ	4 (7.1)	0	0.008
Invasive ductal carcinoma	45 (80.4)	70 (94.6)	
Invasive lobular carcinoma	6 (10.7)	2 (2.7)	
Mixed invasive carcinoma	0	2 (2.7)	
Invasive metaplastic carcinoma	1 (1.8)	0	
Hormone receptor, n (%)			
Estrogen receptor status			
Positive	40 (71.4)	54 (73)	0.846
Negative	16 (28.6)	20 (27)	
Progesterone receptor status, n (%)			
Positive	38 (67.9)	52 (70.3)	0.848
Negative	18 (32.1)	22 (29.7)	
HER-2 status, n (%)			
Positive	10 (17.9)	14 (18.9)	0.986
Negative	46 (82.1)	60 (81.1)	
Molecular subtype, n (%)			
Luminal A	18 (32.1)	27 (36.5)	0.556
Luminal B	22 (39.3)	27 (36.5)	
HER-2 enriched	6 (10.7)	12 (16.2)	
Triple negative	10 (17.9)	8 (10.8)	
Surgical intervention, n (%)			
Yes	12 (21.4)	72(97.3)	0.0001
No	44 (78.6)	2(2.7)	
Treatment protocol, n (%)			
No treatment	56 (100)	0	0.0001
Chemotherapy+hormonal	0	1 (1.4)	
Chemotherapy+radiation	0	12 (16.2)	
Chemotherapy+radiation+hormonal therapy	0	59 (79.7)	
Radiation+hormonal therapy	0	2 (2.7)	

* Differences were considered statistically significant at a two-tailed p-value of p≤0.05; statistical analysis was conducted using the chi-square test with variables represented as frequency and percentage (n, %); DCIS – ductal carcinoma in situ; n – number; % – percentage; SD – standard deviation

Comparative analysis of demographic, hematological, and biochemical characteristics

Table 2 exhibited no significant differences in age (p=0.107) and BMI (p=0.117) among the three groups. The average age was (49.8±11.6) years for the untreated group, (52±9.69 years) for the treated, and (48.6±10.9 years) for the healthy control. The untreated group had a slightly elevated BMI compared with treated and healthy controls (30.5±5.6 vs. 30.1±5.85 and 28.6±3.42 kg/m²). Significant physiological and hematological alterations were observed; healthy controls demonstrated significantly higher RBCs (4.53±0.38 x 10¹²/L) and HB (126±10.3 g/L) compared to both patient groups (p=0.0001).

Untreated patients revealed significantly elevated WBC and neutrophil counts (7.16±1.63x10⁹/L and 4.53±1.08 x 10⁹/L, p=0.0001) relative to the treated and control groups. Furthermore, significant variations were observed in the neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR) across all groups (p=0.0001), with healthy controls exhibiting the highest LMR (5.57±2.54) and lowest NLR (1.79±0.96) ratios. No significant differences were observed in the platelet counts between the groups.

Table 2. Comparative analysis of demographic data, hematological, and biochemical markers among untreated patients, treated patients, and healthy controls

Parameter	Total patients n = 130		Healthy control (n=50)	p
	Untreated (n=56)	Treated (n=74)		
Age (year)	49.8±11.6	52±9.69	48.6±10.9	0.107
BMI (kg/m²)	30.5±5.6	30.1±5.85	28.6±3.42	0.117
HB (g/L)	117.82±23.66	117.0±14.6	126.0±10.3	0.0001
RBC × 10 ¹² /L	4.3±0.78	4.17±0.6	4.53±0.4	0.0001
HCT (L/L)	0.35±0.07	0.35±0.04	0.37±0.024	0.014
MCV (fL)	79.68±13.23	84.13±12.65	83.61±4.28	0.071
MCHC (g/L)	327.57±12.63	331.09±15.87	333.26±16.95	0.051
WBC × 10 ⁹ /L	7.16±1.63	5.98±2.19	6.04±1.57	0.0001
NEU ×10 ⁹ /L	4.53±1.08	3.55±1.76	3.37±1.27	0.0001
LYM ×10 ⁹ /L	1.98±0.72	1.68±0.68	2.07±0.64	0.009
MONO ×10 ⁹ /L	0.47±0.19	0.62±0.38	0.42±0.16	0.006
LMR	4.78±2.79	3.55±2.34	5.57±2.54	0.0001
NLR	2.51±0.89	2.51±1.07	1.79±0.96	0.0001
PLT × 10 ⁹ /L	270.60±69.02	263.77±108.47	268.06±54.17	0.153

* Data are represented as mean±SD. Differences were considered statistically significant at a two-tailed p-value of p ≤ 0.05; the differences in means between the groups were assessed using the Kruskal-Wallis test, Bonferroni correction-adjusted p-value (p<0.002), WBC – white blood cells, MCV – mean corpuscular volume, MCHC – mean corpuscular hemoglobin concentration, NEU – neutrophils, LYM – lymphocytes, MONO – monocytes, LMR – lymphocytes to monocytes ratio, NLR – neutrophils to lymphocytes ratio

To evaluate the impact of the treatment and remission time on the parameter level, the treated group was stratified into three subgroups depending on the time since treatment. The comparative analysis revealed no significant variations in the biomarker levels between patients in active treatment and those with short- and long-term remission times ($p>0.1$). The correlation analysis further confirmed that the elapsed time since treatment did not significantly impact the biomarker levels ($\rho<0.200$, $p>0.1$).

Comparative analysis of serum immunological biomarkers between patients and healthy controls

The serum levels of CCN6, VEGF-A, and NF- κ B p65 demonstrated significant variations (Table 3). The control group exhibited significantly elevated CCN6 levels (19.92 ± 6.92 μ g/L) in comparison with both untreated (2.04 ± 0.17 μ g/L) and treated groups (3.58 ± 1.22 μ g/L), $P=0.0001$, showing a very large effect size (Cohen's $f=1.10$) and 100% statistical power. Conversely, VEGF-A levels were significantly elevated in the untreated group (157.28 ± 83.55 ng/L), Figure 2, compared to both the treated and control groups, $P=0.013$, with a small effect size (Cohen's $f=0.22$ and 74% power. Similarly, NF- κ B p65 levels demonstrated significant elevation in the untreated group (6.85 ± 1.86 μ g/L) relative to the other groups ($P=0.0001$), yielding a large effect size (Cohen's $f=0.51$) and 99% power.

Table 3. Comparative analysis of serum immunological biomarkers among untreated, treated patients and healthy controls*

Parameter	Total patients, n = 130		Healthy control (n=50)	p	e2	(F)	Power
	Untreated (n=56)	Treated (n=74)					
CCN6 (μ g/L)	$\downarrow 2.04\pm0.17$	$\uparrow 3.58\pm1.22$	19.92 ± 6.92	0.0001	0.55	1.10	100%
VEGF-A (ng/L)	$\uparrow 157.28\pm83.55$	$\downarrow 136.09\pm60.37$	143.56 ± 30.29	0.013	0.04	0.22	74%
NF- κ B p65 (μ g/L)	$\uparrow 6.85\pm1.86$	$\downarrow 5.68\pm1.32$	2.78 ± 0.90	0.0001	0.20	0.51	99%

* Data are represented as mean $\pm$ SD; differences were considered statistically significant at a two-tailed p-value of $p\leq0.05$, the differences in means between the groups were assessed using the Kruskal-Wallis test

Bonferroni correction (adjusted $p<0.016$), H – Kruskal-Wallis statistic, F – Cohen's effect size, CCN6 – human WNT1-inducible-signaling pathway protein 3, VEGF-A – vascular endothelial growth factor A, NF- κ B p65 – nuclear factor NF-kappa-B p65 subunit

Association serum levels of CCN6, NF- κ B P65, VEGF-A, and CA15 with disease stages

In the untreated patients, disease stages significantly correlated with circulating biomarker levels (Fig. 3 and 4). Following the application of the Bonferroni correction (adjusted $P=0.008$), CCN6 levels were not significantly varied across stages in the untreated ($p=0.040$), peaking in stage IV (3.73 ± 0.51 μ g/L). In the treated group, the variation was also non-significant ($p=0.037$), and the highest levels were in stage II (5.34 ± 1.06 μ g/L). CA15-3 levels showed significant stage-dependent upregulation in the untreated and treated groups ($P=0.0001$ and $P=0.002$, respectively), reaching maximum levels in advanced-stage disease (IV) (122.49 ± 23.39 U/mL and 50.94 ± 10.9 U/mL, respectively). VEGF-A and NF- κ Bp65 concentrations increased with advancing disease stages in the untreated group, but these variations were not statistically significant in the untreated ($p=0.347$ and $p=0.087$, respectively) and the treated group ($p=0.025$ and $p=0.181$, respectively).

Note: The following tests were performed utilizing the untreated patient cohort.

Assessment of Spearman correlation

The correlation analysis of immunological biomarkers within the untreated group is presented in Table 4. Following the application of the Bonferroni correction (adjusted threshold $p<0.0125$), there was a significant negative correlation between CCN6 and several markers of disease progression, including NF- κ B P65 ($\rho=-0.316$, $p=0.0001$) and CA15-3 ($\rho=-0.499$, $p=0.0001$). No significant relationship could be found between the levels of CCN6 and those of VEGF-A and KI-67. The level of CA15-3 was positively correlated with NF- κ B

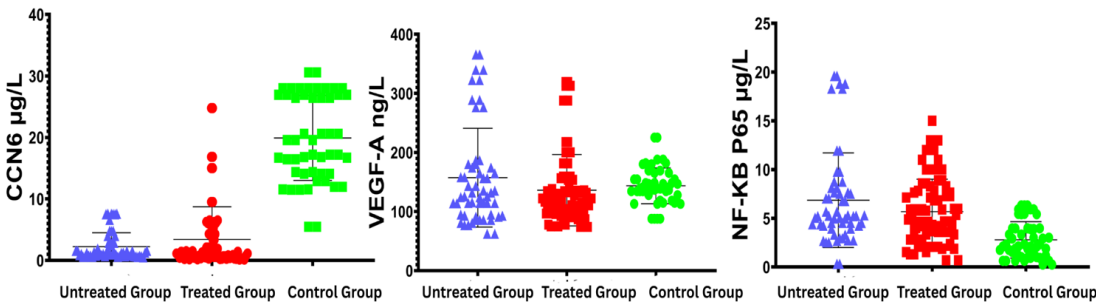


Fig. 2. Scatter plot for the comparative distribution of serum immunological biomarkers among untreated, treated, and healthy controls

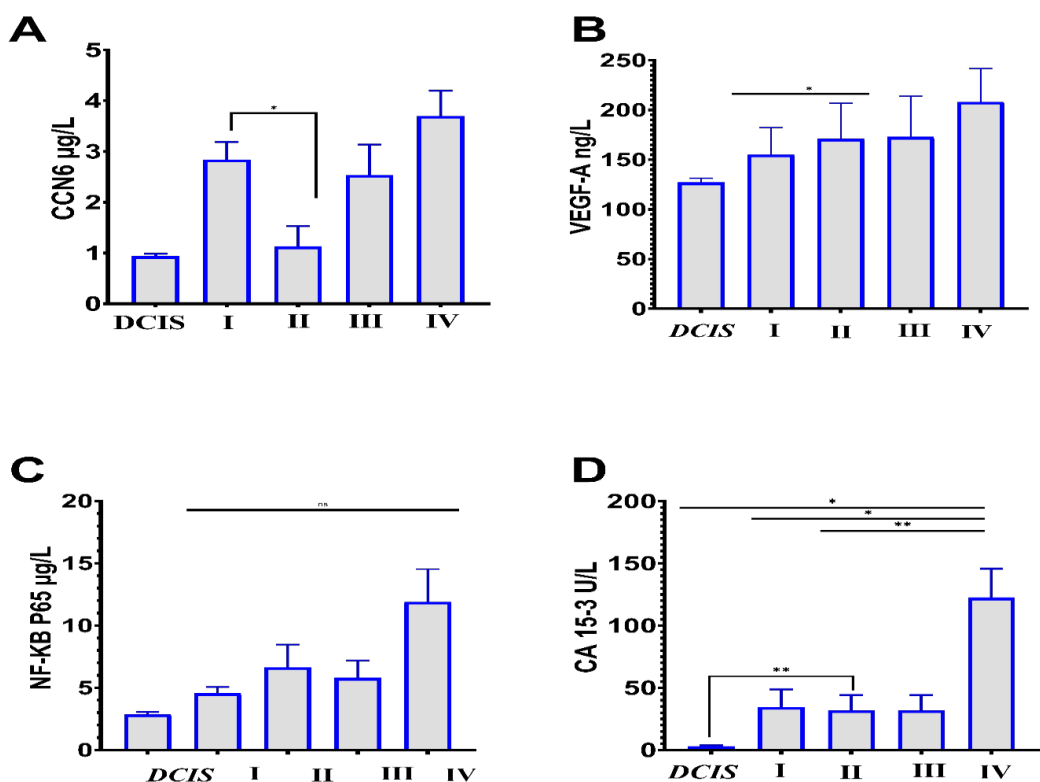


Fig. 3. Association of serum levels of CCN6, NF-κB P65, VEGF-A, and CA15 with disease stages in the untreated group (* – $p<0.05$, ** – $p<0.01$, ns: non-significant >0.05), the differences in means between the groups were assessed using the Kruskal-Wallis test

P65 ($\rho=0.231$, $p=0.002$) and VEGF-A ($\rho=0.420$, $p=0.001$). Furthermore, a synergistic correlation was observed between NF-κB P65 and VEGF-A ($\rho=-0.194$, $p=0.009$).

Table 4. Spearman correlation analysis among serum immunological biomarkers*

Variables	Spearman correlation (r)	p	95% CI		Interpretation
			Lower	Upper	
CCN6 vs NF-κB p65	-0.316 ↓	0.0001 ^Δ	-0.445	-0.174	Significant moderate negative correlation
CCN6 vs. VEGF-A	-0.073 ↓	0.593	-0.337	0.201	No correlation/No significant association
CCN6 vs. CA15-3	-0.499 ↓	0.0001 ^Δ	-0.582	-0.069	Significant moderate negative correlation
CCN6 vs. Ki-67%	-0.214 ↓	0.014	-0.377	-0.039	Significant weak negative correlation
CA15-3 vs. VEGF-A	+0.420 ↑	0.001 ^Δ	0.169	0.620	Significant moderate positive correlation
VEGF-A vs NF-κB p65	-0.194 ↓	0.009 ^Δ	-0.335	-0.045	Significant weak negative correlation
NF-κB p65 vs. CA15-3	+0.231 ↑	0.002 ^Δ	0.083	0.368	Significant moderate positive correlation

* Δ – Correlation is significant at the 0.01 level (2-tailed)

Further analysis was conducted with demographic and clinicopathological features with Bonferroni correction $p<0.01$. As shown in Table 5, downregulated CCN6 was negatively associated with tumor grade. In

Table 5. Spearman correlation analysis between immunological biomarkers, pathological and histological features, and age*

Variables	Spearman Correlation (r)	p	95% CI		Interpretation
			Lower	Upper	
CCN6 vs. age	-0.081 ↓	0.55	-0.320	0.179	No correlation/No significant association
CCN6 vs BMI	0.205 ↑	0.13	-0.065	0.467	Weak positive, non-significant correlation
CCN6 vs. tumor grade	-0.173 ↓	0.049	-0.339	0.005	No correlation/No significant association
VEGF-A vs. age	-0.136 ↓	0.31	-0.438	0.166	Weak negative, non-significant correlation
VEGF-A vs. BMI	0.049 ↓	0.72	-0.245	0.323	No correlation/No significant association
VEGF-A vs. tumor grade	0.179 ↑	0.042	0.002	0.345	No correlation/No significant association
NF-κB p65 vs. age	-0.155 ↓	0.26	-0.411	0.118	Weak negative, non-significant correlation
NF-κB P65 vs. BMI	-0.088 ↓	0.52	-0.347	0.175	No correlation/No significant association
NF-κB P65 vs. tumor grade	0.181 ↑	0.009 ^Δ	0.036	0.375	Significant weak positive correlation
CA15-3 vs. age	-0.051 ↓	0.71	-0.33	0.233	No correlation/No significant association
CA15-3 vs. BMI	-0.013 ↓	0.93	-0.28	0.282	No correlation/No significant association
CA15-3 vs. tumor stage	0.343 ↑	0.0001 ^Δ	0.176	0.490	Significant moderate positive correlation

* Δ – Correlation is significant at the 0.01 level (2-tailed)

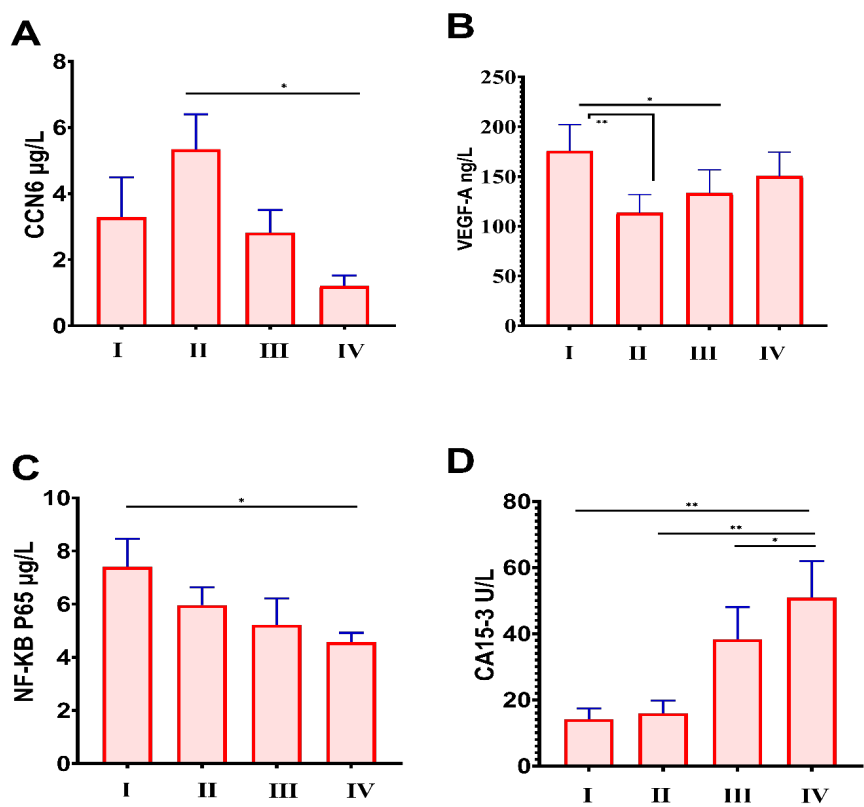


Fig. 4. Association of serum levels of CCN6, NFKB-p65, VEGF-A, and CA15 with disease stages in the treated groups (*– $p < 0.05$, **– $p < 0.01$, ns: non-significant > 0.05); the differences in means between the groups were assessed using the Kruskal-Wallis test

contrast, the elevation of NF- κ BP65 was positively associated with tumor grade ($\rho = 0.181$, $p = 0.009$). Furthermore, CA 15-3 levels correlated positively with tumor stage ($\rho = 0.343$, $p = 0.0001$). Finally, the correlations analysis between the immunological parameters and both age and BMI revealed weak and negative associations, which were not statistically significant.

Receiver operating characteristic of biomarkers’ diagnostic performance

The receiver operating characteristic (ROC) curve technique was employed to evaluate the diagnostic potential of serum biomarkers. As illustrated in the individual ROC curves (Fig. 5 and Table 6), CCN6 exhibited the highest value (AUC=0.995, $p = 0.0001$). With a cutoff of 9.5 $\mu\text{g/L}$ (Table 7), CCN6 achieved 96% sensitivity and 100% specificity, with 98.1% TAR and 1.9% TMR, outperforming the traditional conventional biomarker CA 15-3 (AUC=0.855) and NF- κ BP65 (AUC=0.813). At cut-off of 18.32 U/mL, CA15-3 had a sensitivity of 69.2%, a specificity of 90%, a 79.4% TAR, and a misclassification rate (TMR) of 20.6%. While NF- κ B P65 (cut-off: 4.25 $\mu\text{g/L}$) demonstrated moderate levels of sensitivity and specificity at 75% and 80%, respectively, it resulted in a total agreement rate of 77.4% and a misclassification rate of 22.6%. VEGF-A has the lowest AUC of 0.558; with a cut-off value of 126.8 ng/L, VEGF-A exhibited low sen-

sitivity (50%) but moderate specificity (78%), resulting in an undesirable TAR (63.21%). Internal validation by bootstrapping was conducted to assess the stability of the model; the bootstrap-corrected AUC of CCN6 remains consistent at 0.995 (95% CI: 0.990–1.00), minimizing the likelihood of overfitting.

Table 6. Receiver operating characteristic analysis for biomarkers’ diagnostic performance*

Diagnostic Biomarkers	AUC	Std. Error	p	95% Confidence interval	
				Lower bond	Upper bound
CCN6 (µg/L)	0.995 ^a	0.007	0.0001**	0.982	1
VEGF-A (ng/L)	0.558	0.056	0.321	0.444	0.672
NF-κBP65 (µg/L)	0.813 ^c	0.041	0.0001**	0.732	0.894
CA15-3 (U/mL)	0.855 ^b	0.038	0.0001**	0.795	0.935

* The null hypothesis true area is 0.5, ** – the p was significant at 0.01, ^a – highest AUC value, ^{b, c} – moderate AUC value, AUC – area under the curve

Binary logistic regression model

Individual univariate logistic regression models were evaluated for each biomarker to examine their association with breast cancer presence. As shown in Table 8, part 1, the model revealed that CCN6 demonstrated the strongest association with the outcome, explaining 95.4% of the variance ($\chi^2 = 130$, $p < 0.001$, $R^2_{\text{N}} = 0.954$) with a low information criterion (AIC=20.9, BIC=26.2). The

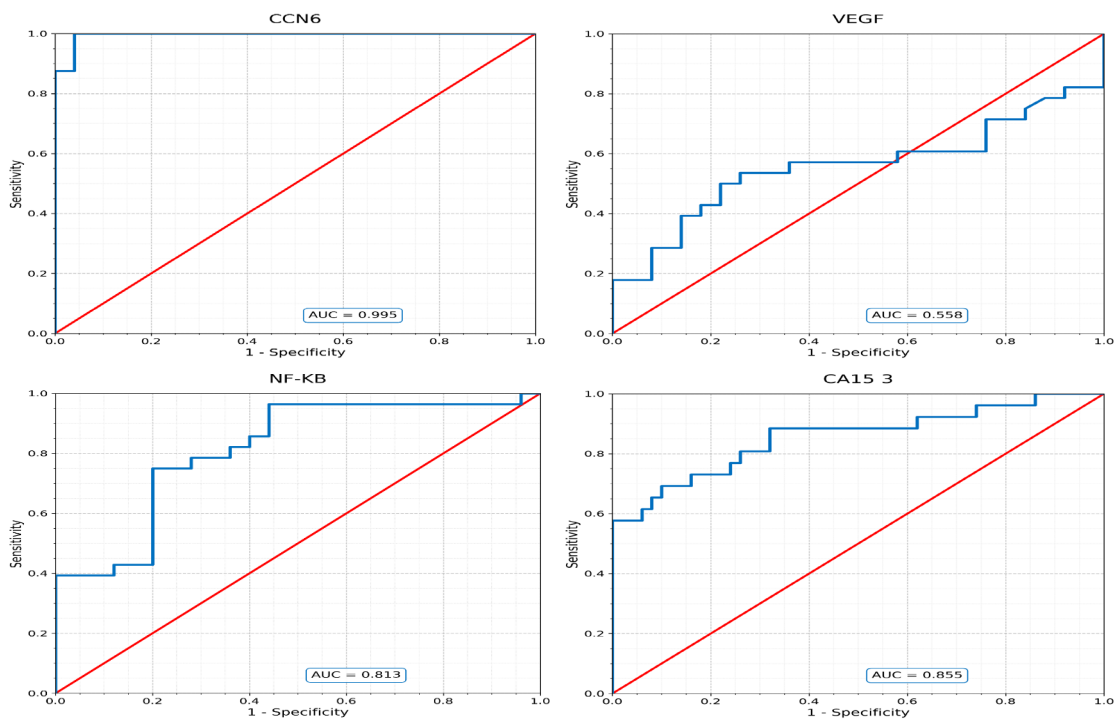


Fig. 5. ROC curve analysis for biomarkers' diagnostic performance

multivariate binary logistic regression model was conducted to assess the predictive power of the biomarkers. Both NF-κB P65 and CA15-3 also demonstrated significant model fit ($R^2N = 0.407$ and $R^2N = 0.534$, respectively). Conversely, VEGF-A failed to establish a significant individual fit ($R^2N = 0.016$).

Table 7. Sensitivity, specificity, positive predictive value, negative predictive value, total agreement rate, and total misclassification rate of serum biomarkers for the diagnosis of breast cancer patients*

Diagnostic biomarkers	Optimal cut-off	Sensitivity %	Specificity %	TAR%	TMR%
CCN6 (μg/L)	9.5	96	100	98.1	1.9
VEGF-A (ng/L)	126.8	50	78	63.21	36.79
NF-κB p65 (μg/L)	4.25	75	80	77.4	22.6
CA-153 (U/mL)	18.32	69.2	90	79.4	20.6

* TAR – total agreement rate, TMR – total misclassification rate

The multivariate model was subsequently conducted to evaluate the combined association of the biomarkers, which excluded CA 15-3, as shown in Table 8, Part 2. The overall multivariate model demonstrated excellent data fit, explaining 95.4% of the variance in the outcome ($R^2_{McF}=0.907, R^2_{N}=0.954$). The overall test confirmed that the combined model was highly significant ($\chi^2=133, df=3, p<0.001$). The multivariable model yielded superior information criteria scores (AIC=21.6, BIC=32.3) compared to the individual model test. Within this final model, CCN6 maintained its significant inverse association with the outcome (B=-0.834, adjusted OR=0.434, 95% CI=0.243–0.755, $p=0.005$). In contrast, both NF-

κB p65 and VEGF-A results were not significant in the multivariate setup ($p>0.2$).

Table 8. Binary logistic regression analysis and model fit diagnostics of tumor-related factors associated with breast cancer status*

Part 1. Models fit measures							
Univariate model fit				Overall model test			
Predictor	AIC	BIC	R ² McF	R ² N	χ ²	df	p
CCN6 (μg/L)	20.9	26.2	0.88	0.954	130	1	0.0001
VEGF-A (ng/L)	149	155	0.0084	0.016	1.24	1	0.266
NF-κB p65 (μg/L)	112	117	0.236	0.407	38.5	1	0.0001
CA-153 (U/mL)	96.4	102	0.370	0.534	54.2	1	0.0001
Overall multivariate model fit*							
	21.6	32.3	0.907	0.954	133	3	0.0001

Part 2. Model coefficients						
Predictor	Univariate coefficients			Multivariate coefficients		
	B	Adjusted OR (95% CI)	p	B	OR (95% CI)	p
CCN6 (μg/L)	-0.821	0.440 (0.276–0.702)	0.0001	-0.834	0.434 (0.243–0.755)	0.005
VEGF-A (ng/L)	0.0034	1.003 (0.997–1.01)	0.276	0.0068	1.007 (0.990–1.024)	0.427
NF-κB p65 (μg/L)	0.552	1.737 (1.336–2.21)	0.0001	0.37	1.454 (0.743–2.244)	0.274
CA-153 (U/mL)	0.175	1.19 (1.001–1.29)	0.0001	-----	-----	-----
Constant				3.505	33.294 (0.167–6654)	0.195

* CA 15-3 was excluded from the multivariate model. Note: models estimated using a sample size of 106 (56 untreated patients and 50 healthy controls), AIC – Akaike Information Criterion, BIC – Bayesian Information Criterion, R^2_{McF} – McFadden's R-squared, R^2_N – Nagelkerke R-squared, χ^2 – Chi-squared, df – degree of freedom, B – log odds coefficients, OR – odds ratio, CI – confidence interval

Discussion

Breast cancer is known to be the most prevalent form of cancer and one of the leading causes of cancer-related mortality among women. This necessitates the identification of precise biomarkers that could help in early detection and serve as potential targets for restoration therapy. While the traditional biomarkers, like CA15-3, remain clinical staples, their limited sensitivity and specificity in early-stage disease emphasize the necessity of a more comprehensive molecular panel. This study provides a multifaceted evaluation of CCN6, VEGF-A, NF- κ Bp65, and CA 15-3, illustrating their significant roles in the systemic landscape of breast cancer and their potential as promising diagnostic tools.

A pivotal finding of this study is the drastic downregulation of CCN6 in breast cancer patients, which exhibited a remarkably large effect size. As this matricellular protein is essential for maintaining epithelial integrity, its loss is associated with epithelial-to-mesenchymal transition (EMT) and tumor invasion.¹⁷ Our data align with the significant negative correlation between CCN6 expression and aggressive features, such as tumor grade, the Ki-67 proliferation index, and CA 15-3 levels. Additionally, the significant inverse correlation between CCN6 and the pro-tumorigenic factor NF- κ Bp65 suggests that reduced CCN6 levels may be associated with increased activation of the NF- κ B pathway, potentially reflecting the antagonistic regulatory role of CCN6 in modulating inflammatory and proliferative pathways.¹⁸

The biomarker profiles in the treated patients were not expected to normalize to healthy control baselines. The persistence of inflammatory (NF- κ B p65) and tumor burden (CA 15-3) markers may reflect the complex interplay of the pharmacological and microenvironmental factors rather than active disease. Systemic chemotherapy has been shown to induce off-target cytotoxicity and systemic inflammation, which may sustain NF- κ B activation. Furthermore, the massive therapy-induced cellular apoptosis and tissue remodeling may continuously shed the intracellular signaling proteins into the bloodstream.¹⁹ The most compelling finding of this study is the exceptional diagnostic performance of CCN6. These findings suggest that CCN6 may serve as a robust diagnostic biomarker, but they should be interpreted critically. The diagnostic accuracy may be subject to overestimation due to single-center design and the inherent characteristics of a cross-sectional case-control cohort, which can produce artificial disease prevalence and potential overfitting. These findings are considered preliminary and require rigorous validation in larger multicenter external cohorts. Consequently, the large negative regression coefficient suggests that further large-scale multicenter prospective histological studies are needed to understand the molecular role of CCN6 in the tumor microenvironment.

VEGF-A is an important pro-angiogenic factor, which promotes vascular growth and permeability, facilitating tumor expansion and metastases.⁹ In the present study, serum VEGF-A levels were elevated in the untreated group and exhibited a recent correlation with tumor grade, suggesting its potential role as a disease-promoting factor, consistent with a previous study.²⁰ Furthermore, the positive correlation between VEGF-A and CA15-3 may reflect a concurrent advancement of VEGF-A-mediated neovascularization and tumor burden (reflected by CA15-3) during tumor progression.²¹ This is practically relevant under hypoxic conditions, where high VEGF-A expression may promote tumor cell survival and correlate well with the microvessel density and adverse clinical outcomes.²² However, despite its biological importance, our findings underscore the limited sensitivity and diagnostic utility of VEGF-A due to its non-specific elevation in various inflammatory and non-malignant conditions. In opposition to our findings, a previous study by Zajkowska et al. identified VEGF-A as a highly promising candidate for cancer diagnosis (particularly in the early stages).²³

NF- κ B is a master transcriptional factor in inflammation and cell survival, facilitating tumor growth by promoting cell proliferation, angiogenesis, and resistance to apoptosis.⁸ The elevation of NF- κ Bp65 in untreated patients may reflect its association with tumor survival and metastatic potential. This is further evidenced by the positive association between NF- κ Bp65 and tumor grade, corroborating the notion that its activation is associated with increased disease severity.²⁴ Furthermore, the high odds ratios (OR) for NF- κ Bp65 suggest a central role in the inflammatory and angiogenic signaling pathways essential for tumor initiation and progression. Its modest diagnostic accuracy and strong association with cancer risk (OR) suggest it may act as a risk-promoting factor in carcinogenesis. These findings align with a recent study by Barnes et al., who reported the association of NF- κ B with tumor grade and its high diagnostic accuracy by breast cancer molecular subtypes.²⁵

Moderate diagnostic performance and weaker risk association (OR) were observed for CA15-3, consistent with Sekacheva et al.'s study²⁶ who mentioned that while CA15-3 lacks sufficient sensitivity for early diagnosis, it remains useful in monitoring disease relapses and treatment response. Suggesting that CA15-3 mirrors the tumor burden, rather than the early molecular events of carcinogenesis. Despite the heterogeneity of the treated group (2-12 years post-treatment). The treated subgroup analysis yielded no significant variations of CCN6, VEGF-A, and NF- κ B p65 between the recent and long-term remissions. This lack of significant association indicates that these biomarkers may reach a sustained biological plateau following therapeutic interventions.

Notably, differences in the menopausal status between treated and untreated patients were identified; the treated group consisted primarily of peri-menopausal women, while the untreated group had a more even distribution of pre-menopausal and post-menopausal women. These findings suggest a potential interaction between metabolic and hormonal elements in the progression of breast cancer. Consequently, breast cancer is more common in aggressive presentations in younger patients, leading to worse outcomes and aggressive subtypes, including triple-negative breast cancer.²⁷

The frequency of poor (grade III) and moderate (grade II) differentiation is consistent with clinical guidelines showing low 10-year survival for high-grade tumors compared to low-grade tumors.²⁸ The prognosis depends on the treatment modality. Multimodal treatment, which includes surgery, chemotherapy, radiotherapy, and hormonal therapy, remains the gold standard, leading to significant improvements in patients' overall survival. Studies nowadays are focused on the molecular basis of breast cancer so that novel diagnostic and treatment approaches can be applied to achieve better patient control and conquer drug resistance-related mechanisms.²⁹

The analysis revealed reduced levels of RBCs, Hb, and HCT in breast cancer patients, which are consistent with anemia of chronic disease or myelosuppression due to chemotherapy. This finding confirms the study by Muthanna et al.³⁰ The elevated WBCs and neutrophils with low lymphocytes and LMR are indicative of systemic inflammation associated with active diseases. A previous study found that low NLR is associated with a good pathological response after neoadjuvant chemotherapy.³¹ The stage-wise analysis of biomarkers in untreated and treated patients provides additional nuance. Serum CCN6 levels in untreated patients exhibited stage-dependent variability, peaking in stage IV. This re-emergence in advanced metastatic disease may represent a compensatory cellular response to vigorous extracellular matrix remodeling or a systemic release during tumor-associated necrosis. Concurrently, progressive elevation of VEGF-A, NF- κ Bp65, and CA 15-3 implies increased angiogenic activity and inflammatory activity, as well as tumor proliferation. An increase in VEGF-A is associated with its established role in facilitating neovascularization that is required for metastatic spread.³² Its upregulation during the different stages of tumor progression suggests that it might thus be an intriguing candidate for tumor progression monitoring or anti-angiogenic treatment response assessment. These findings are consistent with previous reports by Al-Rubaye et al., who reported that VEGF-A levels were significantly high in advanced-stage breast cancer.³³

The stepwise increase in CA 15-3 levels with advancing stage aligns with its well-known function as a marker for tumor burden and metastasis, corroborating the ob-

servation of a recent study by Li et al., where rising CA 15-3 concentrations characterize advanced and metastatic breast cancer, which is associated with poor prognosis.³⁴ This is further evidenced by the significant positive correlation of CA 15-3 levels with tumor stage.³⁴ Treated patients had a decline in serum CCN6, VEGF-A, and NF- κ B P65. Their stage-specific expression suggests a biological regulation of angiogenic and inflammatory pathways. Specifically, pharmacological inhibition of NF- κ B P65 may reduce VEGF expression, thereby potentially inhibiting pathological vascularization.^{8,35} However, serum levels of CA 15-3 remained elevated, which coincides with Kim et al.'s previous study, who observed that this marker may remain elevated after treatment due to residual tumor activity.³⁶ Collectively, our findings underscore a significant association between the immunological biomarkers, reflecting a molecular profile where CCN6 depletion coincides with NF- κ B and VEGF-A elevation, suggesting a coordinated expression pattern linked to inflammation and angiogenesis. Therefore, their tracking as a multi-biomarker panel could enhance disease staging accuracy and even improve prognostic determination in breast cancer treatment.

The lack of significant correlations of age and BMI with the biomarkers suggests that immunological and angiogenic alterations observed are disease-driven rather than age or weight-dependent. It has also been established that VEGF-A and CCN proteins are more influenced by disease biology.^{22,37} NF- κ B has been related to inflammatory pathways associated with aging, but primarily chronic diseases, rather than normal aging.³⁸ Reports indicate that CA 15-3 levels do not depend on age or reproductive factors among normal women, as well as patients with breast cancer.³⁹

This study possesses several strengths, including its novel focus on CCN6, a poorly investigated member of the CCN protein family that could serve as a promising novel biomarker for breast cancer. The simultaneous study of CCN6 with VEGF-A, NF- κ B p65, and the classic marker CA15-3 offers a comprehensive molecular and clinical perspective.

Study limitations

Nevertheless, the study has several limitations. First, the moderate sample size and single-center execution, combined with the absence of an external validation set, restrict the generalizability of the results. Second, the lack of benign breast disease control groups limits the ability to fully evaluate biomarker specificity. Third, our study design precludes the evaluation of long-term clinical outcomes, such as survival and recurrence rates, and prevents the calculation of real-world predictive values (PPV, NPV). Finally, the heterogeneity of the treated group may introduce a confounding variable when evaluating the post-treatment biomarker levels. We suggest

subsequent multicenter prospective validation studies involving larger populations to corroborate and extend these findings.

Conclusion

This study highlights the roles of the CCN6, NF- κ B p65, VEGF-A, and CA15-3 proteins as potential non-invasive diagnostic tools. The sensitivity and specificity of CCN6 are superior to those of other biomarkers for distinguishing patients with breast cancer from healthy controls, whereas the oncogenic role of NF- κ Bp65 in chronic inflammation has been elucidated. The inverse relationship between CCN6 expression and disease outcome aligns with the existing rationale that therapeutic restoration of CCN6 expression could inhibit epithelial-mesenchymal transition (EMT)-induced invasion. However, these findings do not imply immediate clinical applicability. Rigorous validation in larger multicenter prospective studies is warranted to translate this outcome into clinical practice.

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Declarations

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Author contributions

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

Conflicts of interest

The authors declare no conflict of interest.

Data availability

The dataset analyzed during the current study is available from the corresponding author on reasonable request.

Ethics approval

The study protocol received approval from the Research Committee of the Training and Human Development Center, Basra Health Department, Ministry of Health (Resolution No. 629, October 8, 2024).

Use of AI and AI-assisted technologies in the writing process

No AI or AI-assisted tool was used in the preparation of the manuscript.

References

1. Kleer CG. Dual roles of CCN proteins in breast cancer progression. *J Cell Commun Signal.* 2016;10(3):217-222. doi:10.1007/s12079-016-0345-7
2. Birkeness LB, Banerjee S, Quadir M, Banerjee SK. The role of CCNs in controlling cellular communication in the tumor microenvironment. *J Cell Commun Signal.* 2023;17(1):35-45. doi:10.1007/s12079-022-00682-2
3. Tran MN, Kleer CG. Matricellular CCN6 (WISP3) protein: a tumor suppressor for mammary metaplastic carcinomas. *J Cell Commun Signal.* 2018;12(1):13-19. doi:10.1007/s12079-018-0451-9
4. Siegel RL, Giaquinto AN, Jemal A. Cancer statistics, 2024. *CA Cancer J Clin.* 2024;74(1):12-49. doi:10.3322/caac.21820
5. Gonzalez ME, Brophy B, Eido A, et al. CCN6 suppresses metaplastic breast carcinoma by antagonizing Wnt/ β -Catenin signaling to inhibit EZH2-Driven EMT. *Cancer Res.* 2024;84(19):3235-3249. doi:10.1158/0008-5472.CAN-23-4054
6. Huang W, Martin EE, Burman B, et al. The matricellular protein CCN6 (WISP3) decreases Notch1 and suppresses breast cancer initiating cells. *Oncotarget.* 2016;7(18):25180-25193. doi:10.18632/oncotarget.7734
7. McMullen ER, Gonzalez ME, Skala SL, et al. CCN6 regulates IGF2BP2 and HMGA2 signaling in metaplastic carcinomas of the breast. *Breast Cancer Res Treat.* 2018;172(3):577-586. doi:10.1007/s10549-018-4960-2
8. Pavitra E, Kancharla J, Gupta VK, et al. The role of NF- κ B in breast cancer initiation, growth, metastasis, and resistance to chemotherapy. *Biomed Pharmacother.* 2023;163:114822. doi:10.1016/j.biopha.2023.114822
9. Al Kawas H, Saaid I, Jank P, et al. How VEGF-A and its splice variants affect breast cancer development - clinical implications. *Cell Oncol (Dordr).* 2022;45(2):227-239. doi:10.1007/s13402-022-00665-w
10. Ghalehbandi S, Yuzugulen J, Pranjol MZI, Pourgholami MH. The role of VEGF in cancer-induced angiogenesis and research progress of drugs targeting VEGF. *Eur J Pharmacol.* 2023;949:175586. doi:10.1016/j.ejphar.2023.175586
11. Mao H, Zhao X, Sun SC. NF- κ B in inflammation and cancer. *Cell Mol Immunol.* 2025;22(8):811-839. doi:10.1038/s41423-025-01310-w
12. Shaker SA, Alshufta SM, Gowayed MA, et al. Propolis-loaded nanostructured lipid carriers halt breast cancer progression through miRNA-223-related pathways: an in vitro/in vivo experiment. *Sci Rep.* 2023;13(1):15752. doi:10.1038/s41598-023-42709-7
13. Ryu JM, Kang D, Cho J, et al. Prognostic impact of elevation of cancer antigen 15-3 (CA15-3) in patients with early

- breast cancer with normal serum CA15-3 level. *J Breast Cancer*. 2023;26(2):126-135. doi:10.4048/jbc.2023.26.e17
14. Tang Z, Li L, Shen L, Shen X, Ju S, Cong H. Diagnostic value of serum concentration and integrity of circulating cell-free DNA in breast cancer: a comparative study with CEA and CA15-3. *Lab Med*. 2018;49(4):323-328. doi:10.1093/labmed/lmy019
 15. Giuliano AE, Edge SB, Hortobagyi GN. Eighth edition of the AJCC cancer staging manual: breast cancer. *Ann Surg Oncol*. 2018;25(7):1783-1785. doi:10.1245/s10434-018-6486-6
 16. Mohammed Muhibul G, Tariq Ali E, Ali Alrikabi M. Correlation between PD-L1 expression, demographic and pathological characters in patients with breast cancer. *Oncol Radiother*. 2022;16(12):74-81.
 17. Huang W, Zhang Y, Varambally S, et al. Inhibition of CCN6 (Wnt-1-Induced Signaling Protein 3) Down-regulates E-Cadherin in the breast epithelium through induction of snail and ZEB1. *Am J Pathol*. 2008;172(4):893-904. doi:10.2353/ajpath.2008.070899
 18. Ahmed KA, Hasib TA, Paul SK, et al. Potential role of CCN proteins in breast cancer: Therapeutic advances and perspectives. *Curr Oncol*. 2021;28(6):4972-4985. doi:10.3390/currenol28060417
 19. Behranvand N, Nasri F, Zolfaghari Emameh R, et al. Chemotherapy: a double-edged sword in cancer treatment. *Cancer Immunol Immunother*. 2022;71(3):507-526. doi:10.1007/s00262-021-03013-3
 20. Sharma P, Chida K, Wu R, et al. VEGFA gene expression in breast cancer is associated with worse prognosis, but better response to chemotherapy and immunotherapy. *World J Oncol*. 2025;16(1):120-130. doi:10.14740/wjon1993
 21. Habanjar O, Bingula R, Decombat C, Diab-Assaf M, Caldefie-Chezet F, Delort L. Crosstalk of inflammatory cytokines within the breast tumor microenvironment. *Int J Mol Sci*. 2023;24(4):4002. doi:10.3390/ijms24044002
 22. Brogowska KK, Zajkowska M, Mroczko B. Vascular endothelial growth factor ligands and receptors in breast cancer. *J Clin Med*. 2023;12(6):2412. doi:10.3390/jcm12062412
 23. Zajkowska M, Lubowicka E, Malinowski P, Szmitskowski M, Ławicki S. Plasma levels of VEGF-A, VEGF B, and VEGFR-1 and applicability of these parameters as tumor markers in diagnosis of breast cancer. *Acta Biochim Pol*. 2018;65(4):621-628. doi:10.18388/abp.2018_2713
 24. Al-Mutairi MS, Habashy HO. Nuclear Factor- κ B Clinical significance in breast cancer: an immunohistochemical study. *Med Princ Pract*. 2023;32(1):33-39. doi:10.1159/000527828
 25. Barnes P, Mensah A, Derkyi-Kwarteng L, et al. Prognostic significance of nuclear factor Kappa B (p65) among breast cancer patients in Cape Coast Teaching Hospital. *Med Princ Pract*. 2024;33(4):310-320. doi:10.1159/000539241
 26. Sekacheva M, Boroda A, Fatyanova A, Rozhkov A, Bagmet N. Clinical validation of the novel CLIA-CA-62 assay efficacy for early-stage breast cancer detection. *Front Oncol*. 2023;13:1009863. doi:10.3389/fonc.2023.1009863
 27. Henzler M, Willborn KC, Janni W, et al. Oncologic outcomes of young breast cancer patients according to tumor biology. *Cancers (Basel)*. 2025;17(8):1333. doi:10.3390/cancers17081333
 28. Intrieri T, Manneschi G, Caldarella A. 10-year survival in female breast cancer patients according to ER, PR and HER2 expression: a cancer registry population-based analysis. *J Cancer Res Clin Oncol*. 2022;149(8):4489-4496. doi:10.1007/s00432-022-04245-1
 29. Chakraborty S, Banerjee S. Bridging molecular insights and clinical application: non-coding RNAs, targeted drug delivery, and metastatic breast cancer therapy. *Discov Oncol*. 2025;16(1):1413. doi:10.1007/s12672-025-03090-1
 30. Muthanna FMS, Karuppannan M, Abdulrahman E, Uitrakul S, Rasool BAH, Mohammed AH. Prevalence and associated factors of anemia among breast cancer patients undergoing chemotherapy: a prospective study. *Adv Pharmacol Pharm Sci*. 2022;2022:7611733. doi:10.1155/2022/7611733
 31. Yildirim S, Dogan A, Akdag G, et al. The role of laboratory indices on treatment response and survival in breast cancer receiving neoadjuvant chemotherapy. *Sci Rep*. 2024;14(1):1-9. doi:10.1038/s41598-024-63096-7
 32. Madu CO, Wang S, Madu CO, Lu Y. Angiogenesis in breast cancer progression, diagnosis, and treatment. *J Cancer*. 2020;11(15):4474-4494. doi:10.7150/jca.44313
 33. Al-Rubaye RHK, Kh Al-Jumaily RM. High tumor levels of Ki-67, VEGF, and endostatin are associated with progression of breast cancer in Iraqi women. *Egypt J Hosp Med*. 2023;90(1):79-83. doi:10.21608/ejhm.2023.279199
 34. Li Y, Zhou L, San Z, Zhou P. Association of serum CA15-3, CA125, and TPS levels with clinicopathological factors in patients with breast cancer. *J Biol Regul Homeost Agents*. 2024;38(10):5957-5963. doi:10.23812/j.biol.regul.homeost.agents.20243810.475
 35. Li Y, Zhao B, Peng J, et al. Inhibition of NF- κ B signaling unveils novel strategies to overcome drug resistance in cancers. *Drug Resist Updat*. 2024;73:101042. doi:10.1016/j.drug.2023.101042
 36. Kim JY, Jung E, Kim JM, et al. The role of follow-up CA15-3 in prognosis of breast cancer. *Eur J Gynaecol Oncol*. 2025;46(4):19-24. doi:10.22514/ejgo.2025.047
 37. Abdul-Azees PA, Rajesh R, Block TJ, Dean DD, et al. CCN Proteins as matricellular regulators of bone in aging and disease. *Curr Osteoporos Rep*. 2025;23(1):23. doi:10.1007/s11914-025-00915-4
 38. García-García VA, Alameda JP, Page A, Casanova ML. Role of NF- κ B in ageing and age-related diseases: Lessons from genetically modified mouse models. *Cells*. 2021;10(8):1906. doi:10.3390/cells10081906
 39. Cramer DW, Vitonis AF, Fichorova RN, Yamamoto HS, Mudugno F, Finn OJ. Variables affecting CA15.3 tumor antigen expression and antibodies against it in female National Health and Nutritional Survey (NHANES) participants. *Cancer Epidemiol Biomarkers Prev*. 2024;33(9):1211-1219. doi:10.1158/1055-9965.EPI-24-0187



ORIGINAL PAPER

Vaginal and amniotic fluid microbiota in women with preterm labor – a comparative microbiological study

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ABSTRACT

Introduction and aim. This observational comparative study characterized the vaginal and amniotic fluids of women in preterm labor relative to those who delivered at term. A distinctive feature of this work is the paired, quantitative and culture-based assessment of both the vaginal and the amniotic fluid microbiota, while most previous studies have examined the vaginal compartment alone.

Material and methods. Carried out at a single tertiary center, the study enrolled 120 women in preterm labor at 33–35 weeks of gestation together with 89 women delivering at term (≥ 38 weeks). Culture-based identification and quantitative analysis were applied to paired vaginal and amniotic fluid specimens.

Results. Preterm labor was accompanied by a reduced prevalence of *Lactobacillus* spp. and by a markedly higher recovery of *Gardnerella vaginalis* (60.8%; odds ratio [OR] 12.27, 95% confidence interval [CI] 5.78–26.06) and *Ureaplasma* spp. (45.8%; OR 9.91, 95% CI 4.23–23.22), exceeding the term group ($p < 0.001$). Higher vaginal pH and polymicrobial associations occurred more often. The amniotic fluid produced microbial growth in 53.3% of preterm cases and vaginal and amniotic detection of *Ureaplasma* spp. ($\varphi = 0.61$; $p < 0.001$).

Conclusion. Vaginal dysbiosis showed an association with preterm labor that may be compatible with a contribution from ascending microbial colonization, though causality cannot be inferred from the present data.

Keywords. amniotic fluid, bacterial vaginosis, *Gardnerella vaginalis*, preterm labor, *Ureaplasma* spp., vaginal microbiota

Introduction

Preterm labor continues to be among the most prevalent problems in contemporary obstetrics, carrying an increased burden of perinatal morbidity and mortality along with lasting health sequelae in the newborn.^{1–3} Its etiology is multifactorial, arising from an intricate interplay of genetic, environmental, and infectious contributors.

Previous work has shown that women with complicated pregnancies, especially those in preterm labor, frequently display shifts in the microbial makeup of the lower genital tract.^{4,5} Such vaginal microbial changes

can shape the local immune milieu and ease the upward passage of organisms from the lower genital tract into the uterine cavity.

A growing body of evidence links perturbation of the vaginal microbial ecosystem to unfavorable pregnancy outcomes. Overgrowth of opportunistic organisms can undermine the colonization resistance conferred by *Lactobacillus* spp., raise the vaginal pH, and reshape local immune responses.^{6–10} Together, these shifts may promote migration of organisms toward the fetal membranes and amniotic cavity.

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Infections of the genital tract have long been considered an important contributor to premature labor.^{11–13} Bacterial vaginosis and nonspecific vaginitis are commonly documented among affected women.^{14–18} Such conditions typically feature the depletion of protective *Lactobacillus* spp. accompanied by the expansion of anaerobic and opportunistic organisms.

Among these microorganisms, *Gardnerella vaginalis* and *Ureaplasma* spp. have attracted particular interest. Molecular microbiome surveys indicate that these organisms are often recovered in preterm labor and may participate in inflammatory processes on the fetal membranes.^{19,20}

Recent studies have also described microbial presence within the amniotic fluid when pregnancy outcomes are adverse.^{21–24} Although how organisms reach the amniotic cavity is still being clarified, ascending colonization from the lower genital tract is considered a plausible route.

Most current investigations of the vaginal microbiome depend on molecular assays that detect microbial DNA. Culture-based microbiology nevertheless retains clinical value, since it identifies viable organisms and quantifies the microbial burden and can therefore add complementary insight into the microbiological features linked with preterm labor. Quantitative culture data for the amniotic compartment and studies that sample the vaginal and amniotic microbiota in parallel remain comparatively scarce, leaving a gap that culture-based methods are well positioned to address.

Aim

This study aimed to characterize, qualitatively and quantitatively, the vaginal and amniotic fluids of women in preterm labor by culture-based microbiology, and to examine how these are related to microbial presence in the amniotic fluid. When combining quantitative vaginal and amniotic fluid cultures within a single cohort, the study offers a microbiological perspective that complements the molecular microbiome analyses reported in our earlier work.

Material and methods

Study design and participants

The cohort comprised 120 pregnant women in preterm labor (Group I) at 33–35 weeks of gestation and 89 pregnant women delivering at term (38 weeks of gestation) (Group II). Enrollment was limited to primigravidae carrying singleton pregnancies. The exclusion was applied to any woman who reported smoking; recent use of antibiotics, antiseptics, or probiotic use; or a prior preterm birth, miscarriage, or multiple pregnancy. Recruitment was consecutive, covering every eligible woman during the study period, and no a priori sample size calculation was undertaken. The mean participant age was 26.2±1.9 years.

A diagnosis of preterm labor required at least two uterine contractions over a 10-minute interval together with cervical change at 33 to 35 weeks of gestation.

Controls were healthy pregnant women without obstetric, medical, or surgical complications, congenital anomalies, or histological evidence of chorioamnionitis. Routine postpartum histology of the placenta and fetal membranes was undertaken; since no case of histological chorioamnionitis was observed among controls, no woman was excluded for this reason.

All participants underwent a clinical examination, ultrasound evaluation, and microbiological testing.

Sample collection

Immediately before delivery, vaginal discharge was sampled from the mid-vaginal wall with sterile swabs. The smears were spread on glass slides, fixed in 90% ethanol and stained by the Gram method, 1% aqueous methylene blue, and Romanowsky staining.

Amniotic fluid was secured aseptically from the 120 women in the preterm labor group. For the 118 (98.3%) delivered by caesarean section, fluid was drawn directly from the amniotic cavity with a sterile needle once the operative field had been disinfected, immediately after the uterine incision and before the fetal membranes ruptured. In the 2 women (1.7%) who delivered vaginally after membrane rupture, a sterile catheter was used through the cervical canal under strict asepsis was used; neither of these two specimens produced microbial growth.

To limit possible contamination, the initial aliquot of aspirate was discarded; the remaining fluid was placed in sterile transport tubes and transferred to the microbiology laboratory in 2 h for analysis.

Microbiological analysis

Culture-based microbiology was performed on a range of selective and differential media: blood agar, egg yolk salt agar, Endo agar, *Lactobacillus*-selective agar, Sabouraud agar, Chrom *Candida* chromogenic medium, and selective agar for *Gardnerella vaginalis*.

Organisms were identified by their morphological, cultural, and biochemical properties following Bergey's Manual of Determinative Bacteriology, with automated confirmation on the Vitek 2 Compact system (bioMérieux, France). Specimens from both groups were processed under identical standard operating procedures, and microbiologists were blinded to the allocation of the clinical group during the culturing and identification.

The detection of *Ureaplasma* spp. was based on the Mycoplasma IST 2 system (bioMérieux, France), which allows both qualitative and quantitative readout. A count of $\geq 10^4$ colony-forming units (CFU) per sample was taken as clinically significant, and microbial load was reported as log₁₀ CFU/mL.

Bacterial vaginosis was established on the Amsel criteria, at least three homogeneous vaginal discharges, vaginal pH > 4.5, clue cells, and a positive amine test, corroborated by bacterioscopy of stained smears. Vaginal pH was read with indicator strips.

Bacterioscopic assessment

Stained smears were examined under ×40 and ×100 objectives, recording leukocytes, epithelial cells, *Lactobacillus* spp., Gram-positive cocci, Gram-negative bacilli, yeast-like fungi, and clue cells.

Statistical analysis

Data were analyzed in IBM SPSS Statistics version 29 (Armonk, NY, USA). The Shapiro–Wilk test checked normality. Continuous variables appear as mean±standard deviation (SD); normally distributed data were compared by Student’s or Welch’s t test and skewed data by the nonparametric Mann–Whitney U test, as appropriate. Counts and percentages summarized categorical data, which were compared with Pearson’s χ^2 or Fisher’s exact test. Odds ratios (OR) and their 95% confidence intervals (CI) were derived for the link between microbial detection and preterm labor, with a continuity correction where a cell was zero. The ϕ -coefficient gauged the concordance between the detection of vaginal and amniotic fluid of *Ureaplasma* spp. within the preterm labor group.

Because every eligible participant in the study window was enrolled, no a priori sample size calculation was carried out. A post hoc power analysis, based on the observed difference in *Ureaplasma* spp. detection at $\alpha=0.05$, confirmed power above 80%. Due to the number of comparisons, p-values were left unadjusted for multiple testing and are treated as exploratory. Significance was set at $p<0.05$, with exact p-values given except where $p<0.001$. The analyzes were univariate: Given the exploratory, single-centre design, the fixed enrolled sample, and the large number of microbial comparisons, no multivariate regression model adjusting for potential confounders, and associations are therefore reported unadjusted.

Ethical approval

The study adhered to the Declaration of Helsinki and was approved by the Bioethic Committee of the State Institution ‘Center of Maternity and Childhood of the Ukrainian National Academy of Medical Sciences of Ukraine’ (Protocol No. 3, April 20, 2023). Each participant provided written informed consent in writing before entering the study and a completed STROBE checklist is included with the manuscript as supplementary material.

Results

The detection frequencies of opportunistic organisms in vaginal samples from the preterm labor and term delivery groups are given in Table 1.

Table 1. Frequency of detection of opportunistic microorganisms in vaginal samples, n (%)*

Microorganism	Preterm labour (n=120)	Term delivery (n=89)	OR	95% CI	p
<i>Lactobacillus</i> spp.	86 (71.7)	89 (100)	0.01 ^a	0.00–0.23	<0.001
<i>Ureaplasma</i> spp.	55 (45.8)	7 (7.9)	9.91	4.23–23.22	<0.001
<i>Gardnerella vaginalis</i>	73 (60.8)	10 (11.2)	12.27	5.78–26.06	<0.001
<i>Candida</i> spp.	25 (20.8)	10 (11.2)	2.08	0.94–4.59	0.07
<i>Corynebacterium</i> spp.	12 (10.0)	7 (7.9)	1.30	0.49–3.45	0.59
<i>Klebsiella</i> spp.	8 (6.7)	3 (3.4)	2.05	0.53–7.95	0.30
<i>Escherichia coli</i>	22 (18.3)	12 (13.5)	1.44	0.67–3.09	0.35
Haemolytic <i>E. coli</i>	16 (13.3)	0	28.26 ^a	1.67–477.82	0.02
<i>Enterococcus faecalis</i>	22 (18.3)	11 (12.4)	1.59	0.73–3.48	0.24
<i>Streptococcus pyogenes</i>	12 (10.0)	0	20.62 ^a	1.20–353.17	0.03
<i>Streptococcus agalactiae</i>	16 (13.3)	6 (6.7)	2.13	0.80–5.68	0.13
<i>Staphylococcus aureus</i>	8 (6.7)	0	13.52 ^a	0.77–237.51	0.07
<i>Staphylococcus epidermidis</i>	32 (26.7)	10 (11.2)	2.87	1.33–6.22	0.006
Haemolytic <i>S. epidermidis</i>	20 (16.7)	3 (3.4)	5.73	1.65–19.96	0.006

* – Odds ratio computed with a continuity correction for zero cells in the contingency table. For organisms found only in the preterm labor group, these estimates are imprecise and should be read with caution

A reduced prevalence of *Lactobacillus* spp. was evident among women in preterm labor compared with term deliveries. In contrast, *Gardnerella vaginalis* and *Ureaplasma* spp. were recovered more frequently in this group. Within it, 53 cases (44.2%) showed *Ureaplasma* spp. at $\geq 10^4$ CFU/sample, whereas every term delivery stayed below that threshold.

Several additional opportunistic organisms were also more common in the preterm labor group, among them haemolytic *E. coli*, *S. pyogenes*, *S. aureus* and *S. epidermidis*.

Quantitatively, vaginal Gram-positive cocci reached higher loads in the preterm labor group, spanning log₁₀ 4.0 to log₁₀ 5.2 CFU/mL, against log₁₀ 3.0 to log₁₀ 3.8 CFU/mL at term ($p<0.001$).

Enterobacteriaceae were also more prevalent with preterm labor. Recovery of *E. coli*, haemolytic *E. coli*, and *Klebsiella* spp. was greater in this group, with loads of log₁₀ 4.3 to log₁₀ 5.4 CFU/mL versus log₁₀ 3.2 to log₁₀ 3.6 CFU/mL at term ($p<0.001$) (Fig. 1).

Candida spp. frequency was higher with preterm labour than at term. Among *Candida* isolates from the preterm labor group, *C. albicans* was dominant (21 cases, 17.5%), while nonalbicans species occurred less often and comprised *C. glabrata* (3 cases, 2.5%) and *C. tropicalis* (1 case, 0.8%). At term, only *C. albicans* was detected.

Vaginal pH exceeded 4.5 in 101 women (84.2%) of the preterm labor group, whereas at term it fell within 3.8 to 4.5.

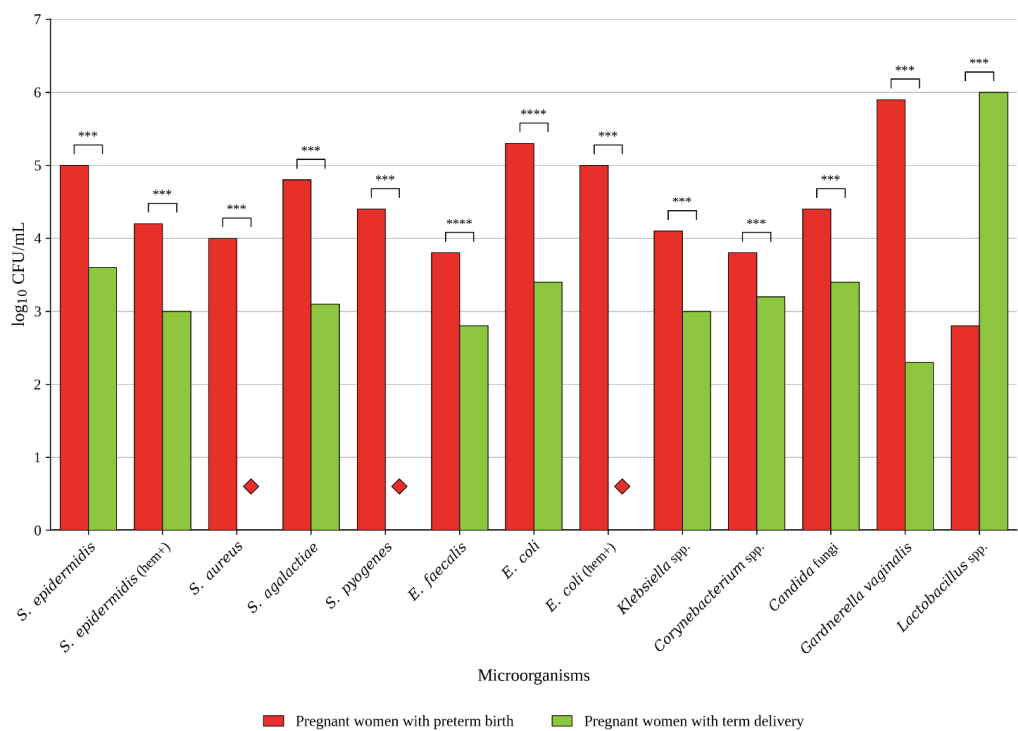


Fig. 1. Vaginal microbial loads in the preterm labor (n=120) and term delivery (n=89) groups, expressed as log₁₀ CFU/mL and compared by the Mann–Whitney U test; *** p<0.001; **** p<0.0001; ♦ organism absent from the term delivery group

Polymicrobial associations arose far more often with preterm labor than at term (61.7% vs. 13.5%). Frequent combinations were *S. epidermidis* with *Candida* spp., hemolytic *S. epidermidis* with *E. coli*, *E. coli* with *S. agalactiae* and *Candida* spp., and *Ureaplasma urealyticum* with *G. vaginalis*.

Bacterioscopy provided a further view of the vaginal microbiota. Smear-type distribution diverged between the preterm labor (n=120) and term (n=89) groups (p<0.001). At term, a normal pattern (normocenosis) prevailed in 86 women (96.6%). In the preterm labor group, by contrast, bacterial vaginosis was the leading finding (72 cases, 60.0%), followed by nonspecific vulvovaginitis (28 cases, 23.3%) and an intermediate type of smear in 20 women (16.7%).

Nonspecific vulvovaginitis featured elevated leukocyte counts (30–100 per field), mostly neutrophils, together with a greater number of Gram-positive cocci, Gram-negative bacilli, and *Candida* spp., in addition to a reduced presence of organisms resembling *Lactobacillus* spp. (Fig. 2).

Where there was bacterial vaginosis, the bacterioscopy showed a predominance of anaerobic morphotypes with moderate leukocyte response (15–30 per field); clue cells were common, as were organisms similar to *Mobiluncus* spp., *Fusobacterium* spp., and *Gardnerella* spp.

At term, bacterioscopy usually revealed a *Lactobacillus* dominated flora with few leukocytes (up to 10–

12 per preparation) and few squamous epithelial cells (5–10 per preparation). Only three women (3.4%) here showed an intermediate smear type (Fig. 2).

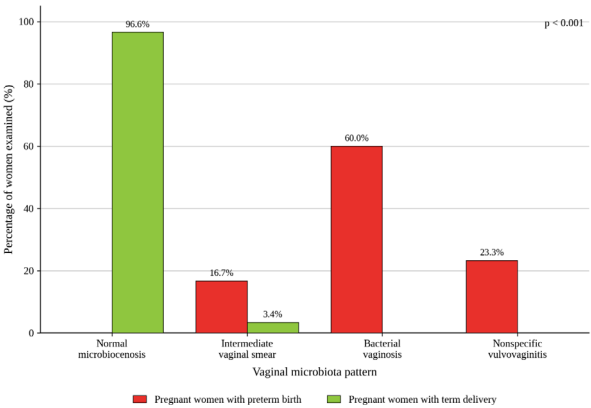


Fig. 2. Principal vaginal microbiota patterns in the preterm labor group and term delivery group; Values are percentages of the women examined

A further aim was to determine the presence and makeup of organisms in amniotic fluid, sampled in all 120 women of the preterm labor group.

The amniotic fluid of the preterm labor group contained several Enterobacteriaceae. *E. coli* occurred in 14 women (11.7%), hemolytic *E. coli* in 9 cases (7.5%), and *E. faecalis* in 13 women (10.8%). Quantitative levels reached log₁₀ 4.2 CFU/mL and log₁₀ 4.4 CFU/mL,

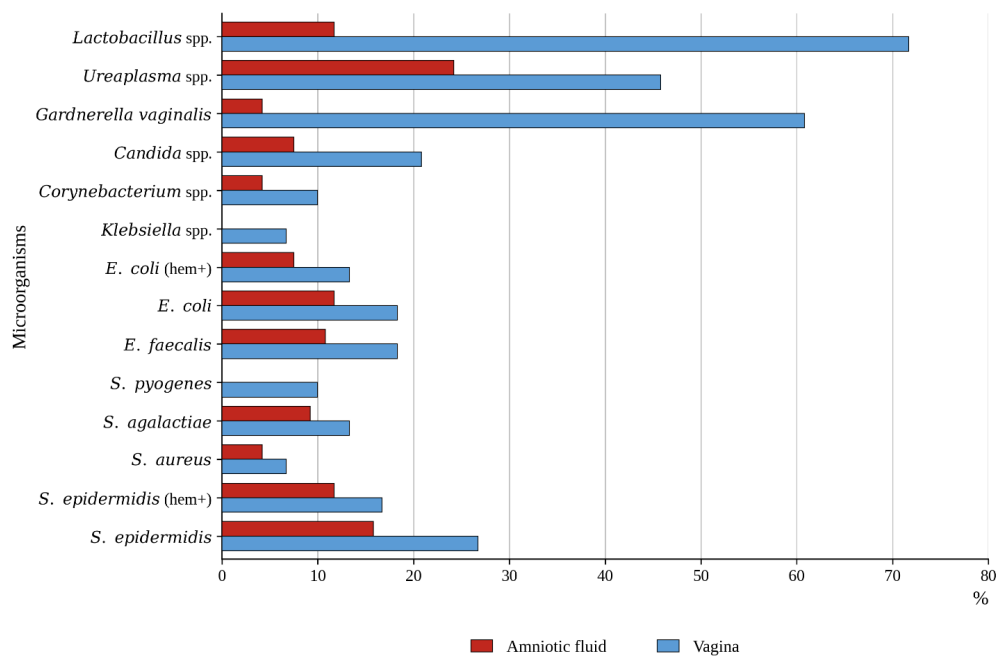


Fig. 3. Range of organisms recovered from vaginal and amniotic fluid specimens in the preterm labor group (n=120); values are percentages of women examined

respectively. *Corynebacterium* spp. appeared in 5 cases (4.2%) at a lower load ($\log_{10}$ 2.2 CFU/mL). None of these were found at term.

Gram-positive cocci were also present in preterm amniotic fluid. *S. epidermidis* was identified in 19 women (15.8%), hemolytic *S. epidermidis* in 14 cases (11.7%), *S. aureus* in 5 cases (4.2%) and *S. agalactiae* in 11 cases (9.2%). Quantitative levels reached $\log_{10}$ 4.4 CFU/mL for *S. epidermidis* and $\log_{10}$ 4.2 CFU/mL for *S. aureus*.

Fungi of the genus *Candida* were recovered from amniotic fluid in 9 women (7.5%), though at loads under $\log_{10}$ 4.0 CFU/mL. *Lactobacillus* spp. were found in 14 cases (11.7%) at $\log_{10}$ 4.2 CFU/mL. *G. vaginalis* occurred in 5 women (4.2%) at $\log_{10}$ 3.8 CFU/mL.

Ureaplasma spp. were present in 29 cases (24.2%); in each, *Ureaplasma* spp. were also present vaginally. Loads reached $\geq 10^4$ CFU/sample in 18 women (15.0%), while 11 cases (9.2%) were below this cut-off point. A positive association linked *Ureaplasma* spp. in vaginal and amniotic fluid specimens ($\phi=0.61$; $p<0.001$).

Amniotic fluid remained sterile in 56 women (46.7%) of the preterm labor group; the other 64 (53.3%) grew organisms as single isolates or polymicrobial mixtures. The most common were *Ureaplasma* spp., *E. coli*, *E. faecalis*, *S. epidermidis*, *S. agalactiae*, and *C. albicans*. At term, the amniotic fluid was sterile in 82 women (92.1%), while 7 (7.9%) showed little growth of *S. epidermidis* at $\log_{10}$ 2.0 CFU/mL.

Comparing vaginal and amniotic fluid isolates within the preterm labor group revealed partial compositional concordance in 63 cases (52.5%) (Figure 3). In summary, three main findings emerged: preterm la-

bor was marked by vaginal dysbiosis, with depleted *Lactobacillus* spp. along with *Gardnerella vaginalis* and *Ureaplasma* spp.; amniotic fluid produced microbial growth in 53.3% of preterm cases, while remaining sterile in the vast majority of term pregnancies; and vaginal and amniotic isolates showed partial concordance, a pattern compatible with ascending colonization.

Discussion

This study examined, by culture-based microbiology, the vaginal and amniotic fluid microbiota accompanying preterm labor. The results point to compositional differences between the preterm labor and term delivery groups.

The preterm labor group showed a lower prevalence of *Lactobacillus* spp. and a correspondingly greater recovery of opportunistic organisms such as *Staphylococcus* spp., Enterobacteriaceae, *Gardnerella vaginalis*, and *Ureaplasma* spp. This is consistent with previous reports that disruption of the vaginal microbial ecosystem often leads to preterm labour.^{13,25}

Numerous large observational and molecular studies have described vaginal dysbiosis - a loss of dominance by *Lactobacillus* spp. with expansion of anaerobic and opportunistic organisms — as a correlate of adverse pregnancy outcomes.²⁵⁻²⁷ Our culture-based data, showing heavier Gram-positive coccal loads in the preterm labor group, align with these reports.

In the present study, *Gardnerella vaginalis* emerged as one of the strongest correlates of preterm labor here, echoing earlier work on the role played by *G. vaginalis* in bacterial vaginosis, biofilm formation, and remodeling

of the vaginal microbial environment.^{19,28} *Ureaplasma* spp. were also recovered often and positively associated with preterm labour in our cohort, in keeping with reports connecting vaginal *Ureaplasma urealyticum* and *Mycoplasma hominis* with preterm delivery.^{29,30}

A vaginal pH above 4.5 was seen more often with preterm labor. Previous work indicates that increasing pH can signal lactobacillus-dominated flora breakdown and overgrowth of opportunistic organisms.^{31,32} Consistent with this, the term group held a physiological pH of 3.8 to 4.5.

Polymicrobial associations were also more frequent with preterm labor, possibly mirroring intricate interspecies interactions within the vaginal ecosystem.³³

The amniotic fluid harbored organisms more often in the preterm labor group than at term, and vaginal and amniotic microbiota showed partial concordance in a percentage of patients. Taken together with the positive association for *Ureaplasma* spp., this may fit previously proposed ascending migration of organisms from the lower genital tract.^{4,34,35} However, given the observational design and the possibility of sampling-related contamination, these results warrant caution and do not prove a causal ascending route.

A notable feature of this work is its reliance on culture. Although much current vaginal microbiome research is molecular, culture retains clinical utility by detecting viable organisms and quantifying load; molecular assays capture a wider set of taxa, including non-culturable ones, yet cannot confirm viability, so the two are best seen as complementary. Recovery of *Lactobacillus* spp. in every term of the participant exceeds the usual figures for molecular studies, most plausibly because culture favors dominant viable populations and because the control group was carefully selected on clinical grounds.

From a clinical perspective, the microbial patterns observed here may be of interest for future research. Because samples were obtained close to delivery, the present study does not assess the predictive value of the vaginal microbiota or the efficacy of any intervention; The findings are hypothesis-generating and may motivate prospective studies by sampling earlier in pregnancy and incorporating inflammatory markers and molecular profiling – to explore whether such patterns could inform screening or preventive strategies. In routine obstetric practice, quantitative culture-based assessment could complement molecular microbiome testing, adding information on viable organisms and microbial load that sequencing alone does not capture, thus supporting future diagnostic or screening approaches in women at risk of preterm labor.

The strengths of the study include its relatively large cohort (n=209), quantitative culture-based analysis, and paired evaluation of vaginal and amniotic fluids.

Study limitations

Several caveats apply. First, the design was observational and cross-sectional, so cause and effect between microbial colonization and preterm labor cannot be drawn. Second, the sample size was not fixed a priori, given the design; a post hoc check, however, showed adequate power (>80%) for the main comparison. Third, culture has limited sensitivity, misses nonculturable or fastidious taxa, and may understate diversity relative to molecular sequencing. Fastidious anaerobes such as *Prevotella*, *Bacteroides*, *Atopobium*, *Mobiluncus*, *Peptostreptococcus*, and *Fusobacterium* spp. went undetected by culture; their absence reflects methodological limits rather than true absence, and bacterioscopy did show matching anaerobic morphotypes where bacterial vaginosis was present. Fourth, no multivariable adjustment was made, so residual confounding remains possible; factors such as antibiotic exposure, timing of membrane rupture, prior genital infection, and smoking were not modelled, and baseline variables were not uniformly captured, so the associations are unadjusted. Fifth, though amniotic fluid was taken aseptically, contamination cannot be wholly excluded, especially after membrane rupture, which bears on the apparent vaginal–amniotic similarity. Sixth, restriction to 33–35 weeks of gestation may curb generalizability to other gestational ages. Finally, inflammatory markers such as interleukin-6 and C-reactive protein were not assayed, so microbial colonization could not be distinguished from an intra-amniotic inflammatory response.

Conclusion

Vaginal dysbiosis – marked by fewer *Lactobacillus* spp., more frequent *G. vaginalis* and *Ureaplasma* spp., raised vaginal pH, and abundant polymicrobial associations – was seen more often with preterm labor than at term.

Amniotic fluid yielded organisms more often in the preterm labor group, with partial vaginal–amniotic concordance in a subset of cases. These observations are compatible with, but do not establish, the previously proposed ascending migration of organisms from the lower genital tract.

Quantitative culture-based evaluation of the vaginal microbiota may add microbiological detail useful for appraising colonization patterns in pregnancy, bearing in mind the limited sensitivity of culture. Prospective longitudinal studies combining culture-based and molecular microbiome analyses are warranted to validate the clinical applicability of these findings.

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Declarations

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Author contributions

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

Conflicts of interest

The authors report no competing interests.

Data availability

Datasets produced or analyzed in this study can be obtained from the corresponding author on reasonable request.

Ethics approval

The study followed the Declaration of Helsinki and received approval from the Bioethics Committee of the State Institution “Ukrainian Center of Maternity and Childhood of the National Academy of Medical Sciences of Ukraine” (Protocol No. 3, April 20, 2023).

Use of AI and AI-assisted technologies in the writing process

In preparing this work, the authors employed AI-assisted language-editing tools to enhance clarity and readability. Having used these tools, the authors then reviewed and revised the text as required and accept full responsibility for the content of the published article.

References

1. Zivaljevic J, Jovandarcic MZ, Babic S, Raus M. Complications of preterm birth—the importance of care for the outcome: a narrative review. *Medicina (Kaunas)*. 2024;60(6):1014. doi:10.3390/medicina60061014
2. Tsonis O, Gkrozou F, Harrison E, Paschopoulos M. Female genital tract microbiota affecting the risk of preterm birth: what do we know so far? *Eur J Obstet Gynecol Reprod Biol*. 2020;245:168–173. doi:10.1016/j.ejogrb.2019.12.005
3. Aguinaga M, Valdespino Y, Medina D, et al. Causal analysis of fetal death in high-risk pregnancies. *J Perinat Med*. 2021;49(6):740–747. doi:10.1515/jpm-2020-0352
4. Rowlands S, Danielewski JA, Tabrizi SN, Walker SP, Garland SM. Microbial invasion of the amniotic cavity in mid-trimester pregnancies using molecular microbiology. *Am J Obstet Gynecol*. 2017;217(1):71.e1–71.e5. doi:10.1016/j.ajog.2017.02.051
5. Romero R, Miranda J, Chaemsathong P, et al. Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. *J Matern Fetal Neonatal Med*. 2015;28(12):1394–1409. doi:10.3109/14767058.2014.958463
6. Saraf VS, Sheikh SA, Ahmad A, Gillevet PM, Bokhari H. Vaginal microbiome: normalcy vs dysbiosis. *Arch Microbiol*. 2021;203(7):3793–3802. doi:10.1007/s00203-021-02414-3
7. Marschalkó M, Ambrus L. Characteristics and physiologic role of female lower genital microbiome. *Orv Hetil*. 2023;164(24):923–930. doi:10.1556/650.2023.32791
8. Dinsdale NK, Castano-Rodriguez N, Quinlivan JA, Mendz GL. Comparison of the genital microbiomes of pregnant aboriginal and non-aboriginal women. *Front Cell Infect Microbiol*. 2020;10:523764. doi:10.3389/fcimb.2020.523764
9. Megli CJ, Coyne CB. Infections at the maternal-fetal interface: an overview of pathogenesis and defense. *Nat Rev Microbiol*. 2022;20(2):67–82. doi:10.1038/s41579-021-00610-y
10. Kumar S, Bagga R, Kumar P, et al. The vaginal microbial signatures of preterm birth delivery in Indian women. *Front Cell Infect Microbiol*. 2021;11:622474. doi:10.3389/fcimb.2021.622474
11. Capros H. *Ureaplasma* in pregnancy. Is there any risk for preterm labor? *One Health Risk Manag*. 2025;6(1):4–15. doi:10.38045/ohrm.2025.1.01
12. Zhao F, Chen Y, Gao J, et al. Characterization of vaginal microbiota in women with recurrent spontaneous abortion that can be modified by drug treatment. *Front Cell Infect Microbiol*. 2021;11:680643. doi:10.3389/fcimb.2021.680643
13. Fettweis JM, Serrano MG, Brooks JP, et al. The vaginal microbiome and preterm birth. *Nat Med*. 2019;25(6):1012–1021. doi:10.1038/s41591-019-0450-2
14. Oerlemans EFM, Wuyts S, Bellen G, et al. The dwindling microbiota of aerobic vaginitis, an inflammatory state enriched in pathobionts with limited TLR stimulation. *Diagnostics (Basel)*. 2020;10(11):879. doi:10.3390/diagnostics10110879
15. Goodfellow L, Verwijs MC, Care A, et al. Vaginal bacterial load in the second trimester is associated with early preterm birth recurrence: a nested case-control study. *BJOG*. 2021;128(13):2061–2072. doi:10.1111/1471-0528.16816
16. Sun S, Serrano MG, Fettweis JM, et al. Race, the vaginal microbiome, and spontaneous preterm birth. *mSystems*. 2022;7(3):e00017–22. doi:10.1128/msystems.00017-22
17. Dos Anjos Borges LG, Pastuschek J, Heimann Y, et al. Vaginal and neonatal microbiota in pregnant women with preterm premature rupture of membranes and consecutive early onset neonatal sepsis. *BMC Med*. 2023;21:92. doi:10.1186/s12916-023-02805-x
18. Schuster HJ, Bos AM, Himschoot L, et al. Vaginal microbiota and spontaneous preterm birth in pregnant women at high risk of recurrence. *Heliyon*. 2024;10(10):e30685. doi:10.1016/j.heliyon.2024.e30685

19. Oyenihni AB, Haines R, Trama J, Faro S, Mordechai E, Adelson ME. Molecular characterization of vaginal microbiota using a new 22-species qRT-PCR test to achieve a relative-abundance and species-based diagnosis of bacterial vaginosis. *Front Cell Infect Microbiol.* 2024;14:1409774. doi:10.3389/fcimb.2024.1409774
20. Urushiyama D, Suda W, Ohnishi E, et al. Microbiome profile of the amniotic fluid as a predictive biomarker of perinatal outcome. *Sci Rep.* 2017;7(1):12171. doi:10.1038/s41598-017-11699-8
21. Ghanbarzadeh N, Mohammadparast-Tabas P, Aramjoo H, et al. An evaluation of antibacterial effects of human amniotic fluid on pathogenic and probiotic bacteria in vitro. *J Reprod Infertil.* 2023;24(2):101-107. doi:10.18502/jri.v24i2.12495
22. Mao Y, Hoffman T, Singh-Varma A, et al. Antimicrobial peptides secreted from human cryopreserved viable amniotic membrane contribute to its antibacterial activity. *Sci Rep.* 2017;7(1):13722. doi:10.1038/s41598-017-13310-6
23. Staude B, Gschwendtner S, Frodermann T, et al. Association of specific microbiota taxa in the amniotic fluid at birth with severe acute and longer-term outcomes of very preterm infants: a prospective observational study. *BMC Med.* 2025;23:431. doi:10.1186/s12916-025-04259-9
24. Leiby JS, McCormick K, Sherrill-Mix S, et al. Lack of detection of a human placenta microbiome in samples from preterm and term deliveries. *Microbiome.* 2018;6(1):196. doi:10.1186/s40168-018-0575-4
25. Huang C, Gin C, Fettweis J, et al. Meta-analysis reveals the vaginal microbiome is a better predictor of earlier than later preterm birth. *BMC Biol.* 2023;21:199. doi:10.1186/s12915-023-01702-2
26. Kosti I, Lyalina S, Pollard KS, Sirota M, Chen LL. Meta-analysis of vaginal microbiome data provides new insights into preterm birth. *Front Microbiol.* 2020;11:476. doi:10.3389/fmicb.2020.00476
27. Vinodhini E, Pegu B, Rengaraj S, Singh R. Vaginal microbiota in women with spontaneous preterm labor compared to spontaneous term labor: a cross-sectional analytical study. *Cureus.* 2025;17(4):e82302. doi:10.7759/cureus.82302
28. Wong YR, Cheah EC, Wong KK, et al. *Gardnerella vaginalis* infection in pregnancy: effects on placental development and neonatal outcomes. *Placenta.* 2022;120:79-87. doi:10.1016/j.placenta.2022.02.018
29. Miyoshi Y, Suga S, Sugimi S, et al. Vaginal *Ureaplasma urealyticum* or *Mycoplasma hominis* and preterm delivery in women with threatened preterm labor. *J Matern Fetal Neonatal Med.* 2022;35(5):878-883. doi:10.1080/14767058.2020.1733517
30. Oh KY, Lee S, Park J, et al. Vaginal microbiota of pregnant women with *Ureaplasma urealyticum* and *Mycoplasma hominis* infections. *Front Cell Infect Microbiol.* 2024;14:1445300. doi:10.3389/fcimb.2024.1445300
31. Weckend MJ, Spineli LM, Grylka-Baeschlin S, Gross MM. Association between increased antenatal vaginal pH and preterm birth rate: a systematic review. *J Perinat Med.* 2019;47(2):142-151. doi:10.1515/jpm-2018-0097
32. Valeriano VD, Lahtinen E, Hwang IC, Zhang Y, Du J, Schuppe-Koistinen I. Vaginal dysbiosis and the potential of vaginal microbiome-directed therapeutics. *Front Microbiomes.* 2024;3:1363089. doi:10.3389/frmbi.2024.1363089
33. Ceccarani C, Foschi C, Parolin C, et al. Diversity of vaginal microbiome and metabolome during genital infections. *Sci Rep.* 2019;9(1):14095. doi:10.1038/s41598-019-50410-x
34. Liu Y, Ma J, Li X, et al. No microorganism was detected in amniotic fluid of healthy pregnancies from the second trimester to the delivery. *Microbiome.* 2025;13:20. doi:10.1186/s40168-024-02024-3
35. Özdemir T, Kasap B, Akın H, Günay T, Sayiner AA. Amniotic fluid microbiome in asymptomatic pregnant at second trimester. *Ege J Med.* 2021;60(1):83-89. doi:10.19161/etd.883161



REVIEW PAPER

Antimalarial drug resistance markers in Southeast Asia (2000–2025) – a systematic review protocol for surveillance and spatial mapping

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ABSTRACT

Introduction and aim. Southeast Asia has historically been the epicenter of drug resistance against *Plasmodium falciparum*. The resistance has markedly reduced the effectiveness of key antimalarial drugs, such as chloroquine, sulfadoxine-pyrimethamine, and artemisinin-based combination therapy (ACTs). This systematic review protocol aims to characterize the spatio-temporal distribution and trends of drug resistance markers, both molecular and phenotypic, in *P. falciparum* malaria across South-east Asian countries from 2000 to 2025 and to identify regional research and surveillance gaps.

Material and methods. This study will follow a systematic search of literature published in English with full text from January 2000 to December 2025, including grey literature from five electronic databases. Data extraction and analysis will strictly adhere to the PRISMA-P guidelines and PECO/PICO framework. Risk of bias will be assessed independently by two reviewers using the Joanna Briggs Institute (JBI) critical appraisal tools matched to study design, including the JBI Analytical Cross-Sectional Checklist for molecular surveillance studies and the JBI Prevalence Checklist for epidemiological surveys.

Expected outcomes. This systematic protocol is expected to synthesize evidence on the prevalence, geographical distribution, and temporal trends of resistance markers and treatment failure associated with drug-resistant *P. falciparum* in Southeast Asia.

Keywords. drug-resistant malaria, Greater Mekong sub-region, *Plasmodium falciparum*, Southeast Asia, systematic review protocol

Introduction

Plasmodium falciparum malaria remains a critical public health challenge in Southeast Asia, and the Greater Mekong Sub-region (GMS)^{1,2} has historically been recognized as a global epicenter for the emergence and spread of antimalarial drug resistance. Resistance to chloroquine and sulfadoxine-pyrimethamine previously led to its major treatment failure across the region by the late 1990s.¹ Resistance to sulfadoxine-pyrimethamine, which was used as a replacement, emerged within a decade of its widespread appearance.^{3,4} The region now confronts its most consequential drug resistance challenge: partial

resistance to artemisinin, the pharmacological foundation of all current WHO-recommended first-line Artemisinin-based combination therapy (ACT).⁵ Molecular markers such as mutations in the *kelch13* (*K13*) propeller domain, *pfcr1*, *pfmdr1*, *pfdhfr*, and *pfdhps* genes have become important indicators for monitoring resistance patterns and therapeutic efficacy.^{2,4}

Preliminary clinical observations suggesting unusually prolonged clearance were recorded from approximately 2003 onwards.^{6,7} The first formal peer-reviewed publications confirming artemisinin partial resistance in GMS appeared in 2008 and 2009.^{6,8} These resistant par-

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asites are characterized primarily by single-nucleotide polymorphisms in the *K13* propeller domain of *P. falciparum*.^{9,10,11} Since initial documentation, *K13*-mutant parasite lineages have been confirmed in Cambodia, Myanmar, Thailand, Laos PDR, and Vietnam, and have remained in sustained circulation across the GMS.^{12,13} Despite substantial reductions in overall malaria incidence, including declines exceeding 70% in Myanmar and 90% in Vietnam,^{14,15} artemisinin-resistant lineages have not been eliminated. Reported increases in cases in certain areas of Myanmar and Cambodia demonstrate that the region has not yet achieved consistent parasite suppression,^{14,16} and this persistence directly threatens the WHO 2030 malaria elimination targets for the GMS.¹⁵

The diverse data can facilitate a precise understanding of the comparative geographical mapping of multi-drug resistance malaria within Southeast Asia. Global and regional environmental pressures challenge the organization of human populations due to administrative boundaries.^{17–20} Although most published studies have focused majorly on resistance markers, therapeutic outcomes, and drug efficacy, the existing body of evidence remains fragmented across geography, time, and methodology.²¹

Aim

This review will examine molecular marker data and phenotypic outcomes, specifically, parasite clearance half-lives and treatment failure rates, as complementary or distinct lines of evidence. Understanding the treatment failure issues, particularly the response rates for each anti-malarial drug, is regarded as critical for addressing this global health threat.^{4,15} The PRISMA-P guided systematic review protocol may address these methodological gaps by synthesizing diverse evidence to map the emergence of drug resistance.² The geographic distribution and spatio-temporal evolution in resistance patterns across Southeast Asia have not been comprehensively addressed within a single surveillance framework. Therefore, this protocol is novel in integration of molecular surveillance data, treatment outcomes, and geospatial evidence to identify regional resistance trends and surveillance gaps. The findings may also reveal methodological limitations and less explored regions, supporting future surveillance strategies and malaria elimination efforts.

Material and methods

Protocol design

This systematic review protocol will follow the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) guidelines and the Population, Exposure, Comparator, Outcome (PECO/PICO) framework, and the updated guidance of the Joanna Briggs Institute's (JBI's) critical appraisal checklist

for systematic reviews and research syntheses.²² This review is expected to systematically synthesize evidence on molecular and phenotypic antimalarial drug resistance in *P. falciparum* across Southeast Asia between January 2000 and December 2025. The protocol was not prospectively registered in PROSPERO or the Open Science Framework (OSF).

The review process will include five gradual stages: (1) identification of research questions, (2) literature search and retrieval, (3) study screening and selection, (4) data extraction and charting, and (5) evidence synthesis and reporting. A preliminary literature search was conducted between August 2025 and December 2025 to refine the search strategy, eligibility criteria, and extraction framework.

The PECO/PICO framework applied in this review is as follows:

- *Population*: Individuals of any age or gender with confirmed infection in Southeast Asia.
- *Exposure/Intervention*: Exposure to antimalarial drugs, including chloroquine, sulfadoxine-pyrimethamine, and ACTs.
- *Comparison*: Geographic regions, temporal trends, and resistance profiles across Southeast Asian countries.
- *Outcome*: Molecular resistance markers, therapeutic efficacy outcomes, treatment failure rates, and parasite clearance patterns.

Stage 1: research questions

The primary research question is, “What is the spatio-temporal distribution and prevalence of drug resistance markers, molecular and phenotypic, in *P. falciparum* malaria across Southeast Asia from 2000 to 2025?”

The following secondary questions will guide the review:

1. How many peer-reviewed articles and grey literature (e.g., WHO reports, Ministry of Health bulletins, etc.) documents on drug-resistant *P. falciparum* malaria in Southeast Asia were published between 2000 and 2025?
2. What is the geographic distribution and temporal trend of key molecular resistance markers (e.g., *K13*, *pfcr*, *pfdhfr*, *pfdhps*, *pfmdr1*) across Southeast Asian countries?
3. What are the reported therapeutic efficacy outcomes, defined as Day 28/42 treatment failure rates and parasite clearance half-lives associated with chloroquine, sulfadoxine-pyrimethamine and ACTs use in *P. falciparum* infections across Southeast Asia?
4. Which geographic areas demonstrate consistently elevated resistance marker prevalence or treatment failure rates, and how have these areas changed over the 25-year study period?

5. What are the documented methodological limitations in existing drug resistance surveillance literature, including limitations in geographic coverage, study design, marker selection, sample size, or outcome reporting standards that future research and surveillance programs need to address?

Search strategy

A comprehensive literature search will be conducted across five electronic databases: PubMed, Scopus, ScienceDirect, EBSCO, and Google Scholar. The search strategy will combine Medical Subject Headings (MeSH), free-text keywords, Boolean operators, and synonyms related to antimalarial drug resistance, molecular markers, and Southeast Asia.

The PubMed search strategy will include:
("Plasmodium falciparum" [MeSH] OR "falciparum malaria")
AND
("Drug Resistance" [MeSH] OR "antimalarial resistance" OR "treatment failure")
AND
("artemisinin" OR "ACT" OR "chloroquine" OR "sulfadoxine-pyrimethamine")
AND
("molecular markers" OR "K13" OR "pfcr" OR "pfmdr1" OR "pfdhfr" OR "pfdhps")
AND
("Southeast Asia" OR Cambodia OR Myanmar OR Thailand OR Vietnam OR Laos).

Equivalent search syntax adapted to indexing systems and operators will be applied for Scopus, ScienceDirect, EBSCO, and Google Scholar databases. Grey literature sources, including WHO reports, governmental publications, and institutional surveillance reports, will also be searched. For Google Scholar, the first 200 results with 100 pages sorted by relevance will be screened to improve feasibility and reproducibility. Only studies

published in English between January 2000 and December 2025 will be considered eligible (Fig. 1).

Specific variables used for the study

The variables will include study identification details (authors and year of publication), methodological characteristics (design type: randomized control trial, observational, molecular surveillance, and in vitro studies), geographic locations of study sites (countries/regions in Southeast Asia), demographic profiles (age groups, gender, clinical setting (outpatient/inpatient/community-based), pharmacological intervention (chloroquine, sulfadoxine-pyrimethamine, and artemisinin-based combination therapies), molecular resistance markers studied (e.g., molecular markers like *pfcr*, *pfdhfr*, *pfmdr1*, and *K13* mutations), therapeutic efficacy outcomes, and epidemiological patterns (incidence and distribution) (Table 1).

Table 1. Specific variables used for the study selection

No	Variables	Identification details
1	Study identification details	Authors, year of publication
2	Methodological characteristics	Design type: randomized control trial, observational, molecular surveillance, in vitro studies
3	Geographic locations of study sites	Countries/regions in Southeast Asia
4	Demographic profiles	Age groups, gender, clinical setting (outpatient/inpatient/community-based)
5	Pharmacological intervention	Chloroquine, sulfadoxine-pyrimethamine, and ACTs
6	Molecular resistance markers studied	<i>pfcr</i> , <i>pfdhfr</i> , <i>pfmdr1</i> , and <i>K13</i> mutations
7	Therapeutic efficacy outcomes, and epidemiological patterns	Incidence, and distribution

Stage 2: retrieving relevant studies

Authors will apply a systematic search strategy to identify scientific journals, grey literature, and peer-reviewed publications aligned with our objectives. This protocol will use a systematic approach: titles, abstracts, and key-

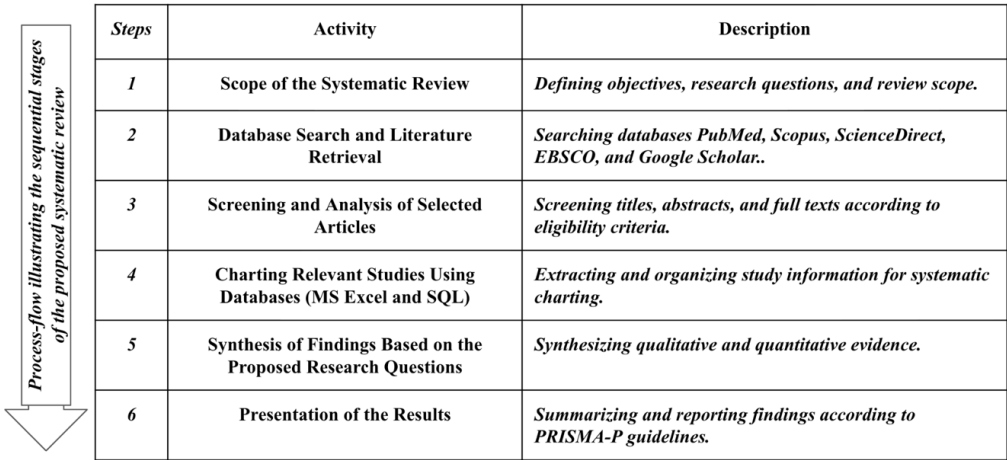


Fig. 1. Conceptual workflow of the proposed systematic review protocol for surveillance and spatial mapping of antimalarial drug resistance in Southeast Asia (2000–2025)

words for searching, screening, and reviewing the studies. Data will be extracted from the studies published between January 2000 and December 2025. In the second phase, we will import the selected list to a systematically featured database (MS Excel or Amazon Aurora DSQL) for further categorizing the references.

Stage 3: study selection

Eligibility criteria

The inclusion and exclusion criteria will be created based on the PECO/PICO framework.

Inclusion criteria

Studies will be included if they satisfy all of the following criteria:

1. This systematic review will include studies from January 2000 to December 2025 in the English language strictly. Reporting at least one of the following: (i) molecular resistance marker data (*K13* codon-specific mutations; *pfprt* K76T; *pfdhfr/pfdhps*; *pfmdr1*); (ii) therapeutic efficacy outcomes (Day 28/42 treatment failure rates, parasite clearance half-lives, or cure rates); or (iii) in vitro drug susceptibility data.
2. Participants of any age or gender with confirmed *P. falciparum* infections (microscopy, RDT, or PCR) will be included from outpatient, inpatient, or community-based clinical settings.
3. Conducted in one or more Southeast Asian countries, including the GMS region (Cambodia, Laos, Myanmar, Thailand, and Vietnam) and other Southeast Asian nations (Indonesia, Malaysia, the Philippines, Singapore, Timor-Leste, and Brunei).
4. Eligible study designs, which include cases with nonrandomized and randomized controlled trials and observational studies (cohort, cross-sectional, and case-control); reports of molecular surveillance, and in vitro susceptibility studies will be taken into account.

Exclusion criteria

1. Studies that focus on other *Plasmodium* species (*P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*) will be excluded. Studies outside Southeast Asia will be removed.
2. Studies that are focused on pathogen biology, diagnostics, or unrelated treatments related to drug-resistant malaria.

Study procedure and selection of the studies

Stage 4: charting the data

The systematic review will follow a standardized variable list using a spreadsheet. Extracted variables will include study identification details, names of the authors' details, publication years, study design, demographic details, drugs investigated, molecular re-

sistance markers, participant characteristics, and therapeutic efficacy outcomes. All retrieved references will be imported into reference management software (i.e., Zotero), and duplicate records will be removed prior to screening. Two independent reviewers will conduct title and abstract screening followed by full-text assessment according to predefined inclusion and exclusion criteria. Disagreements between reviewers will be resolved through discussion and, where necessary, consultation will be done with a third reviewer. The final systematic review will present the study selection process using a PRISMA flow diagram.

Spatial mapping

Resistance distribution maps will be produced as evidence synthesis products, generated from geospatial data extracted from the included literature and not from primary field data collection. Geospatial visualization will be conducted using QGIS (v.2025) to map study site locations and resistance marker prevalence by geographic administrative unit.¹²

Risk of bias assessment

Risk of bias will be assessed independently by two reviewers for each included study, using JBI critical appraisal tools matched to study design.²² The JBI analytical cross-sectional checklist will be applied for molecular surveillance and cross-sectional studies, the JBI prevalence checklist for epidemiological prevalence studies, and the JBI critical appraisal checklist for randomized controlled trials for interventional studies. Disagreement in risk of bias findings will be considered during evidence synthesis and interpretation of study findings.

Stage 5: collating, summarizing, and reporting the results

The purpose of the proposed systematic review is to collect existing information on the pattern and trends, summarize the findings from the included studies, and identify endemic regions that need further research. This systematic review will involve the integration of findings from the included studies and the identification of research gaps for future investigation. These will be collected and synthesized using both descriptive statistics and thematic analysis. The authors will arrange all the processed evidence after tabulation to summarize, analyze, and make a final report on the antimalarial evidence. The reporting of this systematic review will strictly follow PRISMA-P guidelines to ensure the transparency and eligibility of the study.

Ethical considerations

The present study does not require any institutional ethical approval, as it will not involve direct collection of data from human research participants.

Dissemination

This research article containing the outcomes of the systematic review will be submitted to a scientific journal for publication. Study findings will be disseminated via open-access publications in peer-reviewed journals. The findings will be communicated through presentations, relevant meetings, conferences, seminars, and workshops. This review will aim to inform regional and global strategies for the prevention and containment of drug-resistant malaria in endemic regions.

Evidence extraction and analysis

This review will identify studies from January 2000 until December 2025. A search will be conducted for studies that are eligible after retrieving and evaluating the published articles across five major databases: PubMed, Google Scholar, ScienceDirect, EBSCO, and Scopus. The final result with data synthesis and mapping will be presented using PRISMA-P guidelines in the completed systematic review.

Discussion

Resistance to the world's most effective antimalarial drug, artemisinin, and its derivatives (i.e., ACTs) has emerged as a serious threat to global malaria control efforts.^{13,20} This systematic review protocol maps the existing evidence regarding *P. falciparum* resistance to drug patterns across Southeast Asia's GMS using the PICO/PECO framework [Population: Southeast Asian adult population of any age or gender with confirmed *P. falciparum* infection; Exposure/Intervention: antimalarial drugs; Comparison: across Southeast Asia's Greater Mekong Sub-region, 2000-2025; Outcome: Multidrug resistance effect intervention]. By employing a comprehensive search strategy across five databases and grey literature sources, this protocol addresses identified gaps in synthesizing heterogeneous evidence from molecular surveillance and epidemiological studies regarding the key resistance markers, including *pfprt*, *pfdhfr*, *pfdhps*, *pfmdr1*, and *Pfkelch13* mutations.^{23,24} Post-pandemic interruptions in malaria surveillance systems, healthcare accessibility, and therapeutic monitoring may have significantly contributed to gaps in resistance surveillance across endemic Southeast Asian regions.²⁵

Expected contribution of the protocol

This systematic review protocol aims to investigate the incidence and distribution of drug resistance as well as challenges related to drug-resistant *P. falciparum* malaria in endemic regions of Southeast Asia. The severity and mortality of malaria caused by *P. falciparum* are considered crucial public health issues in selected regions of Southeast Asia, including the GMS, despite the rapidly declining rate of malaria cases. As a global epicentre for multi-drug-resistant malaria, especially against arte-

misinin-based therapies, it requires focused, long-term, sustained attention and effective interventions.

Conclusion

Antimalarial drug resistance in *Plasmodium falciparum* remains a major hindrance to malaria eradication policies in Southeast Asia, particularly within the GMS. This systematic review protocol provides a structured framework to synthesize evidence on the spatial and temporal distribution of molecular resistance markers and therapeutic efficacy outcomes reported between 2000 and 2025. By integrating molecular, phenotypic, and geospatial evidence under PRISMA-P guidelines, the review aims to identify resistance zones, surveillance gaps, and multidrug-resistant malaria trends across the region. The findings are expected to support evidence-based treatment policies, strengthen regional surveillance systems, and contribute to malaria elimination strategies in Southeast Asia.

Declarations

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Author contributions

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Conflicts of interest

The authors declare there is no conflict of interest.

Data availability

The datasets generated and analysed during the current study will be available from the corresponding author on reasonable request.

Ethics approval

This systematic review protocol did not involve direct human participation or interaction; hence, ethics approval and consent to participate were not required. Despite this, the study adhered to all institutional protocols (both nationally and internationally) and made use of best practices throughout the study selection, review, and reporting processes when conducting the analysis on the studies included in this proposed systematic review.

References

1. Hyde JE. Drug-resistant malaria - an insight. *FEBS J.* 2007;274(18):4688-4698. doi:10.1111/j.1742-4658.2007.05999.x

2. Amato R, Pearson RD, Almagro-Garcia J, et al. Origins of the current outbreak of multidrug-resistant malaria in southeast Asia: a retrospective genetic study. *Lancet Infect Dis.* 2018;18(3):337–345. doi:10.1016/S1473-3099(18)30068-9
3. Li Q, Liu T, Lv K, et al. Malaria: past, present, and future. *Signal Transduct Target Ther.* 2025;10:188. doi:10.1038/s41392-025-02246-3
4. World Health Organization. Status Report on Artemisinin Resistance and ACT Efficacy. World Health Organization; 2018.
5. MalariaGEN Plasmodium falciparum Community Project. Genomic epidemiology of artemisinin-resistant malaria. *eLife.* 2016;5:e08714. doi:10.7554/eLife.08714
6. Noedl H, Se Y, Schaefer K, et al. Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med.* 2008;359(24):2619–2620. doi:10.1056/NEJMc0805011
7. Hassett MR, Roepe PD. Origin and spread of evolving artemisinin-resistant *Plasmodium falciparum* malarial parasites in Southeast Asia. *Am J Trop Med Hyg.* 2019;101(6):1204–1211. doi:10.4269/ajtmh.19-0379
8. Dondorp AM, Nosten F, Yi P, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med.* 2009;361(5):455–467. doi:10.1056/NEJMoa0808859
9. Arieu F, Witkowski B, Amaratunga C, et al. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature.* 2014;505(7481):50–55. doi:10.1038/nature12876
10. Wang Z, Shrestha S, Li X, et al. Prevalence of K13-propeller polymorphisms in *Plasmodium falciparum* from China-Myanmar border in 2007–2012. *Malar J.* 2015;14:168. doi:10.1186/s12936-015-0672-9
11. Xie SC, Ralph SA, Tilley L. K13, the cytosome, and artemisinin resistance. *Trends Parasitol.* 2020;36(6):533–544. doi:10.1016/j.pt.2020.03.006
12. Imwong M, Dhorda M, Tun KM, et al. Molecular epidemiology of resistance to antimalarial drugs in the Greater Mekong Subregion: an observational study. *Lancet Infect Dis.* 2020;20(12):1470–1480. doi:10.1016/S1473-3099(20)30228-0
13. White NJ, Chotivanich K. Artemisinin-resistant malaria. *Clin Microbiol Rev.* 2024; 37(4):e0010924. doi:10.1128/cmr.00109-24
14. Landier J, Parker DM, Thu AM, et al. Effect of generalised access to early diagnosis and treatment and targeted mass drug administration on *Plasmodium falciparum* malaria in Eastern Myanmar: an observational study of a regional elimination programme. *Lancet.* 2018;391(10133):1916–1926. doi:10.1016/S0140-6736(18)30491-4
15. World Health Organization. *Mekong Malaria Elimination Programme Epidemiology Summary.* Vol 29. World Health Organization; 2025.
16. Pley C, Gibbs H, Sudathip P, et al. Impact of the Global Fund Regional Artemisinin-resistance Initiative on malaria control and elimination in the Greater Mekong Subregion of Southeast Asia. *Lancet Infect Dis.* 2025;25(6):e353–e359. doi:10.1016/S1473-3099(25)00013-1
17. Edwards H, Canavati SE, Rang C, et al. Novel cross-border approaches to optimise identification of asymptomatic and artemisinin-resistant *Plasmodium* infection in mobile populations crossing Cambodian borders. *PLoS One.* 2015;10(9):e0124300. doi:10.1371/journal.pone.0124300
18. Chigozie VU, Aniokete CU, Ogbonna PI, et al. Transforming antimicrobial resistance mitigation: the genomic revolution in one health and public health. *Discov Appl Sci.* 2025;7(10):1187. doi:10.1007/s42452-025-07053-7
19. Hamilton WL, Amato R, van der Pluijm RW, et al. Evolution and expansion of multidrug-resistant malaria in Southeast Asia: a genomic epidemiology study. *Lancet Infect Dis.* 2019;19(9):943–951. doi:10.1016/S1473-3099(19)30392-5
20. Balmer AJ, White NFD, Ünlü ES, et al. Understanding the global rise of artemisinin resistance: insights from over 100,000 *Plasmodium falciparum* samples. *eLife.* 2025;14:e105544. doi:10.7554/eLife.105544
21. Weiss DJ, Dzianach PA, Saddler A, et al. Mapping the global prevalence, incidence, and mortality of *Plasmodium falciparum* and *Plasmodium vivax* malaria, 2000–2022: a spatial and temporal modelling study. *Lancet.* 2025;405:979–990. doi:10.1016/S0140-6736(25)00038-8
22. Joanna Briggs Institute. Critical Appraisal Checklist for Systematic Reviews and Research Syntheses. Joanna Briggs Institute; 2017.
23. Mayxay M, Barends M, Brockman A, et al. In vitro antimalarial drug susceptibility and pfprt mutation among fresh *Plasmodium falciparum* isolates from the Lao PDR (Laos). *Am J Trop Med Hyg.* 2007;76:245–250.
24. Habarugira F, Batamuriza J, Ndahimana R, et al. The global landscape of *Plasmodium falciparum* drug resistance markers, 2005–2025: a systematic review and meta-analysis. *Pathogens.* 2026;15(2):179. doi:10.3390/pathogens15020179
25. Miller MA, Astuti R, Hirsch P, et al. Selective border permeability: governing complex environmental issues through and beyond COVID-19. *Polit Geogr.* 2022;97:102646. doi:10.1016/j.polgeo.2022.102646



REVIEW PAPER

The eye in the era of GLP-1 receptor agonists – a narrative review of current evidence

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ABSTRACT

Introduction and aim. Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are widely used in the treatment of type 2 diabetes and obesity. However, their effects on the visual system remain unclear. This narrative review summarizes current evidence and aims to clarify the association between GLP-1RAs and ocular outcomes.

Literature search. A literature search was conducted using PubMed/MEDLINE, Embase, and ScienceDirect electronic databases. Findings were grouped into thematic categories: diabetic retinopathy, NAION, AMD and other ocular events, and glaucoma.

Analysis of the literature. The available evidence is heterogeneous and sometimes conflicting. GLP-1RAs may be associated with diabetic retinopathy progression, particularly in patients with pre-existing type 2 diabetes, likely related to rapid glycaemic improvement rather than direct drug toxicity. Reports of NAION remain rare, and causality has not been definitively established. The reviewed studies also indicate that GLP-1RAs may reduce AMD risk. Preclinical and observational studies suggest possible neuroprotective and intraocular pressure-lowering effects in glaucoma, while clinical evidence remain limited.

Conclusion. Current evidence does not indicate a consistent increase in major ophthalmic adverse outcomes associated with GLP-1RAs use. However, evidence remains limited and largely observational. Careful ophthalmic assessment and follow-up may be considered in high-risk individuals. Further prospective studies are needed to clarify causal relationships.

Keywords. age-related macular degeneration, diabetic retinopathy, glaucoma, glucagon-like peptide-1 receptor agonists, non-arteritic ischemic optic neuropathy, type 2 diabetes mellitus

Introduction

Glucagon-like peptide-1 receptor agonists (GLP-1RAs) have become important therapeutic agents in the management of type 2 diabetes and obesity, due to their high efficacy in glycemic and weight control.¹ By mimicking the action of endogenous glucagon-like peptide-1, they stimulate insulin secretion in pancreatic β -cells, leading to a reduction in blood glucose levels, as well as influence neurons in the central appetite-regulating pathways, thus reducing appetite.²

Their high clinical effectiveness and proven cardiovascular, renal, and metabolic benefits have resulted in widespread use of GLP-1RAs among various patient populations.^{3–6} In 2024, over 26% of US adults with diabetes reported using injectable GLP-1RAs and 1 in 8 adults in the US report having used GLP-1RAs medication.^{7,8} Due to increasing use of GLP-1RAs, evaluating long-term safety, beyond known metabolic endpoints, including potential visual effects, has become an important clinical and research priority.^{9,10}

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Historically, exenatide was the first GLP-1RA medication approved by the FDA as adjunctive therapy to improve glycemic control in patients with type 2 diabetes mellitus in 2005. Other GLP-1RAs have since entered the market, including liraglutide, dulaglutide, lixisenatide, semaglutide, alongside the dual GIP/GLP-1RA, tirzepatide. As further research progressed, new indications for the use of incretin-based therapies have emerged. Currently, most are approved for glycemic control and to reduce the risk of major cardiovascular events in patients with type 2 diabetes mellitus. Additionally, three of them are also registered for weight loss, including liraglutide, semaglutide, and tirzepatide.

In recent years, researchers have reported various possible links between GLP-1RAs and ocular safety.¹¹ However, recent studies regarding GLP-1RAs use and occurrence of non-arteritic anterior ischemic optic neuropathy have substantially expanded interest in the systemic effects of incretin-based therapies. Emerging data are, however, sometimes conflicting. These opposing signals have contributed to ambiguity in ophthalmological recommendations and underscore a critical research gap.

Additionally, ocular health is a crucial area, especially in patients with metabolic disorders who are inherently predisposed to microvascular and neurovascular eye complications, including diabetic retinopathy and ischemic optic nerve disorders, and even subtle drug-related effects may carry important clinical consequences. This highlights the need to explore potential relationships between GLP-1RAs and ophthalmological aspects. Identifying clinically relevant domains may help integrate ophthalmic considerations into guidelines for GLP-1RAs use, thereby enhancing both safety and efficacy of these agents in routine clinical practice.

Although several reviews addressing ophthalmic outcomes of GLP-1RAs have been published recently, the rapidly evolving literature in this field warrants continual reassessment of the available evidence. In contrast to earlier reviews, the present review incorporates studies published through 2026, including recently reported ophthalmic safety signals and comparative analyses involving newer incretin-based therapies such as tirzepatide. Importantly, this review provides a broad overview of ocular outcomes associated with GLP-1RAs, including retinal, neuro-ophthalmic, glaucomatous, and ocular surface disorders rather than focusing on a single complication or individual agent. The review also emphasizes the hierarchy and limitations of available evidence. Additionally, mechanistic and clinical data are discussed separately to improve evidence interpretation. Given the rapidly growing global use of GLP-1RAs and increasing number of reported ocular outcomes, an updated overview of the evidence remains clinically relevant.

Aim

This narrative review aims to critically evaluate current evidence on the association between GLP-1RAs and ocular outcomes, including diabetic retinopathy, non-arteritic anterior ischemic optic neuropathy, age-related macular degeneration and other structural or functional eye changes, along with the neuroprotective and anti-inflammatory role of GLP-1RAs, particularly in the context of glaucoma. This review strives to clarify the clinical relevance of reported associations and identify the gaps requiring further investigation.

Literature search

This article is a narrative review of the available literature concerning the influence of GLP-1RAs on the visual system. A structured literature search was conducted using PubMed/MEDLINE, Embase and ScienceDirect electronic databases for studies published up to 22 December 2025. To ensure the currency of findings and address the rapidly evolving nature of the literature in this field, an updated search was performed in May 2026. The search methodology combined Medical Subject Headings (MeSH) with relevant free-text terms, including: (“glucagon-like peptide-1” OR “glucagon-like peptide-1 receptor agonists” OR GLP-1RA OR semaglutide OR liraglutide OR dulaglutide OR exenatide OR tirzepatide) AND (“eye diseases” OR retinopathy OR “diabetic retinopathy” OR “optic neuropathy” OR NAION OR “age-related macular degeneration” OR “macular edema” OR AMD OR retina OR papilledema OR glaucoma OR “dry eye” OR “intraocular pressure” OR eye OR ocular OR ophthalmology OR vision OR “visual impairment”).

The combined initial and updated searches yielded 2,409 records. After removing duplicates, 1,565 titles and abstracts were screened, resulting in 150 full-text articles assessed for eligibility. Furthermore, manual screening of reference lists and reassessment of recently published and previously overlooked relevant literature were performed to identify additional studies not captured during the initial database search. Ultimately, 80 studies were included in this review.

Studies were eligible for inclusion if they addressed visual or ocular outcomes associated with the use of GLP-1RAs in the adult human population or explored potential mechanisms of these effects using experimental or animal models. Only articles written in English and published in peer-reviewed journals were included in the analysis. Due to the limited availability of high-quality randomized evidence, a broad range of study designs with varying levels of evidence was included, comprising randomized clinical trials, cohort studies, case-control studies, pharmacovigilance analyses, case reports, and reviews. Preclinical findings were analyzed separately from clinical data, particularly in sections addressing potential biological mechanisms.

Data extraction focused on the type of GLP-1RAs, study design, study population, reported ocular outcomes, relationships between drug exposure and visual findings, potential confounders, and key study limitations. The results were synthesized qualitatively and organized into thematic categories, including diabetic retinopathy (DR), non-arteritic anterior ischemic optic neuropathy (NAION), age-related macular degeneration (AMD) and other ocular events, as well as glaucoma. Given the narrative nature of the review, no formal risk-of-bias assessment or quantitative synthesis was performed. Differences between studies were discussed in the context of study design, evidence level and clinical context.

Analysis of the literature

Diabetic retinopathy

Diabetic retinopathy (DR) is a microvascular complication of diabetes mellitus that may lead to vision-threatening damage to the retina, eventually leading to blindness.¹² The relationship between GLP-1RAs and DR remains complex and is not fully consistent across studies.

Evidence from preclinical studies suggests a potential protective effect. Dulaglutide has been shown to improve retinal structure and vascular function in experimental mouse models with type 2 diabetes (T2D).¹³ While preclinical models suggest potential benefits at the retinal level, bridging the translational gap to human clinical practice remains challenging, reflecting a multifaceted clinical reality.

Evidence from randomized clinical trials (RCTs) did not consistently support a direct association. A meta-analysis of 61 RCTs (n=188,463) found no significant difference in risk of DR between GLP-1RAs and comparator therapies.¹⁴ In contrast, a second meta-analysis including 13 RCTs indicated possible association with DR worsening. Yoshida et al. identified a higher incidence of rapidly worsening DR in four major RCTs with CV benefits in T2D. The significant association between GLP-1RAs and DR was also observed in subgroups of RCTs with a duration exceeding 52 weeks and when compared with placebo. A similar association was observed in patients with diabetes treated for ≥ 10 years, however it did not reach statistical significance. The presented associations were observed for liraglutide, semaglutide and dulaglutide.¹⁵

Observational studies in humans provide heterogeneous findings. A Swedish cohort study of patients with incident T2D reported a lower incidence of DR among GLP-1RA-treated patients compared with the non-exposed group (5.97 vs 12.85 per 1000 person-years).¹⁶ Similarly, a retrospective cohort study in Saudi Arabia suggested improvement in DR status in patients treated with liraglutide. However, the subgroup represented only a minority of the total population, and most pa-

tients showed no change in retinopathy status.¹⁷ Furthermore, recent population based study observed that tirzepatide was associated with a lower incidence of new or progressive DR and fewer complications.¹⁸ Another retrospective observational study of adults with T2D and moderate cardiovascular risk, without history of advanced diabetic retinal complications, focused on comparing four antidiabetic medications groups in terms of the risk of DR at 2 and 5 years of follow-up. GLP-1RAs treatment was associated with a comparable risk of vision-threatening retinopathy compared with DPP-4is and sulphonylureas, whereas SGLT2is therapy was associated with lower risk.¹⁹ Additionally, in a separate cohort study of 981 patients, GLP-1RAs were not associated with DR progression compared with SGLT2is.²⁰

Conversely, other large observational analyses reported an increased incidence of DR among patients receiving GLP1-RAs.^{21,22} Wai et al. also observed a higher rate of progression of proliferative diabetic retinopathy after 1 and 3 years in a GLP1-RAs cohort compared with SGLT2i-treated patients.²³ Similarly, increased risk of progression of pre-existing DR was reported in patients receiving GLP-1RAs in additional cohort study, with tractional retinal detachment identified as a major driver of this association.²⁴ A small cohort study, consisting of 4,500 patients with DR, observed DR progression in 3 of 19 treated with GLP-1RAs, whereas no progression was noted in the control group.²⁵

An increase in the risk of DR complications compared to placebo was also observed in SUSTAIN-6, a pre-registration trial of cardiovascular outcomes. Importantly, this signal was not observed across the SUSTAIN 1 to 5 and Japanese trials. Furthermore, the SUSTAIN-6 findings were not replicated in the cardiovascular outcome trials of liraglutide (LEADER) or dulaglutide (REWIND), nor in the PIONEER 6 trial for oral semaglutide. The results of the SUSTAIN-6 study were attributed to the magnitude and rapidity of HbA1c reduction during the first 16 weeks of treatment, particularly in patients with pre-existing DR and poor glycemic control which may partially explain discrepancies in the results.²⁶ To address these discrepancies, the ongoing prospective FOCUS trial (NCT03811561) is specifically investigating the long-term effects of semaglutide on DR.

Consistent with this interpretation, Berkovic and Strollo, analyzing the effect of semaglutide on patients with diabetes and obesity, suggested that DR events observed with semaglutide therapy may be related with rapid glycemic improvement rather than a direct drug-related effect.²⁷ Additionally, comparative analyses between different GLP-1RAs representatives (liraglutide, dulaglutide, exenatide) did not demonstrate significant differences in DR risk.²⁸ The significance of the route of administration was also evaluated, with higher

rate of retinal adverse events, including DR, reported after subcutaneous compared with oral semaglutide.²⁹

Overall, the available evidence, largely derived from observational studies with inconsistent findings, suggests heterogeneous effects of GLP-1RAs on DR and may depend on baseline patient characteristics, glycaemic control and study design.

Heterogeneity in DR definitions, study design, and lack of standardized ophthalmic endpoints likely contributed to the inconsistent findings across studies. Pre-clinical studies focused mainly on precise molecular and retinal structural changes, whereas clinical studies evaluated heterogeneous outcomes, including incident DR, DR progression, proliferative or vision-threatening DR, retinal adverse events and need for retinal interventions. These methodological differences limit direct comparison and interpretation of the available evidence.

Non-arteritic anterior ischemic optic neuropathy

Non-arteritic anterior ischemic optic neuropathy (NAION) is characterized by acute ischemic damage of the optic nerve head resulting in abrupt, painless, usually unilateral vision loss and optic disc edema.^{30,31} The potential association between the use of GLP-1RAs and NAION has recently attracted attention. However, evidence remains inconsistent and methodologically limited.

Evidence from randomized clinical trials is limited and does not support a clear association between GLP-1RA use and NAION. A meta-analysis by Silverii et al. assessed available RCTs and found no significant difference in NAION risk between patients treated with GLP-1RAs and comparator groups.³² Recent meta-analysis including >1.5 million patients, reported the association between GLP-1RAs and elevated NAION risk (OR 1.70), however the absolute risk difference remained low (0.037%).³³ Furthermore, a dedicated safety evaluation of placebo-controlled clinical trials, demonstrated comparable NAION incidence between GLP-1RAs and placebo groups.³⁴ However, it should be explicitly stated that NAION is a rare outcome and was not a primary endpoint in any of the analyzed RCTs, limiting their statistical power to detect such events.

Observational studies have reported signals suggesting a possible association, although their findings are heterogeneous. The issue was first brought to public attention by Hathaway et al. in a retrospective cohort study including patients with T2D or obesity treated with semaglutide, without a prior history of NAION. The study reported a higher incidence of NAION among semaglutide-treated patients compared with individuals receiving other antidiabetic or anti-obesity medications.³⁵ Similarly, a large Danish nationwide cohort study including all individuals with T2D in Denmark (n=424,152) observed more than a twofold increase in

NAION risk after five years of semaglutide use. Moreover, the study noted that the record-high incidence of first-time NAION episodes in 2019-2023 was temporally coincident with the introduction of semaglutide on the Danish market in November 2018.³⁶ These outcomes were mirrored in a study comparing semaglutide with SGLT2is, which included data from national health registers in Denmark and Norway, where the final pooled hazard ratio was 2.81.³⁷ Similarly, a recent large cohort study among US Veterans demonstrated an increased NAION risk among semaglutide initiators compared with SGLT2is users.³⁸ This trend was further reflected in a retrospective cohort study concerning patients with T2D initiated on GLP-1RA therapy compared with DPP-4is-treated patients.³⁹ Notably, data regarding the temporal relationship, indicates that the timing and duration of GLP-1RAs treatment may significantly influence clinical outcomes.⁴⁰ This aspect emphasizes the complexity of the association and potentially explains the discrepant findings across previous literature, as different studies varied in drug exposure definition and the specific treatment timelines. Importantly, despite hazard ratios of concern reported in several studies, the absolute incidence increase remains low.

A multi-database study, including 37.1 million individuals with T2D, found only a slight increase in NAION risk associated with semaglutide exposure in a self-controlled case series analysis. Notably, the hazard ratio for NAION was higher only when compared to empagliflozin and showed no difference to any other non-GLP-1RAs interventions when a sensitive NAION definition was applied.⁴¹ In other retrospective cohort study including patients with T2D without a known history of any eye disorder, the incidence of NAION was higher in semaglutide and tirzepatide cohort compared with other antidiabetic drugs, however the absolute risk remained very low (35 patients [0.04%] vs 19 patients [0.02%], HR 1.76).⁴² This is further illuminated by other large-scale global and multi-databases cohort studies, which reported only a modest increased risk of NAION associated with GLP-1RAs exposure.⁴³⁻⁴⁵ Consistently, a real-world cohort analysis incorporating sensitivity analyses did not confirm a statistically significant increase in risk after adjustment for confounders.⁴⁶ Similarly, Ramsey et al. observed no statistically significant change in NAION risk among patients receiving GLP-1RAs therapy in their cohort study.²¹ Regional real-world data from a Polish cohort did not observe an increased risk of NAION associated with GLP-1RAs therapy, suggesting that risk signals diminish after rigorous adjustment for baseline metabolic parameters.⁴⁷

Crucially, recent retrospective cohort analysis included over 1.2 million individuals. After adjustment for comorbidities, the authors observed significantly

decreased risk of NAION in patients with T2D treated with semaglutide (OR=0.36).⁴⁸

Pharmacovigilance data from the FAERS database have suggested higher risk of NAION associated with semaglutide use compared with other antidiabetic or anti-obesity medications.⁴⁹ However, such reporting systems are inherently limited by underreporting, reporting bias and incomplete clinical information, thus restricting the ability to establish a definitive association.

Case reports provide an additional signal but represent the lowest level of clinical evidence. Several case reports describe cases of NAION associated with GLP-1RAs therapy.^{50–53} These include a case of 47-year-old woman without known risk factors and with a BMI=27.92 who developed NAION after GLP-1RAs therapy, including liraglutide and semaglutide, for weight loss. The temporal relationship between drug initiation and disease onset and clinical course, suggested a potential role of GLP-1RAs therapy in the development and progression of NAION.⁵¹ However, such reports are inherently limited by the absence of a control group and are insufficient to confirm a direct drug-related effect. Additionally, case reports often concern patients with established risk factors, such as hypertension and hyperlipidemia⁵⁰ or anatomical predispositions like small Bruch's membrane opening diameter and crowded optic disc.⁵²

Glaucoma - a neuroprotective and anti-inflammatory role of GLP-1RAs

Evidence regarding GLP-1RAs and glaucoma is derived primarily from observational studies, with limited high-quality clinical data. Current evidence may be broadly divided into observational studies evaluating glaucoma incidence, clinical studies assessing intraocular pressure (IOP) and mechanistic and preclinical studies. Available cohort analyses suggest that GLP-1RAs use may be associated with reduced risk of glaucoma development.^{54–59} However, these findings should be interpreted with caution, as they are subject to residual confounding.

Clinical incidence data

Some observational studies reported a greater risk reduction with GLP-1RAs compared with other antidiabetic medications.^{54–56} Importantly, a nationwide study observed that prolonged GLP-1RA treatment extending beyond 3 years further reduced the risk.⁵⁹ No significant influence of ethnicity, race, sex, education or income on the association between GLP-1RAs use and glaucoma risk was established, although greater risk reduction was observed in patients aged >60 years and in those with coexisting T2D.⁵⁷ Furthermore, more recent evidence has also evaluated tirzepatide, a dual GIP/GLP-1 receptor agonist. Retrospective cohort analyses suggested that

tirzepatide treatment was associated with lower rates of primary open-angle glaucoma and ocular hypertension compared with selective GLP-1RAs.^{60,61} However, these findings remain observational and require prospective confirmation.

Intraocular pressure (IOP) data

Reduction of intraocular pressure (IOP) is one of the most frequently proposed mechanisms. In observational analyses, Hallai et al. reported that GLP-1RAs were associated with greater IOP reduction compared with other antidiabetic medications, with more pronounced effect in patients with baseline IOP ≥ 15 mmHg and in those with pre-existing glaucoma. Additionally, no significant difference between the injectable and oral form of semaglutide was observed.⁶²

Mechanistic and preclinical evidence

Beyond IOP reduction, experimental studies have suggested that GLP-1RAs exert anti-inflammatory and neuroprotective effects on retinal ganglion cells (RGCs), which are progressively lost in glaucoma. Elevated IOP was associated with increased levels of pro-inflammatory cytokines, including C1q, TNF- α , IL-1 α , which may contribute to neurotoxic astrocyte activation. It was also observed that reducing IOP to physiological ranges did not restore cytokines levels to baseline. In preclinical models, GLP-1RAs such as NLY01 were found to reduce cytokine levels by modulating microglial cells activity (responsible for C1q, TNF- α and IL-1 α production).⁵⁸

Additional *in vitro* and animal studies, have suggested that high glucose conditions induces mitochondrial damage and increased reactive oxygen species overproduction, driving RGCs degeneration through mitophagy, which plays a crucial role in neurodegenerative disorders, including glaucoma.^{63,64,65} Experimental studies suggested that GLP-1RAs may mitigate the ultrastructural mitochondrial damage and reduce levels of glucose-induced ROS.⁶⁶ Additionally, both *in vitro* and *in vivo* diabetic rat models demonstrated that GLP-1RAs improved RGC viability by attenuating excessive mitophagy, specifically through down-regulation of LC3A/B, increased p62 levels and inhibition of the PINK1/Parkin pathway.⁶⁵ Further mechanistic evidence from experimental models suggests that semaglutide may protect RGCs against neurodegenerative injury through modulation of glucose metabolism and mitochondrial homeostasis.⁶⁷ However, these findings derive from preclinical studies and their clinical relevance remains uncertain.

Overall, while observational and preclinical evidence suggests a potential protective role of GLP-1RAs in glaucoma, clinical evidence remains limited and does not allow definitive association.

AMD and other ocular events

Reported ocular manifestations associated with GLP-1RAs may be broadly categorized into retinal and macular disorders, ocular surface disorders, and neuro-ophthalmic or inflammatory findings.

Retinal and macular disorders

Age-related macular degeneration (AMD) is a neuro-degenerative disorder and one of the leading causes of vision loss in individuals over 60. It progressively damages the central retina and choroid, leading to central vision impairment and, in advanced stages, severe visual loss.^{68,69}

The potential impact of GLP-1RAs on AMD has been explored, given the growing prevalence and public health impact.⁷⁰ These findings are derived exclusively from observational studies and therefore do not allow definitive conclusions. Some cohort analyses suggest a protective association. In a recent retrospective cohort study of non-diabetic adults receiving GLP-1RAs for obesity treatment, exposure to GLP-1RAs was associated with a lower risk of both nonexudative and overall AMD compared with other weight-loss pharmacotherapies.⁷¹ McLaughlin et al. reported that in patients with T2D, GLP-1RAs therapy was associated with a relative risk reduction of 15% in nonexudative and 20% in exudative AMD, as well as lower likelihood of progression to intermediate or advanced stages compared with other glucose-lowering therapies (27–29%).⁷² Similar findings were reported in other cohort studies, with stronger associations observed with longer duration of exposure.^{73,74} However, these studies are limited by their observational design and differences in baseline metabolic and vascular risk profiles.

In contrast, another population-based study identified an approximately two-fold increased risk of neovascular AMD among patients receiving GLP-1RAs in a large cohort study. Although this study accounted for several comorbidities and socioeconomic factors, the authors noted that the underlying mechanisms remain unclear, highlighting the need for further research.⁷⁵ However, this finding was not replicated in more recent large-scale cohort analyses, which did not confirm an increased risk of neovascular AMD.⁷⁶

Additional evidence from pharmacovigilance systems, including FAERS and VigiBase, has reported signals of various vitreoretinal and ocular adverse events temporally associated with GLP-1RAs use, including retinal hemorrhage, vitreous hemorrhage, vitreous detachment, retinal detachment, papilledema and macular edema and macular hole, compared with metformin.⁷⁷

Observational evidence regarding diabetic macular edema (DME) remains inconsistent. Wai et al. suggested an increased incidence of DME among patients receiving GLP-1RAs at multiple follow-up time points.²³ How-

ever, large database analyses including approximately two million patients with T2D did not confirm an increased risk of DME associated with GLP-1RAs therapy after adjustment for confounders, while SGLT2is were associated with a reduced risk.²² Similar neutral findings were further reported by Phu et al. in comparative analyses of glucose-lowering therapies.⁷⁸

Ocular surface disorders

Evidence regarding dry eye disease (DED) is also inconsistent. A large retrospective cohort study conducted by Su et al. reported a lower risk of clinically diagnosed DED in patients treated with SGLT2is compared with GLP-1 RAs, based on electronic medical records and prescription records for dry eye medications.⁷⁹ By comparison a small prospective study reported short-term improvements in tear film stability and tear production in GLP-1RA-treated patients. However, the study was limited by small sample size ($n = 35$) and short follow-up duration and did not assess clinically diagnosed DED incidence.⁸⁰ These discrepancies likely reflect differences in study design and outcome definitions.

Neuro-ophthalmic and inflammatory findings

Rare ocular events, including diabetic papillitis, papilledema, optic neuritis, scleritis, and reversible bilateral central scotomas, were described, sometimes coinciding with rapid glycemic improvement.^{53,81,82} However, these events were more likely related to underlying diabetic eye disease, rapid metabolic changes, or detection bias rather than a direct pharmacologic effect of GLP-1RAs. Conversely, a large retrospective cohort study involving over 36 000 patients treated with various GLP-1RAs reported a low incidence of papilledema and optic neuritis, without demonstrating a clear association with GLP-1RA therapy.⁴⁷ Overall, these findings suggest that currently available evidence regarding the above-mentioned neuro-ophthalmic adverse events associated with GLP-1RAs remains inconsistent, and no definitive association has been established.

A detailed summary of key studies evaluating ocular outcomes associated with GLP-1RAs was presented in Table 1.

Discussion

Diabetic retinopathy

DR remains a leading cause of vision loss in both working-age and older populations, and is strongly associated with longer diabetes duration, suboptimal glycemic control, and coexisting hypertension.⁸³

Evidence regarding the association between GLP-1RA therapy and DR is inconsistent across study designs. While several observational studies reported a possible increased risk of DR, randomized clinical trials and meta-analyses have not confirmed a consistent

Table 1. Summary of selected studies on ocular outcomes associated with GLP-1 receptor agonists*

Study	Design	Population	Exposure/comparator	Outcome	Main results
Diabetic Retinopathy					
Majszczak et al., 2024 ⁴	Systematic review and meta-analysis	T2D (n=188,463)	GLP-1RA vs placebo	DR	OR 1.08 (95% CI 0.94–1.23)
Yoshida et al., 2022 ⁵	Systematic review and meta-analysis (13 RCTs)	T2D	GLP-1RA vs other antidiabetic drugs or placebo	Incident DR/DR complications	RCIs with CV benefits in T2D: OR 1.23 (95% CI 1.05–1.44) RCIs > 52 weeks: OR 1.2 (95% CI 1.00–1.43) RCIs using placebo as a comparator: OR 1.22 (95% CI 1.05–1.42)
Zheng et al., 2023 ⁶	Cohort with Mendelian randomization	T2D (n=14,119)	GLP-1RAs vs metformin or sulfonylureas	DR	HR 0.42 (95% CI 0.29–0.61)
Shah et al., 2026 ⁸	Retrospective cohort	T2D/obesity (n=173,846)	Tirzepatide vs no exposure to weight-loss drugs	Mild NPDR	RR 0.864 (95% CI 0.758–0.985, p=0.029)
				PDR	RR 0.705 (95% CI 0.564–0.882, p=0.002)
				Moderate/severe NPDR	No statistical significance
Barkmeier et al., 2024 ⁹	Retrospective cohort	T2D with moderate CV risk (n=371,698)	GLP-1RA vs DPP-4i	Treatment for DR (DME/PDR)	HR 1.07 (95% CI 0.85–1.35)
			GLP-1RA vs sulfonylurea		HR 0.83 (95% CI 0.67–1.03)
			SGLT2i vs GLP-1RA		HR 0.73 (95% CI 0.55–0.97)
Ramsey et al., 2025 ²¹	Retrospective cohort	T2D (n=185,066)	GLP-1RA vs non-exposed	DR	HR 1.07 (95% CI 1.03–1.11; p=0.001)
Eleftheriadou et al., 2024 ²²	Retrospective cohort	T2D (n=2,305,755)	GLP-1RA + insulin vs insulin	DR	HR 1.308 (95% CI 1.261–1.357)
			SGLT2i + insulin vs insulin	DME	HR 1.013 (95% CI 0.960–1.069)
				DR	HR 1.076 (95% CI 1.027–1.127)
				DME	HR 0.835 (95% CI 0.780–0.893)
			GLP-1RA + insulin vs SGLT2i + insulin	DR	HR 1.205 (95% CI 1.153–1.259)
				DME	HR 1.130 (95% CI 1.056–1.208)
Wai et al., 2024 ²³	Retrospective cohort	NPDR (n=12,962)	GLP-1RAs vs SGLT2is	Progression to proliferative DR	after 1 year of therapy: RR 1.26 (95% CI 1.04–1.51, p=0.017) after 3 years: RR 1.284 (95% CI 1.1–1.499, p=0.002)
Lin et al., 2024 ²⁴	Retrospective cohort	Pre-existing DR (n=4,551) Without pre-existing DR (n=28,647)	GLP-1RA vs SGLT2i	New-onset PDR, vitreous hemorrhage, tractional RD	HR 1.50 (95% CI 1.01–2.23; p=0.043) (mainly increased risk of tractional RD)
				Newly diagnosed DR, vitreous hemorrhage, tractional RD	No statistical significance
Non-arteritic anterior ischemic optic neuropathy					
Nogueira et al., 2026 ³	Meta-analysis	T2D (n>1,500,000)	GLP-1RA vs control	NAION	OR 1.70 (95% CI 1.23–2.36)
Hathaway et al., 2024 ⁵	Retrospective cohort	T2D (n=710)	semaglutide vs non-GLP-1RA antidiabetic medications	NAION	HR 4.28 (95% CI, 1.62–11.29)
		Obesity (n=979)	semaglutide vs non-GLP-1RA anti-obesity medications		HR 7.64 (95% CI, 2.21–26.36)
Grauslund et al., 2024 ⁶	Retrospective cohort	T2D (n=424,152)	Once-weekly semaglutide vs non-exposed	NAION	HR 2.19 (95% CI 1.54–3.12)
Simonsen et al., 2025 ⁷	Retrospective cohort	T2D (n=61,377)	Semaglutide vs SGLT2i	NAION	HR 2.81 (95% CI 1.67–4.75)
Heberer et al., 2026 ³⁸	Retrospective cohort	T2D (n=102,361)	Semaglutide vs SGLT2i	NAION	HR 2.33 (95% CI, 1.54–3.54; p<0.001)
Cai et al., 2025 ⁴¹	Retrospective cohort (SCCs)	T2D (n=37,100,000)	GLP-1RA vs non-GLP-1RAs medications	NAION	IRR 1.32 (95% CI, 1.14–1.54; p<0.001)
Wang et al., 2025 ⁴²	Retrospective cohort	T2D (n=1,511,637)	Semaglutide/tirzepatide vs other antidiabetic medications	NAION	HR 1.76 (95% CI, 1.01–3.07)
				Other optic nerve disorders	HR 1.65 (95% CI 1.18–2.31)

Lieberman et al., 2026 ⁴⁸	Retrospective cohort	T2D (n=973,529) Obesity (n=239,246)	Semaaglutide vs non-GLP-1RAs medications	NAION	OR 0.36 (95% CI 0.25-0.51) No statistical significance
Glaucoma					
Sterling et al., 2021 ⁵⁴	Retrospective cohort	T2D (n=6332)	GLP-1RAs vs other antidiabetic medications	Glaucoma	HR 0.56 (95% CI 0.36-0.89; p=0.01)
Nazi et al., 2024 ⁴⁹	Nationwide cohort	T2D (n=10,422)	GLP-1RA vs other antidiabetic medication	Glaucoma	HR 0.81 (95% CI 0.70-0.94; p=0.006)
Hong et al., 2025 ⁵⁰	Retrospective cohort	T2D (n=83,698)	Tirzepatide vs selective GLP-1RA	POAG	RR 0.50 (95% CI 0.34-0.74)
				OHTN	RR 0.59 (95% CI 0.40-0.88)
				Glaucoma treatment	RR 0.54 (95% CI 0.45-0.64)
AMD and other ocular events					
Wai et al., 2024 ²³	Retrospective cohort	NPDR (n=12,962)	GLP-1RAs vs SGLT2s	Development of DME	at 3 months: RR 1.192 (95% CI 1.059-1.276; p=0.002) at 6 months: RR 1.22 (95% CI 1.13-1.32; p<0.001) at 1 year: RR 1.24 (95% CI 1.15-1.33; p<0.001) at 3 years: RR 1.29 (95% CI 1.21-1.38; p<0.001)
Joo et al., 2025 ³	Retrospective cohort	T2D (aged ≥50) (n=30,224)	GLP-1RAs vs insulin GLP-1RAs vs metformin GLP-1RAs vs SGLT2i	Nonexudative AMD	RR 0.28 (95% CI 0.15-0.46) RR 0.25 (95% CI 0.14-0.41) RR 0.42 (95% CI 0.22-0.74)
Lakhani et al., 2025 ⁷	Pharmacovigilance	T2D and/or obesity	Semaaglutide/tirzepatide vs metformin, empagliflozin, dulaglutide, insulin or phentemine- topiramate	Retinal hemorrhage Vitreous hemorrhage Vitreous detachment Retinal detachment	FAERS: OR 2.75; Vigibase: OR 5.49 FAERS: OR 5.89; Vigibase: OR 9.00 FAERS: OR 4.17; Vigibase: OR 20.91 FAERS: OR 2.44; Vigibase: OR 8.60
Su et al., 2022 ⁹	Retrospective cohort study	T2D (n=11,115)	SGLT2i vs GLP-1RA	DED	HR 0.78 (95% CI 0.68-0.89)

* T2D – type 2 diabetes mellitus, GLP-1RA – glucagon-like peptide-1 receptor agonist, SGLT2i – sodium-glucose cotransporter 2 inhibitor, DPP-4i – dipeptidyl peptidase-4 inhibitor, DR – diabetic retinopathy, NPDR – non-proliferative diabetic retinopathy, PDR – proliferative diabetic retinopathy, POAG – primary open-angle glaucoma, OHTN – ocular hypertension, DME – diabetic macular edema, RD – retinal detachment, NAION – non-arteritic anterior ischemic optic neuropathy, AMD – age-related macular degeneration, DED – dry eye disease, HR – hazard ratio, 95% CI – 95% confidence interval, SCCS – self-controlled case series, RR – relative risk, IRR – incidence rate ratio, OR – odds ratio

signal. Interpretation of available studies is limited by heterogeneous DR definitions, incomplete ophthalmic data, variable ophthalmic endpoints, short follow-up periods, and potential confounding related to baseline metabolic control.^{25,29}

Rapid and substantial HbA1c reduction, particularly in patients with poor baseline glycemic control, likely represents an important confounding factor underlying early DR worsening signals.²⁶ This phenomenon is well described in the context of intensive glycemic control and is generally considered transient, with risk attenuation over longer follow-up.⁸³

Overall, current evidence suggests that association between GLP-1RAs and DR is more likely related to magnitude and rapidity of glycemic improvement rather than a direct drug-specific effect. However, the quality of evidence remains limited. Given the heterogeneity of study designs and patient populations, residual uncertainty remains. Large and long-term prospective studies with detailed ophthalmic evaluation are needed to define risk profiles and underlying mechanisms.

NAION

Despite numerous studies suggesting an increased risk of NAION with GLP-1RAs use,^{32,35-39} these findings should be interpreted cautiously, as several limitations have been highlighted. The study by Hathaway et al. was based on a single-center neuro-ophthalmology referral population, potentially increasing susceptibility to selection bias and limits generalizability of the findings, as highlighted in a real-world evidence appraisal by Klonoff, Hui and Gombur.⁴⁶ Additionally, the observed association may be related to rapid HbA1c-lowering effect of semaglutide and tirzepatide rather than a direct drug effect.⁸⁴

The interpretation of these findings is further complicated by the incompletely understood pathophysiology of NAION,⁸⁵ and its multifactorial etiology, thus several risk factors may contribute to its development. Patients receiving incretin-based therapies frequently present with obesity, poorly controlled diabetes, and cardiovascular diseases, all of which are independent NAION risk factors and may contribute to confounding by indication.^{30,86,87} Consequently, it becomes exceedingly difficult to definitively isolate whether a newly diagnosed NAION episode is a pharmacological side effect, or simply the natural progression of the patient's baseline metabolic syndrome. This methodological impediment highlights why interpreting observational data in this specific patient population requires extreme caution. Additional risk factors remain inconsistently supported, thereby hindering the ability to obtain reliable data on the influence of GLP-1RAs and to design an accurate study addressing this issue. A crowded optic disc, small Bruch's membrane opening diameter, low cup-to-disc ratio, sleep apnea, surgery, infections and vacci-

nations, vitreopapillary traction, increased intraocular pressure, optic disc edema (i.e. in cases of papilledema) have been reported in various studies as factors contributing to NAION development, however, their definitive role remains debated.^{30,31,52,86}

Importantly, while several studies report concerning relative risks, these numbers must be interpreted in the context of low baseline incidence of the disease. Given the rarity of this condition with the annual incidence of NAION ranging from 2.3 to 10.2 per 100000 in the US, even a more than twofold increase in risk, translates into a small absolute incidence rate increase (from 0.093 to 0.228 per 1000 person-years).^{30,35} Moreover, the low event rate limits the ability to reliably identify associations with specific patient characteristics and to exclude unidentified confounding factors that may influence the observed results in a sample of this size.

While a potential association between GLP-1RAs and NAION cannot be excluded, current evidence in the absence of prospective studies, does not allow to establish a definitive association. From a clinical perspective, given the irreversible nature of NAION, cautious monitoring may be warranted, particularly in high-risk individuals. However, well-designed prospective studies are needed to clarify this relationship.

Glaucoma

Available evidence, largely observational, suggests a possible association between GLP-1RAs use and reduced risk of glaucoma. However, these findings are subject to residual confounding and cannot establish a definitive conclusion. Differences in baseline metabolic control or co-medications may partially explain the observed associations.

Proposed biological mechanisms are primarily based on experimental and animal studies. While such findings provide mechanistic plausibility and may seem promising, their direct clinical relevance remains uncertain.

Overall, while GLP-1RAs show potential as modulators of pathways relevant to glaucoma pathogenesis, current evidence is insufficient to support a therapeutic role. Further clinical studies are needed to determine whether these findings translate into clinical outcomes.

AMD and other ocular events

The potential of GLP-1RAs in AMD is gaining increasing attention. Some observational studies suggest that these drugs may be associated with preserved retinal function and slower disease progression, potentially through anti-inflammatory and neuroprotective mechanisms.⁶⁹⁻⁷³ However, the available evidence is heterogeneous, with large cohort studies reporting mixed findings, including neutral associations for neovascular AMD and a potential protective signal for non-neovascular AMD across both diabetic and non-diabetic pop-

ulations, while other reports have suggested a possible increased risk of neovascular AMD. These discrepancies highlight uncertainty regarding the direction and magnitude of the association.^{70,74,75} Additionally, most current evidence derives from observational studies, which are inherently susceptible to residual confounding and cannot establish definitive association. Well-designed clinical studies are needed to clarify the role of GLP-1RAs in AMD pathogenesis and progression.

Beyond AMD, evidence regarding other ocular adverse events remains limited and heterogeneous. It is crucial to emphasize that spontaneous reporting systems are prone to bias and may over-represent high-risk populations, including patients with long-standing diabetes, suboptimal prior glycemic control, and established microvascular complications, thereby limiting definitive conclusions.

Most non-retinopathy ocular adverse events with GLP-1RAs are rare and likely attributable to diabetic eye disease rather than a direct drug effect. From a clinical perspective, these findings do not warrant changes in prescribing practices but support baseline ophthalmic evaluation and regular follow-up in high-risk patients.

Integrated clinical implications and future directions

GLP-1 receptor expression in retinal tissue provides biological plausibility for potential direct ocular effects of incretin-based therapies. Experimental models suggest that GLP-1 receptor activation may attenuate oxidative stress, inflammation, endothelial dysfunction, and apoptosis, while improving blood-retinal barrier integrity and antioxidant defense. These findings point to potential neuroprotective and vasculoprotective effects rather than direct retinal toxicity.⁸⁸⁻⁹⁰ Nevertheless, the available evidence is derived from in vitro and animal models, and its clinical relevance in human clinical practice remains uncertain.

Given the rapidly growing clinical use of dual GIP/GLP-1 receptor agonists, evaluating the specific ophthalmic safety profile of tirzepatide has become highly relevant. Emerging evidence suggests that its dual incretin activity may enhance retinal neuronal resilience, potentially offering broader neuroprotective and anti-inflammatory benefits compared with selective GLP-1RAs. Clinically, tirzepatide demonstrated greater reductions in HbA1c and cardiometabolic risk factors.⁹¹ As rapid glycemic improvement itself may contribute to transient DR worsening; careful ophthalmologic monitoring remains warranted. However, large-scale real-world evidence indicates that over an extended follow-up, tirzepatide leads to a long-term reduction in DR complications.¹⁸ At present, available evidence does not allow definitive conclusions regarding superiority or inferiority of tirzepatide with respect to ocular safety outcomes.

From a clinical perspective, current evidence does not support modification of prescribing practices sole-

ly because of concerns regarding ocular adverse effect. Nevertheless, careful ophthalmic monitoring may be warranted in patients with pre-existing risk factors. Further prospective studies with standardized ophthalmologic endpoints are needed to clarify association between incretin-based therapy and ocular outcomes.

Conclusion

Current evidence does not consistently demonstrate an increased risk of major ophthalmic adverse outcomes associated with GLP-1RAs use. However, the available data are heterogeneous and predominantly observational and therefore do not allow firm definitive conclusions. Signals of DR progression have been reported in some patients treated with different GLP-1RAs. This phenomenon appears to be more likely related to rapid and substantial reductions in HbA1c, rather than a direct drug-specific toxic effect. Reports of NAION remain rare and derive mainly from observational studies. A definitive association with GLP-1RAs exposure has not been established. For AMD, the evidence is limited and inconsistent. Some studies suggest that GLP-1RAs may reduce the risk of AMD or slow its progression, while other reports suggest a potential increase in risk of neovascular AMD. The clinical relevance of these findings remains uncertain. Most other ocular adverse events are uncommon and are likely attributable to underlying disease, detection bias, or rapid metabolic changes rather than a direct effect of the medication. Evidence regarding glaucoma is derived largely from pre-clinical and observational studies, suggesting potential neuroprotective and intraocular pressure-lowering effects; however, clinical evidence remains limited.

From a clinical perspective, baseline ophthalmic assessment and appropriate follow-up may be considered, particularly in individuals with pre-existing retinopathy, advanced diabetes, or additional vascular risk factors. Treatment decisions should therefore be based on individualized risk-benefit assessment, taking into account the well-established metabolic and cardiovascular advantages of GLP-1RAs.

This narrative review highlights the need for further well-designed prospective studies with standardized ophthalmic endpoints, to clarify association, elucidate underlying mechanisms, and identify patient subgroups at increased risk of ocular complications.

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Author contributions

Conceptualization, H.R., J.K., W.D.; Methodology, H.R.; Validation, N.M.; Formal Analysis, H.R., J.K., N.M.; Investigation, H.R., J.K.; Data Curation, H.R., J.K., N.M.;

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Conflict of interest

The authors declare no competing interests.

Data availability

All data analyzed in this study were derived from previously published studies and are included in this published article.

Ethics approval

Not applicable.

Use of AI and AI-assisted technologies in the writing process

AI-tools (ChatGPT) were used solely for language editing, text reduction, and improving the readability of the manuscript. The content was verified and edited by the authors. The authors take full responsibility for the content of the published article.

References

1. Husain M, Birkenfeld AL, Donsmark M, et al. Oral Semaglutide and Cardiovascular Outcomes in Patients with Type 2 Diabetes. *N Engl J Med*. 2019;381(9):841-851. doi:10.1056/NEJMoa1901118

2. Liu QK. Mechanisms of action and therapeutic applications of GLP-1 and dual GIP/GLP-1 receptor agonists. *Front Endocrinol (Lausanne)*. 2024;15:1431292. doi:10.3389/fendo.2024.1431292

3. Kristensen SL, Rørth R, Jhund PS, et al. Cardiovascular, mortality, and kidney outcomes with GLP-1 receptor agonists in patients with type 2 diabetes: a systematic review and meta-analysis of cardiovascular outcome trials. *Lancet Diabetes Endocrinol*. 2019;7(10):776-785. doi:10.1016/S2213-8587(19)30249-9

4. Tuttle KR, Bain SC, Bosch-Traberg H, et al. Effects of Once-Weekly Semaglutide on Kidney Disease Outcomes by KDIGO Risk Category in the SUSTAIN 6 Trial. *Kidney Int Rep*. 2024;9(7):2006-2015. doi:10.1016/j.ekir.2024.04.028

5. Wu R, Xing B, Huang Y, et al. Effect of semaglutide on arrhythmic, major cardiovascular, and microvascular outcomes in patients with type 2 diabetes: a systematic review and meta-analysis. *Front Endocrinol (Lausanne)*. 2025;16:1554795. doi:10.3389/fendo.2025.1554795

6. Sheth K, Kim S, Porterfield L, Virani SS, Wadhwani S, Vaughan EM. The Expanding Scope of GLP-1 Receptor Agonists: Six Uses Beyond Diabetes. *Curr Atheroscler Rep*. 2025;27(1):76. doi:10.1007/s11883-025-01319-6

7. Vahratian A, Warren A. GLP-1 Injectable Use Among Adults With Diagnosed Diabetes: United States, 2024. 2025 Aug. In: NCHS Data Briefs [Internet]. Hyattsville (MD): National

Center for Health Statistics (US); 2024 Jul-. No. 537. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK617443/>. doi:10.15620/cdc/174616. Accessed January 20, 2026

8. Montero A, Sparks G, Presiado M, Hamel L. The Public's Use and Views of GLP-1 Drugs. KFF; 2024 May 10. Available from: <https://www.kff.org/health-costs/kff-health-tracking-poll-may-2024-the-publics-use-and-views-of-glp-1-drugs/>. Accessed January 20, 2026

9. Ukhanova M, Wozny JS, Truong CN, Ghosh L, Krause TM. Trends in glucagon-like peptide 1 receptor agonist prescribing patterns. *Am J Manag Care*. 2025;31(8):e228-e234. doi:10.37765/ajmc.2025.89778

10. Mailhac A, Pedersen L, Pottegård A, et al. Semaglutide (Ozempic®) Use in Denmark 2018 Through 2023 – User Trends and off-Label Prescribing for Weight Loss. *Clin Epidemiol*. 2024;16:307-318. doi:10.2147/CLEP.S456170

11. Luo ZY, Li X, Chen CT, et al. Ocular adverse events associated with GLP-1 receptor agonists: a real-world study based on the FAERS database and network pharmacology. *Expert Opin Drug Saf*. 2025;24(3):287-296. doi:10.1080/14740338.2024.2419989

12. Shukla UV, Tripathy K. Diabetic Retinopathy. [Updated 2023 Aug 25]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2025 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK560805/>. Accessed January 20, 2026

13. Wei L, Mo W, Lan S, et al. GLP-1 RA Improves Diabetic Retinopathy by Protecting the Blood-Retinal Barrier through GLP-1R-ROCK-p-MLC Signaling Pathway. *J Diabetes Res*. 2022;2022:1861940. doi:10.1155/2022/1861940

14. Małyszczak A, Przeździecka-Dolyk J, Szydelko-Paśko U, Misiuk-Hojło M. Novel Antidiabetic Drugs and the Risk of Diabetic Retinopathy: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *J Clin Med*. 2024;13(6):1797. doi:10.3390/jcm13061797

15. Yoshida Y, Joshi P, Barri S, et al. Progression of retinopathy with glucagon-like peptide-1 receptor agonists with cardiovascular benefits in type 2 diabetes - a systematic review and meta-analysis. *J Diabetes Complications*. 2022;36(8):108255. doi:10.1016/j.jdiacomp.2022.108255

16. Zheng D, Li N, Hou R, et al. Glucagon-like peptide-1 receptor agonists and diabetic retinopathy: nationwide cohort and Mendelian randomization studies. *BMC Med*. 2023;21(1):40. doi:10.1186/s12916-023-02753-6

17. Mahzari MM, Alanazy AM, Feroz Z, et al. Retinopathy risk factors in patients with type 2 diabetes on liraglutide. *Medicine (Baltimore)*. 2024;103(29):e39026. doi:10.1097/MD.00000000000039026

18. Shah J, Razavi P, Festok M, et al. Tirzepatide and Reduced Risk of Diabetic Retinopathy and Related Complications: A Multicenter US Cohort Study. *Ophthalmology*. 2026;133(6):728-732. doi:10.1016/j.ophtha.2026.01.013

19. Barkmeier AJ, Herrin J, Swarna KS, et al. Comparative Effectiveness of Glucagon-Like Peptide-1 Receptor Agonists, Sodium-Glucose Cotransporter 2 Inhibitors, Dipep-

- tidyl Peptidase-4 Inhibitors, and Sulfonylureas for Sight-Threatening Diabetic Retinopathy. *Ophthalmol Retina*. 2024;8(10):943-952. doi:10.1016/j.oret.2024.05.003
20. Joo JH, Sharma N, Shaia J, et al. The Effect of Glucagon-Like Peptide-1 Receptor Agonists on Diabetic Retinopathy at a Tertiary Care Center. *Ophthalmol Sci*. 2024;4(6):100547. doi:10.1016/j.xops.2024.100547
 21. Ramsey DJ, Makwana B, Dani SS, et al. GLP-1 Receptor Agonists and Sight-Threatening Ophthalmic Complications in Patients With Type 2 Diabetes. *JAMA Netw Open*. 2025;8(8):e2526321. doi:10.1001/jamanetworkopen.2025.26321
 22. Eleftheriadou A, Riley D, Zhao SS, et al. Risk of diabetic retinopathy and diabetic macular oedema with sodium-glucose cotransporter 2 inhibitors and glucagon-like peptide 1 receptor agonists in type 2 diabetes: a real-world data study from a global federated database. *Diabetologia*. 2024;67(7):1271-1282. doi:10.1007/s00125-024-06132-5
 23. Wai KM, Mishra K, Koo E, et al. Impact of GLP-1 Agonists and SGLT-2 Inhibitors on Diabetic Retinopathy Progression: An Aggregated Electronic Health Record Data Study. *Am J Ophthalmol*. 2024;265:39-47. doi:10.1016/j.ajo.2024.04.010
 24. Lin DSH, Lo HY, Huang KC, Lin TT, Lee JK, Lin LY. Incidence and progression of diabetic retinopathy in patients treated with glucagon-like peptide-1 receptor agonists versus sodium-glucose cotransporter 2 inhibitors: A population-based cohort study. *Diabetes Obes Metab*. 2024;26(10):4386-4396. doi:10.1111/dom.15788
 25. Michaeli T, Khateb S, Levy J. The Effect of Glucagon-like-Peptide-1 Receptor Agonists on Diabetic Retinopathy Progression, Central Subfield Thickness, and Response to Intravitreal Injections. *J Clin Med*. 2024;13(20):6269. doi:10.3390/jcm13206269
 26. Vilsbøll T, Bain SC, Leiter LA, et al. Semaglutide, reduction in glycated haemoglobin and the risk of diabetic retinopathy. *Diabetes Obes Metab*. 2018;20(4):889-897. doi:10.1111/dom.13172
 27. Cigrovski Berkovic M, Strollo F. Semaglutide-eye-catching results. *World J Diabetes*. 2023;14(4):424-434. doi:10.4239/wjd.v14.i4.424
 28. Barkmeier AJ, Deng Y, Swarna KS, et al. Risk of Sight-Threatening Diabetic Retinopathy with Glucagon-Like Peptide-1 Receptor Agonist Use in Routine Clinical Practice: Comparative Effectiveness of Semaglutide, Dulaglutide, Liraglutide, and Exenatide. *Ophthalmol Retina*. 2025;10(2):142-151. doi:10.1016/j.oret.2025.07.019
 29. Zhao T, Zheng L, Feng Y, Lao M, Huang Y, Wu G. Association between various dosage forms of semaglutide and ocular adverse events in a real-world setting. *BMC Ophthalmol*. 2025;25(1):248. doi:10.1186/s12886-025-04096-7
 30. Salvatat ML, Pellegrini F, Spadea L, Salati C, Zeppieri M. Non-Arteritic Anterior Ischemic Optic Neuropathy (NA-AION): A Comprehensive Overview. *Vision (Basel)*. 2023;7(4):72. doi:10.3390/vision7040072
 31. Atkins EJ, Bruce BB, Newman NJ, Biousse V. Treatment of nonarteritic anterior ischemic optic neuropathy. *Surv Ophthalmol*. 2010;55(1):47-63. doi:10.1016/j.survophthal.2009.06.008
 32. Silverii GA, Pala L, Cresci B, Mannucci E. Glucagon-like peptide 1 (GLP1) receptor agonists and risk for ischemic optic neuropathy: A meta-analysis of randomised controlled trials. *Diabetes Obes Metab*. 2025;27(2):1005-1009. doi:10.1111/dom.16076
 33. Nogueira A, Rassi TNO, Iqbal A, et al. Association Between GLP-1 Receptor Agonists and Ischemic Optic Neuropathy: A Meta-analysis. *Diabetes Care*. 2026;49(5):724-729. doi:10.2337/dc25-1238
 34. Vilsbøll T, Arun Kumar D, Aiello LP, Rönnebeck C, Stellfeld M, Larsen LM. Non-arteritic anterior ischaemic optic neuropathy incidence in placebo-controlled clinical trials of liraglutide or semaglutide. *Br J Ophthalmol*. 2026;bjo-2025-328729. doi:10.1136/bjo-2025-328729
 35. Hathaway JT, Shah MP, Hathaway DB, et al. Risk of Nonarteritic Anterior Ischemic Optic Neuropathy in Patients Prescribed Semaglutide. *JAMA Ophthalmol*. 2024;142(8):732-739. doi:10.1001/jamaophthalmol.2024.2296
 36. Grauslund J, Taha AA, Molander LD, et al. Once-weekly semaglutide doubles the five-year risk of nonarteritic anterior ischemic optic neuropathy in a Danish cohort of 424,152 persons with type 2 diabetes. *Int J Retina Vitreous*. 2024;10(1):97. doi:10.1186/s40942-024-00620-x
 37. Simonsen E, Lund LC, Ernst MT, et al. Use of semaglutide and risk of non-arteritic anterior ischemic optic neuropathy: A Danish-Norwegian cohort study. *Diabetes Obes Metab*. 2025;27(6):3094-3103. doi:10.1111/dom.16316
 38. Heberer K, Bress AP, Cogill S, et al. New-Onset Nonarteritic Anterior Ischemic Optic Neuropathy and Initiators of Semaglutide in US Veterans With Type 2 Diabetes. *JAMA Ophthalmol*. 2026;144(3):259-264. doi:10.1001/jamaophthalmol.2025.6262
 39. Noh Y, Yin H, Ben Ghezala I, Yu OHY, Suissa S, Azoulay L. Glucagon-Like Peptide 1 Receptor Agonists and Risk of Nonarteritic Anterior Ischemic Optic Neuropathy in Patients With Type 2 Diabetes. *Diabetes Care*. 2026;49(5):763-771. doi:10.2337/dc25-2577
 40. Niazi S, Gnesin F, Jawad BN, et al. Timing and duration of glucagon-like peptide-1 receptor agonist use and risk of nonarteritic anterior ischaemic optic neuropathy. *Acta Ophthalmol*. 2026. doi:10.1111/aos.70168
 41. Cai CX, Hribar M, Baxter S, et al. Semaglutide and Nonarteritic Anterior Ischemic Optic Neuropathy. *JAMA Ophthalmol*. 2025;143(4):304-314. doi:10.1001/jamaophthalmol.2024.6555
 42. Wang L, Volkow ND, Kaelber DC, Xu R. Semaglutide or Tirzepatide and Optic Nerve and Visual Pathway Disorders in Type 2 Diabetes. *JAMA Netw Open*. 2025;8(8):e2526327. doi:10.1001/jamanetworkopen.2025.26327

43. Choi T, Al-Aly Z, Xie Y. GLP-1 Receptor Agonists or SGLT2 Inhibitors and Nonarteritic Anterior Ischemic Optic Neuropathy. *JAMA Netw Open*. 2026;9(4):e269917. doi:10.1001/jamanetworkopen.2026.9917
44. Chen JW, Yu FT, Chen HA, Huang TF, Chen HS, Wu TE. Glucagon-like peptide-1 receptor agonists and the risk of nonarteritic anterior ischemic optic neuropathy: Evidence from a global real-world cohort. *Diabetes Metab*. 2026;52(2):101740. doi:10.1016/j.diabet.2026.101740
45. Barnett MJ, Ibe F, Lam J. Sight Unseen: Glucagon-Like Peptide-1 (GLP-1) Agonism Therapy and Nonarteritic Anterior Ischemic Optic Neuropathy. *Cureus*. 2026;18(1):e100953. doi:10.7759/cureus.100953
46. Klonoff DC, Hui G, Gombar S. Real-World Evidence Assessment of the Risk of Nonarteritic Anterior Ischemic Optic Neuropathy in Patients Prescribed Semaglutide. *J Diabetes Sci Technol*. 2024;18(6):1517-1518. doi:10.1177/19322968241268050
47. Bahit MC, Gibson CM, Kuleta M, et al. Vision impairment following glucagon-like peptide-1 receptor agonist use: is it harmful? *Pol Arch Intern Med*. 2025;135:16987. doi:10.20452/pamw.16987
48. Lieberman RA, Korona-Bailey J, Banaag A, Furhman B, Corrado R, Koehlmoos TP. Semaglutide Decreases Risk of Non-Arteritic Anterior Ischemic Optic Neuroapthy in Type 2 Diabetic Patients. *Mil Med*. 2026;191(5-6):e1020-e1025. doi:10.1093/milmed/usaf522
49. Massy M, Marti S, Hammer H, Hoepner R. Increased vision impairment reports linked to semaglutide: analysis of FDA adverse event data. *BMC Med*. 2025;23(1):203. doi:10.1186/s12916-025-04031-z
50. Lešin Gaćina D, Vidović T, Vlajić Oreb N, Matković L, Jandroković S. Is Semaglutide Linked to NAION? A Case Report on a Rare Ocular Complication. *Reports*. 2025;8(3):149. doi:10.3390/reports8030149
51. Lixi F, Calabresi V, Cukurova F, Giannaccare G. Non-Arteritic Anterior Ischemic Optic Neuropathy in an Otherwise Healthy Young Adult Patient Treated with Liraglutide and Semaglutide for Weight Loss: A Cautionary Tale. *Int Med Case Rep J*. 2025;18:991-995. doi:10.2147/IMCRJ.S531930
52. Ahmadi H, Hamann S. Anterior ischemic optic neuropathy in patients treated with semaglutide: report of four cases with a possible association. *BMC Ophthalmol*. 2025;25(1):132. doi:10.1186/s12886-025-03958-4
53. Katz BJ, Lee MS, Lincoff NS, et al. Ophthalmic Complications Associated With the Antidiabetic Drugs Semaglutide and Tirzepatide. *JAMA Ophthalmol*. 2025;143(3):215-220. doi:10.1001/jamaophthalmol.2024.6058
54. Sterling J, Hua P, Dunaief JL, Cui QN, VanderBeek BL. Glucagon-like peptide 1 receptor agonist use is associated with reduced risk for glaucoma. *Br J Ophthalmol*. 2023;107(2):215-220. doi:10.1136/bjophthalmol-2021-319232
55. Asif M, Asif A, Rahman UA, et al. Incidence of Glaucoma in Type 2 Diabetes Patients Treated With GLP-1 Receptor Agonists: A Systematic Review and Meta-Analysis. *Endocrinol Diabetes Metab*. 2025;8(4):e70059. doi:10.1002/edm2.70059
56. Mouhammad ZA, Vohra R, Horwitz A, et al. Glucagon-Like Peptide 1 Receptor Agonists - Potential Game Changers in the Treatment of Glaucoma? *Front Neurosci*. 2022;16:824054. doi:10.3389/fnins.2022.824054
57. Chuang CC, Wang K, Chang CK, et al. Prescription of glucagon-like peptide 1 agonists and risk of subsequent open-angle glaucoma in individuals with type 2 diabetes mellitus. *Int J Med Sci*. 2024;21(3):540-546. doi:10.7150/ijms.90273
58. Sterling JK, Adetunji MO, Guttha S, et al. GLP-1 Receptor Agonist NLY01 Reduces Retinal Inflammation and Neuron Death Secondary to Ocular Hypertension. *Cell Rep*. 2020;33(5):108271. doi:10.1016/j.celrep.2020.108271
59. Niazi S, Gnesin F, Thein AS, et al. Association between Glucagon-like Peptide-1 Receptor Agonists and the Risk of Glaucoma in Individuals with Type 2 Diabetes. *Ophthalmology*. 2024;131(9):1056-1063. doi:10.1016/j.ophtha.2024.03.004
60. Hong AT, Lin F, Baxter S, Weinreb RN. Tirzepatide is Associated With Reduced Risk of Primary Open-Angle Glaucoma and Ocular Hypertension in Patients With Type 2 Diabetes. *Am J Ophthalmol*. 2026;283:120-128. doi:10.1016/j.ajo.2025.12.003
61. Pan SY, Weng CH, Sheen YJ, et al. Association of tirzepatide versus GLP-1 receptor agonists with incident glaucoma in patients with type 2 diabetes: a retrospective cohort study. *Ophthalmol Sci*. 2026;6:101245. doi:10.1016/j.xops.2026.101245
62. Hallaj S, Halfpenny W, Chuter BG, Weinreb RN, Baxter SL, Cui QN. Association Between Glucagon-Like Peptide-1 Receptor Agonists Exposure and Intraocular Pressure Change: GLP-1 Receptor Agonists and Intraocular Pressure Change. *Am J Ophthalmol*. 2025;269:255-265. doi:10.1016/j.ajo.2024.08.030
63. Picard M, Turnbull DM. Linking the metabolic state and mitochondrial DNA in chronic disease, health, and aging. *Diabetes*. 2013;62(3):672-678. doi:10.2337/db12-1203
64. Kim I, Rodriguez-Enriquez S, Lemasters JJ. Selective degradation of mitochondria by mitophagy. *Arch Biochem Biophys*. 2007;462(2):245-253. doi:10.1016/j.abb.2007.03.034
65. Palikaras K, Tavernarakis N. Mitophagy in neurodegeneration and aging. *Front Genet*. 2012;3:297. doi:10.3389/fgene.2012.00297
66. Zhou HR, Ma XF, Lin WJ, et al. Neuroprotective Role of GLP-1 Analog for Retinal Ganglion Cells via PINK1/Parkin-Mediated Mitophagy in Diabetic Retinopathy. *Front Pharmacol*. 2021;11:589114. doi:10.3389/fphar.2020.589114
67. Mouhammad ZA, Tribble JR, Nicol A, et al. Semaglutide Protects Retinal Ganglion Cells Against Rotenone-Induced Degeneration via Improved Glucose Metabolism. *Invest Ophthalmol Vis Sci*. 2026;67(2):25. doi:10.1167/iovs.67.2.25

68. Ayoub T, Patel N. Age-related macular degeneration. *J R Soc Med.* 2009;102(2):56-61. doi:10.1258/jrsm.2009.080298
69. Thomas CJ, Mirza RG, Gill MK. Age-Related Macular Degeneration. *Med Clin North Am.* 2021;105(3):473-491. doi:10.1016/j.mcna.2021.01.003
70. Rein DB, Wittenborn JS, Burke-Conte Z, et al. Prevalence of Age-Related Macular Degeneration in the US in 2019. *JAMA Ophthalmol.* 2022;140(12):1202-1208. doi:10.1001/jamaophthalmol.2022.4401
71. Myers WK, Heath G, Rohrer B. Rate of Age-Related Macular Degeneration in Patients Prescribed Glucagon-Like Peptide-1 Receptor Agonists or Other Weight Loss Therapies. *Ophthalmol Retina.* 2026;10(5):471-479. doi:10.1016/j.oret.2025.12.016
72. McLaughlin SA, Davila N, Shields C, Bineshfar N, Banaee T, Williams BK Jr. Impact of GLP-1 receptor agonists for type 2 diabetes mellitus on the development and progression of age-related macular degeneration. *Retina.* 2025;45(12):2241-2251. doi:10.1097/IAE.0000000000004616
73. Joo JH, Zhao AH, Chalasani M, Allan KC, Rachitskaya AV. Impact of Glucagon-Like Peptide-1 Receptor Agonists on Age-Related Macular Degeneration at a Tertiary Ophthalmology Center. *Ophthalmol Retina.* 2026;10(5):480-489. doi: 10.1016/j.oret.2025.12.014
74. Allan KC, Joo JH, Kim S, et al. Glucagon-like Peptide-1 Receptor Agonist Impact on Chronic Ocular Disease Including Age-Related Macular Degeneration. *Ophthalmology.* 2025;132(7):748-757. doi:10.1016/j.ophtha.2025.01.016
75. Shor R, Mihalache A, Noori A, et al. Glucagon-Like Peptide-1 Receptor Agonists and Risk of Neovascular Age-Related Macular Degeneration. *JAMA Ophthalmol.* 2025;143(7):587-594. doi:10.1001/jamaophthalmol.2025.1455
76. Allan KC, Cohn EF, Bala S, et al. Glucagon-Like Peptide-1 Receptor Agonist Use and Risk of Neovascular Age-Related Macular Degeneration in a National Cohort Study. *Ophthalmol Retina.* 2026;10(4):362-372. doi: 10.1016/j.oret.2025.10.020
77. Lakhani M, Kwan ATH, Mihalache A, et al. Association of Glucagon-Like Peptide-1 Receptor Agonists With Optic Nerve and Retinal Adverse Events: A Population-Based Observational Study Across 180 Countries. *Am J Ophthalmol.* 2025;277:148-168. doi:10.1016/j.ajo.2025.05.007
78. Phu A, Banghart M, Bahrainian M, Liu TYA, Wolf RM, Channa R. Dipeptidyl peptidase 4 inhibitors, sodium glucose cotransporter 2 inhibitors, and glucagon-like peptide 1 receptor agonists do not worsen diabetic macular edema. *J Diabetes Complications.* 2024;38(8):108808. doi:10.1016/j.jdiacomp.2024.108808
79. Su YC, Hung JH, Chang KC, et al. Comparison of Sodium-Glucose Cotransporter 2 Inhibitors vs Glucagonlike Peptide-1 Receptor Agonists and Incidence of Dry Eye Disease in Patients With Type 2 Diabetes in Taiwan. *JAMA Netw Open.* 2022;5(9):e2232584. doi:10.1001/jamanetworkopen.2022.32584
80. Ottonelli G, Gaeta A, Montericchio N, et al. GLP-1R Agonists Improve Ocular Surface Parameters in Type 2 Diabetes Mellitus. *Clin Ophthalmol.* 2025;19:3829-3836. doi:10.2147/OPTH.S547776
81. Maher CB, Guo B, Dunlop AA. Inflammatory optic neuritis and scleritis following commencement of semaglutide: a case report. *J Surg Case Rep.* 2025;2025(11):rjaf937. doi:10.1093/jscr/rjaf937
82. Bracha P, Johnson W, Chu S, Davison J. Reversible bilateral central scotoma under scotopic conditions associated with oral semaglutide. *Am J Ophthalmol Case Rep.* 2024;36:102121. doi:10.1016/j.ajoc.2024.102121
83. Bain SC, Klufas MA, Ho A, Matthews DR. Worsening of diabetic retinopathy with rapid improvement in systemic glucose control: A review. *Diabetes Obes Metab.* 2019;21(3):454-466. doi:10.1111/dom.13538
84. Tesfaye H, Paik JM, Wexler DJ, et al. GLP-1RA and the risk of non-arteritic anterior ischaemic optic neuropathy in patients with type 2 diabetes: A population-based study. *Diabetes Obes Metab.* 2026;28(2):1517-1528. doi:10.1111/dom.70200
85. Berry S, Lin WV, Sadaka A, Lee AG. Nonarteritic anterior ischemic optic neuropathy: cause, effect, and management. *Eye Brain.* 2017;9:23-28. doi:10.2147/EB.S125311
86. Liu B, Yu Y, Liu W, Deng T, Xiang D. Risk Factors for Non-arteritic Anterior Ischemic Optic Neuropathy: A Large Scale Meta-Analysis. *Front Med (Lausanne).* 2021;8:618353. doi:10.3389/fmed.2021.618353
87. Dal Canto E, Ceriello A, Rydén L, et al. Diabetes as a cardiovascular risk factor: An overview of global trends of macro and microvascular complications. *Eur J Prev Cardiol.* 2019;26(2):25-32. doi:10.1177/2047487319878371
88. Puddu A, Maggi D. Anti-Inflammatory Effects of GLP-1R Activation in the Retina. *Int J Mol Sci.* 2022;23(20):12428. doi:10.3390/ijms232012428
89. Hebsgaard JB, Pyke C, Yildirim E, Knudsen LB, Heegaard S, Kvist PH. Glucagon-like peptide-1 receptor expression in the human eye. *Diabetes Obes Metab.* 2018;20(9):2304-2308. doi:10.1111/dom.13339
90. Xie Z, Yang Z, Tian D, Chen Y. Unlocking the potential of GLP-1 receptor agonists in ocular therapeutics: from molecular pathways to clinical impact. *Front Pharmacol.* 2025;16:1618079. doi:10.3389/fphar.2025.1618079
91. Nicholls SJ, Pavo I, Bhatt DL, et al. Cardiovascular Outcomes with Tirzepatide versus Dulaglutide in Type 2 Diabetes. *N Engl J Med.* 2025;393(24):2409-2420. doi:10.1056/NEJMoa2505928



CASE REPORT

Anaphylaxis to antivenom in the background of alpha-gal syndrome – a case report

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ABSTRACT

Introduction and aim. Alpha-gal syndrome is an IgE-mediated allergy to galactose- α -1,3-galactose, typically associated with delayed hypersensitivity reactions following ingestion of mammalian meat. Severe immediate hypersensitivity reactions to equine-derived antivenom in patients with previously unrecognized alpha-gal syndrome are rarely reported. We report one of the first cases from South Asia of refractory anaphylaxis following equine-derived antivenom in a patient with previously undiagnosed alpha-gal syndrome, highlighting an important but under-recognized clinical challenge.

Description of the case. A 45-year-old Sri Lankan woman developed refractory anaphylaxis shortly after administration of equine-derived antivenom for a Russell's viper bite. She required intravenous adrenaline and noradrenaline infusions, fluid resuscitation, and high-dependency care, with subsequent full recovery. Further evaluation revealed a history suggestive of alpha-gal syndrome, including recurrent urticaria and wheezing following consumption of mammalian meat and dairy products, as well as possible anaphylaxis to wasp stings. Serum alpha-gal-specific IgE levels were markedly elevated.

Conclusion. This case highlights the potential for severe hypersensitivity reactions to mammalian-derived antivenoms in patients with alpha-gal syndrome.

Keywords. alpha-gal syndrome, anaphylaxis, antivenom hypersensitivity

Introduction

Alpha-gal syndrome (AGS), an immunoglobulin E (IgE) mediated allergy to the oligosaccharide galactose- α -1,3-galactose (alpha-gal), classically presents with delayed urticaria, angioedema, or anaphylaxis following ingestion of mammalian meat or meat-derived products.¹ This condition is commonly associated with sensitization triggered by bites from certain hard ticks, particularly *Amblyomma americanum* in the USA. A unique and evolving facet of this syndrome is the recognition of anaphylactic reactions to medications and bio-

logics that contain mammalian-derived glycoproteins.² However, evidence linking AGS to hypersensitivity reactions following equine-derived antivenom remains limited, with only a few cases reported worldwide and very little published experience from South Asia, where snakebite is a major public health problem.

Aim

We report this case to highlight a biologically plausible association between previously unrecognized AGS and severe immediate hypersensitivity following equine-de-

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rived antivenom, and to increase clinical awareness of this rare but potentially life-threatening complication in regions where snakebite and alpha-gal syndrome may coexist.

Description of the case

A 45-year-old female presented 30 minutes after sustaining a Russell’s viper (*Daboia russelii*) bite to her left big toe. The incident occurred while she was gardening at home. The dead specimen was brought to the hospital and identified by the medical officer. On admission, she complained of severe local pain, vomiting, shortness of breath, and wheezing (Table 1). She was drowsy, with a blood pressure of 150/80 mmHg, a pulse rate of 80 beats per minute, and bilateral widespread rhonchi on lung auscultation. Examination of the bite site revealed two distinct fang marks, local bleeding, and tenderness, but no oedema. The 20-minute whole blood clotting test was noncoagulopathic (Table 2).

In view of having a non-specific systemic envenomation feature (vomiting), along with the positively identified snake specimen, 20 vials of Indian polyvalent antivenom serum (Bharat SV Ltd, manufactured in 12/2024, lot number 18224078) were administered 60 minutes after the bite at the emergency treatment unit, following the national guideline for snakebite management. Subcutaneous Adrenaline 1:1000 dilution (0.25 mg) was given as premedication. Within five minutes of starting antivenom serum, the patient developed acute worsening of dyspnea and wheezing, accompanied by faintness and hypotension (blood pressure, 70/40 mmHg, pulse oximeter oxygen saturation 94%). Anaphylaxis to antivenom was diagnosed and was treated immediately with three intramuscular doses of adrenaline 1:1000 dilution, 0.5 mg. As hypotension persisted with features of shock, the reaction was classified as refractory anaphylaxis, and she was commenced on intravenous adrenaline 0.1 µg/kg/minute and noradrenaline infusion at 0.4 µg/kg/minute. Antivenom administration was completed with vasopressor support within 60 minutes. Additional management included fluid resuscitation and nebulized bronchodilators. Following antivenom administration and anaphylaxis management, her respiratory distress resolved, with clear lung fields on auscultation.

Following stabilization, the patient was transferred to the medical casualty ward four hours after admission and subsequently managed in the high dependency unit. She made a full recovery and was discharged after four days of inpatient care. The patient had no evidence of developing venom-induced consumption coagulopathy with a normal international normalized ratio (Table 2), neuromuscular paralysis or acute kidney injury.

The patient reported a previous episode of possible anaphylaxis to a wasp sting four years earlier. Over the

past five years, she had experienced recurrent urticarial rashes and wheezing following ingestion of mammalian meat and milk products.

Table 1. Timeline of clinical presentation, therapeutic interventions, and patient outcomes following Russell’s viper envenomation and antivenom-induced anaphylaxis

Time elapsed	Clinical event and intervention	Clinical status and key metrics
0 minutes	Russell’s viper bite to left big toe while gardening at home.	Dead specimen brought for identification.
30 minutes	Admission to emergency department. Dead snake positively identified as <i>Daboia russelii</i> .	Drowsy, severe local pain, vomiting, dyspnea, wheezing. BP: 150/80 mmHg, PR: 80 bpm. Lungs: Bilateral widespread rhonchi. 20-WBCT: Noncoagulopathic.
60 minutes	Premedication and antivenom initiation. Given 0.25 mg subcutaneous adrenaline. Started infusion of 20 vials of Indian polyvalent antivenom serum.	Non-specific systemic features (vomiting).
65 minutes	Severe anaphylaxis onset. Antivenom halted. Treated with 3 doses of IM adrenaline 0.5 mg. Classified as refractory anaphylaxis; initiated IV adrenaline and noradrenaline infusions.	Acute worsening of dyspnea, wheezing, faintness, and shock. BP: 70/40 mmHg, SpO ₂ :94%.
120 minutes	20 vials of antivenom administered under continuous vasopressor and inotropic support.	Stabilization achieved. Respiratory distress resolved; lung fields clear on auscultation.
4 hours	Patient stabilized and transferred to the medical casualty ward.	Hemodynamically stable.
Days 1–3	Inpatient monitoring. Serial testing performed.	No evidence of VICC, neuromuscular paralysis, or acute kidney injury.
Day 4	Discharge home.	

There was a strong family history of allergic disorders and anaphylaxis. Her mother died at approximately 60 years of age following a possible anaphylactic reaction to wasp stings. Her elder sister suffers from urticaria triggered by fish, meat, insect bites, and even water exposure. Additionally, the elder sister’s two daughters also experience urticarial reactions following insect bites. Notably, male family members are unaffected. Assessment of physical urticaria subtypes revealed dermatographia in both the patient and her sister. The sister additionally demonstrated aquagenic and cold urticaria, while other subtypes (cholinergic, delayed pressure, vibratory, solar, and exercise-induced) were absent in both individuals.

Serum testing revealed a markedly elevated alpha-gal-specific IgE level of 17.1 kUA/L (immunoCAP class 3, high positive: 3.50–17.49 kUA/L), supporting the diagnosis of alpha-gal syndrome. This was performed using the allergen-specific IgE fluorescent enzyme immunoassay (Phadia ImmunoCap, Phadia 100). Her total IgE level was 468.3 IU/L, with an alpha-gal-specific IgE-to-total IgE ratio of 3.6%, which is associated with a high likelihood of clinically significant meat allergy.

There is no universally accepted reference range for the ratio.³ However, these values are considered clinically meaningful for risk stratification and diagnostic purposes.^{3,4} Serum trypase was not measured due to unavailability.

Table 2. Laboratory investigations performed during hospitalization*

Investigations	Reference range	Day 1	Day 3
White cell count	4–10 ×10 ⁹ /L	14.53	12.49
Neutrophils	50–70%	95.3	90.6
Lymphocytes	20–40%	2.3	8.1
Eosinophils	1.0–6.0%	0.0	0.0
Haemoglobin	11–17 g/dL	11.4	11.8
Platelets	150–400 ×10 ⁹ /L	347	380
Blood urea	2.8–7.7 mmol/L	4.8	NR
Serum creatinine	60–120 µmol/L	94	84
Sodium	135–148 mmol/L	146	146
Potassium	3.6–5.0 mmol/L	3.2	4.1
C-reactive protein	<5 mg/L	3.99	NR
Prothrombin time	12.3–16.3 s	15.9	16.3
International normalised ratio	<1.5	1.13	0.92
Activated partial thromboplastin time	25–38 s	22.8	NR
Urine full report	protein trace; pus cells 10–12/HPF; red cells 50–60/HPF		
Blood picture	reactive changes due to inflammation, no evidence of microangiopathic hemolytic anemia		

* NR – not repeated, HPF – high power field

Discussion

Refractory anaphylaxis developed immediately after antivenom administration in this patient, requiring dual vasopressor support. The temporal relationship between antivenom administration and the onset of anaphylaxis, together with markedly elevated alpha-gal-specific IgE levels, suggests a biologically plausible association between underlying alpha-gal sensitization and severe immediate hypersensitivity following equine-derived antivenom. However, in the absence of definitive immunological confirmation, a causal relationship cannot be established. While similar reactions have been reported previously, this case adds to the limited literature by describing severe refractory anaphylaxis following Russell’s viper antivenom in South Asia, in a patient with previously unrecognized alpha-gal syndrome and a female-predominant familial clustering of allergic manifestations. The case also highlights the diagnostic and therapeutic challenges encountered in resource-limited emergency settings.

Previous reports have described immediate hypersensitivity reactions to equine-derived antivenom in patients with alpha-gal syndrome and have demonstrated that alpha-gal epitopes present on equine F(ab’)₂ antivenoms can trigger IgE-mediated reactions.^{10,13,16} However, published cases remain few, and most originate from North America or Europe. In contrast, our pa-

tient developed refractory anaphylaxis following Indian polyvalent Russell’s viper antivenom in the setting of previously unrecognized alpha-gal syndrome in South Asia. This case therefore extends the existing literature by illustrating the relevance of alpha-gal syndrome in a region where snakebite is common, and access to specialized allergy investigations is limited.

Hard tick species (Ixodidae), recognized as vectors associated with alpha-gal syndrome in other regions, are present in Sri Lanka. Recent evidence also supports the occurrence of alpha-gal syndrome in Sri Lanka, with *Amblyomma testudinarium* proposed as a possible vector.^{5,6} This case underscores an underrecognized risk in snakebite management in tick-endemic regions, where alpha-gal syndrome may be prevalent yet largely undiagnosed. Alpha-gal syndrome is an IgE-mediated allergy to galactose-α-1,3-galactose, classically associated with delayed reactions after mammalian meat ingestion, whereas intravenous exposure to alpha-gal-containing products can trigger immediate anaphylaxis.^{7,8}

Some snake venoms contain terminal alpha-galactosyl residues, such as those from some cobra species (genus *Naja*).⁹ However, whether Russell’s viper venom contains this epitope is yet unknown. Antivenoms derived from mammalian serum, such as horse serum, contain the galactose-α-1,3-galactose (alpha-gal) epitope, which is a known target of IgE-mediated hypersensitivity in patients with alpha-gal syndrome. Pepsin digestion removes the Fc portion but does not eliminate N-linked glycans on Fab fragments that carry the alpha-gal epitope. This epitope binds pre-existing human anti-alpha-gal IgE, triggering basophil activation and anaphylaxis, with higher reaction rates observed in F(ab’)₂ antivenom compared to Fab products.^{10,11} Researchers have identified the alpha-gal epitope as a target of IgE-mediated reactivity in equine antivenoms, thereby explaining how individuals with alpha-gal syndrome can experience immediate anaphylaxis upon first administration. Consequently, the presence of these epitopes in Indian polyvalent antivenom is a biologically plausible cause of such reactions in sensitized patients, as this specific antivenom (Bharath SV Ltd) has been confirmed to contain alpha-gal and elicit high levels of basophil activation in clinical studies. However, it is noteworthy that conflicting findings have been reported, with specific IgE against α-gal not being a major trigger of early anaphylactic reactions in Laotian snakebite patients who received Thai antivenom.^{12,13}

Familial clustering and emerging data on intrinsic host risk factors (including non-B-antigen ABO blood groups and a family history of alpha-gal or other allergies) suggest that, beyond tick exposure alone, some individuals may have an underlying susceptibility to the clinical manifestations of alpha-gal syndrome.¹⁴ Hereditary α-trypsaemia is a well-described familial trait that

increases baseline tryptase and can amplify anaphylaxis risk and severity, so it is a biologically plausible contributor to multigenerational clustering of severe reactions; it does not replace tick exposure as the main driver of α -gal IgE sensitisation.¹⁵

The management of snakebite in a patient with alpha-gal syndrome creates a therapeutic dilemma. The standard of care, the administration of polyclonal antivenom derived from horse serum, carries a high alpha-gal load and poses a risk of anaphylaxis.¹⁶ Pre-medication protocols, graded challenge, or consideration of novel, low-IgE antivenoms are options for preventing such complications.^{16,17}

This case has several limitations. First, the indication for antivenom administration was not unequivocal, as the patient did not develop definitive evidence of systemic envenomation such as coagulopathy, neurotoxicity, or progressive systemic manifestations beyond persistent vomiting. This represents an important clinical consideration, as unnecessary antivenom exposure may increase the risk of severe hypersensitivity reactions. Although snake venom itself may have contributed to the allergic reaction, the anaphylactic episode occurred shortly after antivenom administration, making antivenom the more likely trigger. Furthermore, several specialized investigations were unavailable, including serial serum tryptase measurements, antivenom-specific IgE assays, and basophil activation testing. Therefore, although alpha-gal syndrome is biologically plausible, definitive immunological confirmation could not be established. In addition, serial follow-up measurements of alpha-gal-specific IgE were unavailable; therefore, changes in sensitization over time following avoidance of mammalian products and subsequent tick exposure could not be assessed.

Patient's perspective

This patient was concerned about her medical condition, as she had lost her mother due to an anaphylactic reaction, and she was also having similar reactions. Therefore, she wants to know how to prevent recurrences, what other tests are available to aid in diagnosis, and what treatment options are available. She was happy with our diagnosis and the instructions, and she and her family members will be more cautious about future exposures that could trigger additional allergic reactions.

Conclusion

A high index of clinical suspicion for alpha-gal syndrome is needed in patients presenting with unexplained anaphylaxis, recurrent reactions to insect stings, mammalian meat allergy, or severe reactions to biologic products, including antivenom. An important clinical learning point from this case is that the indication for antivenom administration was not unequivocal, as the

patient did not develop objective evidence of systemic envenomation such as coagulopathy, neurotoxicity, or progressive systemic manifestations beyond persistent vomiting. This highlights the importance of assessing the risk-benefit balance before administering antivenom, particularly in patients with a history suggestive of severe allergic disease. Future studies are warranted to determine whether routine risk assessment or alternative antivenom strategies could improve patient safety.

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Author contributions

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

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Ethics approval

Patient's consent was obtained for the publication of this case report and accompanying clinical details. Written informed consent for publication was obtained from the patient in Sinhala, the local language. The signed consent form is uploaded as a supplementary document. This is a single case report. Formal ethics committee approval was not required under institutional policy; patient consent for publication was obtained as noted above.

Use of AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Vera Health & ChatGPT only to check for errors and perform proofreading in some parts of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the published article.

References

1. Commins SP, Platts-Mills TAE. Delayed anaphylaxis to red meat in patients with IgE specific for galactose α -1,3-galactose (alpha-gal). *Curr Allergy Asthma Rep*. 2013;13(1):72-77. doi:10.1007/s11882-012-0315-y

2. Platts-Mills TAE, Commins SP, Biedermann T, et al. On the cause and consequences of IgE to galactose- α -1,3-galactose: A report from the National Institute of Allergy and Infectious Diseases Workshop on understanding IgE-Mediated mammalian meat allergy. *J Allergy Clin Immunol*. 2020;145:1061-1071. doi:10.1016/j.jaci.2020.01.047
3. Mabelane T, Basera W, Botha M, Thomas HF, Ramjith J, Levin ME. Predictive values of alpha-gal IgE levels and alpha-gal IgE: Total IgE ratio and oral food challenge-proven meat allergy in a population with a high prevalence of reported red meat allergy. *Pediatr Allergy and Immunol*. 2018;29(8):841-849. doi:10.1111/pai.12969
4. Iglesia EGA, Kwan M, Virkud Y V, Iweala OI. Management of food allergies and food-related anaphylaxis. *JAMA*. 2024;331(6):510-521. doi:10.1001/jama.2023.26857
5. Dasch GA, Eremeeva ME, Zambrano ML, Premaratna R, Kularatne SAM, Rajapakse RPVJ. Molecular characterisation of Rickettsial agents in ticks (Acari: Ixodidae) from Sri Lanka. *Am J Trop Med Hyg*. 2022;106(6):1613-1623. doi:10.4269/ajtmh.21-0995
6. Dasanayake D, Karunatilake C, Karunaratne C, Fernando N, Iddagoda J, de Silva R. Alpha-gal allergy in a South Asian country. *BMC Immunol*. 2025;26(1):37. doi:10.1186/s12865-025-00717-4
7. Román-Carrasco P, Hemmer W, Cabezas-Cruz A, Hordžić A, de la Fuente J, Swoboda I. The α -Gal syndrome and potential mechanisms. *Front Allergy*. 2021;2:783279. doi:10.3389/falgy.2021.783279
8. Chung CH, Mirakhur B, Chan E, et al. Cetuximab-induced anaphylaxis and IgE specific for galactose- α -1,3-galactose. *N Engl J Med*. 2008;358:1109-1117. doi:10.1056/NEJMoa074943
9. Gowda DC, Davidson EA. Structural features of carbohydrate moieties in snake venom glycoproteins. *Biochem Biophys Res Commun*. 1992;182(1):294-301. doi:10.1016/S0006-291X(05)80144-5
10. Wai M, Filip AB, Abbott A, Martin A, Donnell R, Sutton J. Anaphylaxis in Alpha-Gal patients treated with F(ab')₂ for snake bite venom. *Toxicol Commun*. 2024;8(1):2392459 doi: 10.1080/24734306.2024.2392459
11. Filip AB, Mahmood L, Tilly C, et al. Prevalence of adverse drug reactions: Comparing Crotalidae immune polyvalent antivenoms F(ab')₂ and F(ab) in a galactose-alpha-1,3-galactose (alpha-gal) endemic area. *Ann Emerg Med*. 2026;87(3):292-302. doi:10.1016/j.annemerg-med.2025.06.618
12. Blessmann J, Hanlodsomphou S, Santisouk B, et al. Serum IgE against galactose-alpha-1,3-galactose is common in Laotian patients with snakebite envenoming, but not the major trigger for early anaphylactic reactions to antivenom. *Toxicon X*. 2020;7:100054. doi:10.1016/j.toxcx.2020.100054
13. Fischer J, Eberlein B, Hilger C, et al. Alpha-gal is a possible target of IgE-mediated reactivity to antivenom. *Allergy*. 2017;72(5):764-771. doi: 10.1111/all.13073
14. Taylor ML. Intrinsic risk factors for alpha-gal syndrome in a case-control study, 2019 to 2020. *Ann Allergy Asthma Immunol*. 2024;132(6):759-764.e2. doi:10.1016/j.anai.2024.01.029
15. Kačar M, Rijavec M, Šelb J, Korošec P. Clonal mast cell disorders and hereditary α -tryptasemia as risk factors for anaphylaxis. *Clin Exp Allergy*. 2023;53(4):392-404. doi:10.1111/cea.14264
16. Banner W, Edelen K, Epperson LC, Moore E. Hypersensitivity reactions due to North American pit viper antivenom administration and confirmed elevation of alpha-gal IgE. *Toxicol Commun*. 2024;8(1):2314314. doi:10.1080/24734306.2024.2314314
17. De Silva HA, Ryan NM, De Silva HJ. Adverse reactions to snake antivenom, and their prevention and treatment. *Br J Clin Pharmacol*. 2016;81(3):446-452. doi:10.1111/bcp.12739



CASE REPORT

Feasibility of an evidence-based multidisciplinary care framework in a Pompe disease carrier pregnancy – a case report

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ABSTRACT

Introduction and aim. Pompe disease-carrier pregnancies present complex management challenges that require integrated genetic, biochemical, and psychosocial interventions. The current literature lacks comprehensive management frameworks that address the multifaceted needs of these high-risk pregnancies.

Description of the case. We report the treatment of a 31-year-old South Asian woman with three previous pregnancies affected by Pompe disease using a systematic multidisciplinary protocol developed following the current European Reference Network guidelines. Both partners were compound heterozygous carriers with documented pathogenic variants. The protocol integrated early genetic diagnosis through sampling of the chorionic villus, systematic biochemical monitoring, structured psychological support, and coordinated multidisciplinary care involving eight specialist services. Maternal psychological scores showed substantial improvement with the Edinburgh Postnatal Depression Scale decreasing from 14 to 2 (86% improvement) and the Generalized Anxiety Disorder Scale decreasing from 12 to 1 (92% improvement) after protocol-based psychological support without concurrent medication or therapy. Six-month follow-up confirmed sustained clinical and psychological benefits with normal infant development.

Conclusion. This case demonstrates the feasibility of implementing a systematic multidisciplinary care framework for Pompe disease-carrier pregnancy. Although preliminary results are encouraging, validation through larger cohort studies is necessary before widespread implementation, considering resource availability, genetic diversity, and cultural factors that may influence reproducibility in different healthcare settings.

Keywords. lysosomal storage disorders, multidisciplinary care, Pompe disease, pregnancy management, prenatal diagnosis

Introduction

Pompe disease, or glycogen storage disease type II, is a rare autosomal recessive lysosomal storage disorder caused by acid alpha-glucosidase (GAA) deficiency, leading to glycogen accumulation in cardiac and skeletal muscle.¹ Global birth prevalence is estimated at 2.0 cases (95% CI: 1.5–2.4) per 100,000 live births.² When both partners carry pathogenic *variants of GAA*, subsequent pregnancies carry a 25% risk of recurrence and substan-

tial psychological burden on the family.^{2,3} Current literature on managing such high-risk pregnancies remains limited, consisting largely of isolated case reports rather than structured evidence-based management frameworks.⁴ Pregnant women with rare diseases have been identified as a particularly vulnerable population, with recent reviews emphasizing the need to address physical, emotional, and psychological dimensions of care concurrently.⁵ In 2024, the European Reference Network

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for Metabolic Diseases (MetabERN) published clinical pathway recommendations for Pompe disease that emphasize coordinated multidisciplinary care, but practical guidance on applying these recommendations within an actual pregnancy care pathway remains limited.⁶

This report addresses that gap by describing the systematic implementation of an evidence-based multidisciplinary care framework, rather than simply presenting a rare clinical scenario. We present the case of a Pompe disease carrier couple with three previous affected pregnancies to demonstrate the feasibility and real-world practical implementation of MetabERN-aligned multidisciplinary care in guiding a subsequent pregnancy to a favorable outcome.

Aim

The objective of this case report is to demonstrate the feasibility and practical implementation of a comprehensive, evidence-based multidisciplinary care framework for the management of pregnancy carriers of Pompe disease, addressing existing gaps between published clinical guidelines and their application in routine clinical practice.

Description of the case

Demographics and history of the patient

A 31-year-old South Asian woman presented at 8 weeks of gestation for specialized pregnancy management. She had been married for nine years, with a reproductive history of three previous pregnancies, all affected by Pompe disease, each ending in the child’s death.

Genetic background

Both partners were confirmed compound heterozygous carriers of pathogenic GAA variants (Table 2 summarizes specific variants), conferring a 25% risk of recurrence for each pregnancy.

Previous pregnancy history

- First pregnancy (2018): twin gestation, intrauterine fetal death at 28 weeks’ gestation; autopsy showed bilateral cardiomegaly with glycogen deposition
- Second pregnancy (2019): male infant, progressive symptoms at 4 months of age despite enzyme replacement therapy; died at 7 months of cardiorespiratory failure
- Third pregnancy (2020): prenatal diagnosis confirmed affected status; delivered at 34 weeks of gestation due to severe polyhydramnios and fetal cardiomegaly; child died at 18 months despite ongoing treatment

Current pregnancy: diagnostic and clinical course

Written informed consent was obtained from the patient; as this report describes the application of existing

clinical guidelines rather than experimental research, formal approval from the ethics committee was not required.

At 5 weeks of gestation, the patient underwent genetic counseling and a baseline psychological evaluation, which showed an Edinburgh Postnatal Depression Scale (EPDS) score of 14, a generalized anxiety disorder-7 (GAD-7) score of 12, and a perceived stress scale score of 28, consistent with significant antenatal distress. Structured psychological support was initiated without concurrent medication or psychotherapy (further details on the multidisciplinary care structure are provided in the discussion).

Transcervical chorionic villus sampling was performed at 11 weeks of gestation, showing fetal GAA enzyme activity of 18.2 nmol/hour/mg protein (normal range of 10–25 nmol/hour/mg protein). Definitive molecular testing at 13 weeks of gestation identified heterozygosity for only the maternal c.525delT variant, confirming carrier status and excluding the four parental pathogenic variants.

Maternal GAA enzyme levels remained stable (2.0–2.3 nmol/hour/mg protein, coefficient of variation 6.8%) throughout pregnancy; the clinical significance of this stability in carriers is not established, but provided reassurance alongside baseline data. The fetal anatomical survey at 16 weeks of gestation and fetal echocardiography at 20 weeks of gestation showed normal development.

In the third trimester, EPDS and GAD-7 scores had decreased from baseline, coinciding with ongoing genetic reassurance and structured psychological support; This improvement is reported descriptively and cannot be causally attributed to any single intervention in this observation of a single patient.

Delivery and neonatal outcomes

Elective induction at 38 weeks and 2 days gestation resulted in vaginal delivery of a healthy female neonate (2,850 g; Apgar 8 at 1 minute, 9 at 5 minutes). Cord blood GAA enzyme activity was normal at 22.1 nmol/hour/mg protein. Neonatal genetic testing and echocardiography confirmed the prenatal findings.

Follow-up

At 6 months of age, the infant showed age-appropriate developmental milestones (weight 7.2 kg, 50th percentile; length 66 cm, 45th percentile). Maternal EPDS and GAD-7 scores remained low in this assessment. No emergency visits or antepartum hospitalizations occurred during this pregnancy, in contrast to the previous course of pregnancy course; these observations are based on a single 6-month follow-up and should be interpreted as preliminary.

Discussion

Framework implementation and preliminary outcomes

This case documents a comprehensive, evidence-based multidisciplinary approach to the treatment of pregnant women with Pompe disease. Although further validation is necessary, its successful implementation suggests potential improvements in clinical outcomes and psychological well-being compared to family previous pregnancy experiences.

Integration of structured psychological support throughout pregnancy addresses the needs identified in the recent literature on rare disease and high-risk pregnancies.⁷ In this case, coordinated input from eight specialist services was associated with favorable maternal and neonatal outcomes, consistent with reports that multidisciplinary care models improve pregnancy outcomes in other rare and high-risk maternal conditions.⁸

Psychological impact and intervention

A substantial improvement in maternal psychological well-being occurred after structured psychological support alone, without concurrent medication or therapy. The Edinburgh Postnatal Depression Scale improved from 14 to 2, and the Generalized Anxiety Disorder Scale from 12 to 1, both sustained through 6-month follow-up. Non-pharmacological psychological interventions have been associated with measurable reductions in anxiety, depression, and stress in other high-risk pregnancy populations, although in this single case attribution of improvement specifically to our framework requires confirmation through controlled studies.

Clinical outcomes and diagnostic accuracy

This approach ensured concordance between prenatal and postnatal testing in this case. When parental variants are known, combined molecular and biochemical tests represent a recommended diagnostic approach for the diagnosis of Pompe disease prenatal and postnatal, providing comprehensive diagnostic information as demonstrated in our case.⁹

Limitations and considerations

The principal limitations of this single case report include a significantly constrained evidence level due to the absence of a control group and limited generalizability arising from specific genetic variants, family history, and institutional resources. Cultural factors, the genetic diversity of Pompe disease, and differences in healthcare system resources can substantially affect reproducibility of this approach in different settings.

Only preliminary long-term outcome data are available at six months of follow-up; larger cohorts and extended follow-up periods are needed to determine long-term benefits or complications. Future considerations include the possibility that framework modifi-

cations may be required for specific genetic variants; implementation requires specialized multidisciplinary teams and substantial resources; and cultural and healthcare system adaptations will be necessary for different populations. Cost-effectiveness analysis is needed before a larger implementation, and healthcare personnel would need training in rare disease pregnancy.

Integration with current guidelines

Our approach aligns with the recommendations of the 2024 MetabERN clinical pathway for Pompe disease, which emphasize multidisciplinary care coordination.⁶ Adapting these guidelines for pregnancy management requires consideration of pregnancy-specific factors and resource availability in different healthcare settings.

Future research directions

Despite the potential advantages of systematic multidisciplinary approaches, this preliminary report underscores the need for:

- Prospective multicenter cohort studies evaluating effectiveness in diverse populations
- Development of validated outcome measures specifically for rare disease pregnancies
- Cost-effectiveness analysis comparing systematic frameworks to standard care
- Investigation of the optimal timing and duration of psychological interventions
- Evaluation of long-term family impact beyond the immediate postpartum period

Conclusion

This case demonstrates that the implementation of a structured multidisciplinary framework for Pompe disease carrier pregnancy management is feasible. In this single case, the framework resulted in favorable clinical outcomes and notable improvements in psychological well-being despite three previous devastating losses.

Initial outcomes, including coordinated multidisciplinary care, structured psychological support, and successful prenatal diagnosis, are encouraging; however, this single case cannot establish the efficacy of the framework. The observed improvements may be attributable to various factors beyond the framework itself, including previous experiences, specific genetic variants involved, and available institutional resources.

The most significant contributions demonstrated were the structuring of multidisciplinary coordination and the integration of systematic psychological support with clinical care. Framework development and implementation required substantial resources and specialized expertise, which can challenge utilization across different healthcare settings. Larger, controlled, multicenter studies, alongside cost-effectiveness analysis

and adaptation for diverse populations and healthcare systems, are necessary to determine the true effectiveness and generalizability of this approach. This case represents an initial step toward the development of an evidence-based framework for rare disease pregnancies, which requires substantial further validation.

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Declarations

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Author contributions

Conceptualization, S.P. and S.K.R.; Methodology, S.K.R. and S.T.; Protocol Development, S.K.R. and S.T.; Investigation, S.P.; Writing – Original Draft, S.P.; Writing – Review & Editing, S.K.R. and S.T.; Supervision, S.K.R. and S.T.

Conflicts of interest

The authors declare that they have no conflict of interest.

Data availability

The data sets generated during this study are available from the corresponding author on reasonable request.

Ethics approval

Written informed consent for publication was obtained from the patient. This case report describes the implementation of existing clinical guidelines and does not constitute experimental research that requires approval from the Ethics Review Board. However, we acknowledge the institutional Ethics Review Committee for their guidance on the development of clinical protocols.

References

1. Meena NK, Raben N. Pompe disease: new developments in an old lysosomal storage disorder. *Biomolecules*. 2020;10(9):1339. doi:10.3390/biom10091339
2. Waters D, Adeloye D, Woolham D, Wastnedge E, Patel S, Rudan I. Global birth prevalence and mortality from in-born errors of metabolism: a systematic analysis of the evidence. *J Glob Health*. 2018;8(2):021102. doi:10.7189/jogh.08.021102
3. Taglia A, Picillo E, D'Ambrosio P, Cecio MR, Viggiano E, Politano L. Genetic counseling in Pompe disease. *Acta Myol*. 2011;30(3):179-181.
4. Witt S, Schuett K, Wiegand-Grefe S, Boettcher J, Quitmann J. Living with a rare disease - experiences and needs in pediatric patients and their parents. *Orphanet J Rare Dis*. 2023;18(1):242. doi:10.1186/s13023-023-02837-9
5. Talay L, Vickers M, Lu D. Nurse practitioner and general practitioner error rates in a large digital health service: a retrospective cohort analysis. *Nurs Rep*. 2024;14(4):3407-3416. doi:10.3390/nursrep14040246
6. Parenti G, Fecarotta S, Alagia M, et al. The European reference network for metabolic diseases (MetabERN) clinical pathway recommendations for Pompe disease (acid maltase deficiency, glycogen storage disease type II). *Orphanet J Rare Dis*. 2024;19(1):408. doi:10.1186/s13023-024-03373-w
7. Yoo H, Ahn S. Effects of nonpharmacological interventions on the psychological health of high-risk pregnant women: a systematic review and meta-analysis. *Korean J Women Health Nurs*. 2021;27(3):180-195. doi:10.4069/kjwhn.2021.09.17
8. Añón-Oñate I, Cáliz-Cáliz R, Rosa-Garrido C, Pérez-Galán MJ, Quirosa-Flores S, Pancorbo-Hidalgo PL. Multidisciplinary unit improves pregnancy outcomes in women with rheumatic diseases and hereditary thrombophilias: an observational study. *J Clin Med*. 2021;10(7):1487. doi:10.3390/jcm10071487
9. Van den Veyver IB, Chandler N, Wilkins-Haug LE, Wapner RJ, Chitty LS; ISPD Board of Directors. International Society for Prenatal Diagnosis updated position statement on the use of genome-wide sequencing for prenatal diagnosis. *Prenat Diagn*. 2022;42(6):796-803. doi:10.1002/pd.6157



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When you submit a manuscript to the Eur J Clin Exp Med, we will take it to imply that the manuscript has not already been published or submitted elsewhere. If similar or related work has been published or submitted elsewhere, then you must provide a copy of this work with the submitted manuscript. You may not submit your manuscript elsewhere while it is under consideration in the Eur J Clin Exp Med. If the manuscript includes personal communications, please provide a written statement of permission from any person who is quoted. Permission by email is acceptable.

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.

Author responsibilities

Authorship provides credit for a researcher's contributions to a study and carries accountability. Authors are expected to fulfill 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, although reviewers are aware of the authors' identities as part of the single-blind peer review process. 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 accepted manuscripts, 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 journal's Archiving policy. This confidentiality requirement does not restrict authors from sharing their own work as preprints or accepted manuscripts in accordance with the journal's Archiving policy.

Referee suggestions

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 journal verifies reviewer identity and affiliation; the use of non-institutional e-mail addresses for suggested reviewers may be declined. 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. The ethics statement must include the name of the approving ethics committee, approval or decision number, and date of approval; studies involving human participants must confirm compliance with the Declaration of Helsinki, and animal studies must comply with the ARRIVE guidelines and the principles of the 3Rs (Replacement, Reduction, Refinement).

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 form their own judgments of potential bias, authors must declare any competing financial and/or non-financial interests related to the work described. Competing interests are defined as financial or non-financial relationships or circumstances that could directly influence, or be perceived to influence, the objectivity, integrity, or interpretation of the reported research.

Examples of potential competing interests include, but are not limited to, employment, consultancies, stock ownership or options, honoraria, paid expert testimony, patents or patent applications, and other personal or institutional relationships. Sources of research funding must be disclosed separately in the Funding statement, regardless of whether they are considered a competing interest.

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.
- Competing interests: Dr X's work has been funded by A. He has received compensation as a member of the scientific advisory board of B and owns stock in the company. He has also consulted for C and received compensation. Dr Y and Dr Z declare no competing interests.

Funding information must be reported in a separate Funding statement and should not be included in the Competing interests declaration.

Application to Editors or Editorial Board Members

Editors and Editorial Board Members of the *Eur J Clin Exp Med* are required to disclose any conflicts of interest and may be excluded from the peer review process if such conflicts exist. They should also recuse themselves from handling manuscripts where a conflict of interest is present. This may include, but is not limited to, having previously co-published with one or more of the authors or being affiliated with the same institution as one or more of the authors.

If an Editor or Editorial Board Member of *Eur J Clin Exp Med* is listed as an author on a manuscript, it is recommended that they declare this in the competing interests section of the submission. If they are an author or have any other conflict of interest regarding a specific manuscript, another editor will be assigned to oversee the peer review process. These submissions will undergo the same review process as any other manuscript.

Editors and Editorial Board Members of *Eur J Clin Exp Med* are welcome to submit their own papers to the journal. These submissions receive no special priority, and the status of the Editor or Editorial Board Member does not influence the editorial consideration.

Peer-reviewers

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 data in repositories or by publishing the data and files as supplementary information in this journal. The journal may request access to underlying data, protocols, or original materials for editorial assessment. Where data cannot be publicly shared due to privacy, ethical, or legal restrictions, authors must describe the restrictions and provide a mechanism for qualified access where feasible.

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large datasets 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 re3data.org and DataCite resources. The data repository name, link to the dataset (URL) and accession number, DOI or handle number of the dataset must be provided in the paper. The journal Data also accepts submissions of dataset 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 restric-

tions 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].

Declaration of use of AI and AI-assisted technologies in the writing process

Authors are required to disclose the use of generative AI in the writing of scientific papers upon submission. This guidance applies solely to the writing process and does not pertain to the use of AI tools for data analysis or drawing insights as part of the research process.

Generative AI and AI-assisted technologies should be used in the writing process solely to enhance the readability and language of the manuscript. These tools must be employed under human supervision, with authors thoroughly reviewing and editing the output, as AI can produce seemingly credible content that may be inaccurate, incomplete, or biased. Ultimately, authors are responsible and accountable for the content of the work.

Authors should not list generative AI and AI-assisted technologies as an author or co-author, nor cite AI as an author. Authorship entails responsibilities and tasks that can solely be assigned to and carried out by humans. The Eur J Clin Exp Med does not allow the use of generative AI or AI-assisted tools to create or modify images in submitted manuscripts. This includes activities such as enhancing, obscuring, moving, removing, or introducing specific elements within an image or figure. Adjustments to brightness, contrast, or color balance are permitted as long as they do not obscure or remove any information present in the original. Image forensics tools or specialized software may be used to detect suspected irregularities in images within submitted manuscripts. Authors must not upload confidential, personal, or patient-identifiable information to generative AI tools.

The only exception to this policy is when AI or AI-assisted tools are integral to the research design or methods, such as in AI-assisted imaging approaches used to generate or interpret the underlying research data, particularly in fields like biomedical imaging. In such cases, the use of AI must be described in a reproducible manner within the methods section. This description should include details on how the AI or AI-assisted tools were applied in the image creation or alteration process, along with the name of the model or tool, version and extension numbers, and the manufacturer.

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- Title of new section: Use of AI and AI-assisted technologies in the writing process.
- Example of a statement: “During the preparation of this work the author(s) used [NAME TOOL/SERVICE] in order to [REASON]. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.”

The declaration does not apply to the use of basic tools, such as those used for checking grammar, spelling, and references. If there is nothing to disclose, no statement is required.

NOTE: to protect authors’ rights and the confidentiality of their research, the Eur J Clin Exp Med does not currently allow the use of Generative AI or AI-assisted technologies such as ChatGPT or similar services by reviewers or editors in the peer review and manuscript evaluation process. Suspected breaches may result in reviewer removal and rejection of the manuscript or other editorial actions. Moreover, editors may decline to move forward with manuscripts if AI is used inappropriately.

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 into one of four categories: Publisher Correction (formerly Erratum), Author Correction (formerly Corrigendum), Retraction or Addendum.

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.

Expression of Concern. A notice issued when serious concerns have been raised about a publication, but an investigation is ongoing and conclusive evidence is not yet available.

Corrections, retractions, and expressions of concern are published as separate notices and are permanently linked to the original article. The online article and PDF are clearly labeled to reflect the amendment status.

Archiving policy

Articles published in Eur J Clin Exp Med are long-term deposited in the repository of the University of Rzeszów. Authors of articles are permitted to self-archive the submitted (preprint) version of the article at any time, and may self-archive the accepted (peer-reviewed) version. The authors may also deposit the published version of the article. On submission of the manuscript, authors may deposit the submitted version in their personal, institutional, or online preprint repository. The first page of the manuscript must clearly display the following wording: “*This paper is a preprint of a paper submitted to European Journal of Clinical and Experimental Medicine (ISSN: 2544-1361)*”. If the paper is rejected, authors must remove all mention of the journal.

The author may deposit the accepted manuscript of the paper (accepted version of the manuscript after peer-review and content amendments, but before copyediting, typesetting and proof correction) to the author’s personal website, provided that it is non-commercial, and to the repository of the author’s institution with acknowledgement of the Journal (acknowledgement should be made as follows: “*This is an accepted peer-reviewed version of the paper. The published version of the article is available at European Journal of Clinical and Experimental Medicine (ISSN: 2544-1361), at [https://doi.org/\[DOI of the article\]](https://doi.org/[DOI of the article])*”). Published version of an open-access article. The author may deposit the published version of the paper (final edited and typeset version that is made publicly available by the Publisher and can be considered an article) to any institutional repository, and distribute and make it publicly available in any way with acknowledgement to the Journal (acknowledgement should be made as follows: “*This is a published version of the paper, available at European Journal of Clinical and Experimental Medicine (ISSN: 2544-1361), at [https://doi.org/\[DOI of the article\]](https://doi.org/[DOI of the article])*”). The journal ensures long-term digital preservation of the version of record through institutional repository archiving and persistent identifiers (DOIs).

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The Eur J Clin Exp Med permits the posting of manuscripts on recognized preprint servers prior to submission

or during the peer review process. Authors must disclose the existence of any preprint version at the time of submission and provide the preprint DOI or URL.

Preprints are not considered peer-reviewed publications and must not be cited as references in manuscripts submitted to the Eur J Clin Exp Med.

Following publication in the journal, authors are encouraged to update the preprint record by providing a clear link to the final published version of record (VoR), including the article DOI.

Sponsorship and advertising

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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.

Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts may be rejected at the editorial screening stage due to: out-of-scope content, insufficient novelty or methodological rigor, ethical concerns, suspected misconduct, poor reporting quality, or failure to comply with author guidelines. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission.

Peer review

Once a manuscript passes the initial checks, it is assigned to at least two independent experts for peer review. The reviewers access the manuscript securely through our online system. The Eur J Clin Exp Med applies a single-blind peer review process, in which the reviewers are aware of the authors' identities, but the authors do not know the identity of the reviewers.

Reviewers are selected by the editorial team based on their subject expertise and the absence of any conflict of interest with the authors or the submitted work. Suggested reviewers must not have co-authored publications with the authors in the last five years or be affiliated with the same institutions.

All peer review comments are treated as confidential and will only be disclosed with the explicit consent of the

reviewer. Reviewers are expected to evaluate manuscripts objectively, fairly, and constructively, and to refrain from any personal criticism of the authors.

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. 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. You can use a template for responding to the reviewers' comments

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 <https://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.

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ORCID

The Eur J Clin Exp Med supports the use of ORCID. The Eur J Clin Exp Med mandates ORCID iDs for all submitting authors; this is published on the final article to promote discoverability and credit. Please provide the ORCID iDs of the authors in the title page.

Submission guidelines

Submission process

Manuscripts for the Eur J Clin Exp Med should be submitted online at <https://mc04.manuscriptcentral.com/pmur>. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the submission website. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

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

Use the Microsoft Word template to prepare your manuscript [[download](#)]

Authors must use Microsoft Word to prepare their manuscript. LaTeX submissions are not accepted. 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 characters on a page).
- The main text should be no more than 4,500 words (not including Abstract, References and Figure legends).
- The title should be no more than 20 words.
- The abstract should be no more than 200 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.

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.

Research manuscripts should comprise:

- Title page: Title, Author list, Affiliations.
- Research manuscript sections: Abstract, Keywords, 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.

- *Materials 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).

The best way to ensure you have obtained appropriate consent for publication in Eur J Clin Exp Med is to use our Eur J Clin Exp Med consent form.

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. We recommend adding as primary the affiliation where most of the research was conducted or supported, but please check with your institution for any contractual agreement requirements. For each affiliation provide the details in the following order: department, institution, city, country.

It is very important that author names and affiliations are correct. Incorrect information can mean a lack of proper attribution or incorrect citation and can even lead to problems with promotion or funding. After the publication of an article, updates or corrections to the author’s address or affiliation may not be permitted.

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 sec-

tion. Please also provide the ORCID iDs of the authors in the title page.

Independent Researcher: If one or all the authors are not currently affiliated with a university, scientific institution or company, or have not been during the development of the manuscript, they should list themselves as an “Independent Researcher”.

Abstract

The abstract should be a total of about 200 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; *Materials 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: Authors must disclose all sources of funding and the role of the funder(s) in study design, data collection, analysis, interpretation, and manuscript preparation. If the funder had no role, authors should explicitly state this.

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).”

Use of AI and AI-assisted technologies in the writing process: Example of a statement: “During the preparation of this work the author(s) used [NAME TOOL/SERVICE] in order to [REASON]. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.”

The declaration does not apply to the use of basic tools, such as those used for checking grammar, spelling, and references. If there is nothing to disclose, no statement is required.

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.

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— **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. <https://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. <https://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.

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