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ul. Kopisto 2A, 35-959 Rzeszów, Poland
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**ORIGINAL PAPER**

# Impact of combat trauma on motivational types in military personnel facing life-threatening danger

Ihor Prykhodko [1], Yanina Matsehora [1], Stanislav Larionov [1], Kateryna Marushchenko [2], Yurii Rumiantsev [2], Anastasiia Bolshakova [3], Olena Bilyk [3], Olena Servachak [4]

[1] National Academy of the National Guard of Ukraine, Kharkiv, Ukraine
[2] Ukrainian Military Medical Academy, Kyiv, Ukraine
[3] Kharkiv State Academy of Culture, Kharkiv, Ukraine
[4] Donetsk National Technical University, Drohobych, Ukraine

**ABSTRACT**

**Introduction and aim.** The motivation of soldiers actively fighting during war is very important. Long-term participation in battles do not have a positive effect on maintaining motivation. The purpose of this study was to determine the motivational types of military personnel with different attitudes toward danger in conditions of a real threat of death after participating in long-term intensive battles.

**Material and methods.** A cross-sectional, descriptive study was conducted among military personnel from the Ukrainian Defense Forces (225 males, 40.73±9.81 years). The Mississippi Scale for Combat-Related Posttraumatic Stress Disorder (MSCRPTSD) was used to diagnose posttraumatic stress disorder (PTSD), the "Disadaptation Express Questionnaire" (DEQ) to identify signs of a violation of the adaptability of the soldier's personality, the 'Resilience to Combat Mental Trauma Questionnaire" (RCMTQ) was used to assess the impact of combat stress, as well as the 'Perspectives assessment of professional motivation in Military Personnel Questionnaire" (APPMMPQ) for a comprehensive assessment of military personnel, including the attitude to danger in conditions of a real threat of death.

**Results.** The relationships between motivational characteristics of conscious attitude towards danger of military personnel and indicators of posttraumatic stress and resistance to combat mental trauma were determined. Two groups (motivational types) of CAD were identified, to which 214 (95.11%) of the participants were assigned: group 1 with low profile 191 (84.89%) and group 2 with a medium-high profile 23 (10.22%). The profile of negative emotional reactions and states of group 2 was located mainly in the range of 1.5–3.5 points with peaks on the scales of "Anxiety", "Unwillingness to communicate" and "Irritability". The profile of subgroup 1.2 was located in the range of 2-4 points and was quite close to the profile of group 2 with peaks on the scales of 'Anxiety' and "Distrust of commanders".

**Conclusion.** A high level of motivation for a conscious attitude toward danger can reduce the risk of injury to military personnel. Unformed or depleted motivation for a conscious attitude to danger under the conditions of a real threat of death of military personnel increases the risk of mental trauma.

**Keywords.** military personel, motivation, post-traumatic stress

**Corresponding author:** Ihor Prykhodko, e-mail: iprikhodko1966@gmail.com

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## Introduction

The Russian war against Ukraine, which began on February 24, 2022, requires a growing mobilization of the Ukrainian civilian population for military service. Combat operations are always associated with a high level of danger to the health and life of military personnel, and many experience combat stress.[1-4] However, direct participation in the battles is not the only threat to the life and health of personnel, the occurrence of post-traumatic stress (PTS), or a necessary and sufficient cause of mental disorders.[5,6] It is important to take into account life-threatening military incidents during military deployment, psychological characteristics of the personality, as well as the conditions of the social environment of post-traumatic recovery.[7,8]

The experience of Israeli specialists has shown that a high existential threat leads to an increased willingness of military personnel to engage in life-threatening behavior.[9,10] It was found that soldiers with combat experience, when under threat of death, were more concerned about not letting down their comrades and subordinates than about fear of death.[11] But for soldiers without combat experience, these results were the opposite. This paradox can be explained using terror management theory (TMT), which attempts to explain a wide range of human behavior in terms of the defense mechanisms by which people protect themselves from fear of their own death.[12,13] The authors of TMT argued that a person's ability to predict the future and self-reflectively lead to an awareness of one's mortality, which, in turn, can manifest itself emotionally in increased anxiety and fear.[14,15]

The concept of appeasement indicates that some people have 'supersocial activity' that allows them to take control of their emotions when faced with a real threat to life, suppress the natural 'fight / flight / freeze' stress response, and formulate interactions in such a way as to reduce the likelihood of provoking aggression toward themselves and help de-escalate the situation.[16]

One of the factors in the formation of PTS and its chronicity is the personality of a serviceman, which influences the results of professional activity through motivational processes.[17] Such a specific type of professional motivation as a conscious attitude to danger indicates the ability of an individual to realize motivation in conditions that threaten health and life.[13] This motivation is important for the formation of resilience to combat mental trauma, is associated with the identification of the role of a serviceman in protecting his country, and forms a sense of duty. Studying the motivational characteristics of military personnel in life-threatening danger will allow adjustment of military and psychological training activities. In the future, this will increase the psychological resilience of the soldiers and reduce the psychogenic losses of the military personnel during combat operations. However, the role of such motivation when military personnel participate in long-term, intensive combat operations has not been studied.

## Aim

The purpose of this study was to determine the motivational types of military personnel with different attitudes toward danger in conditions of a real threat of death after participating in long-term intensive battles.

## Material and methods

### *Study design and participants*

A a cross-sectional, descriptive study was conducted among military personnel from Ukrainian Defense Forces (225 males, between 18 and 59 years of age, 40.73±9.81 years). All of included participants were sent after participating in intensive combat operations to undergo a two-week psychological recovery program at a rehabilitation center.[18] The rehabilitation center was created in June 2022 in one of the sanatoriums of the Kharkiv region for the psychological recovery of military personnel after participating in hostilities. The main objective was to relieve the negative impact of combat stress on the psyche of combatants, strengthen their mental health and mobilize their psychological resources, improve adaptation to combat conditions, and quickly return to combat activities. After completion of this program, all servicemen returned to the combat zone to continue performing tasks.

All participants gave their informed consent to be included before participating in the study. The approval of the ethics committee was obtained before the initiation of the study (meeting date; 17/04/2024, decision number; 2024/7). All procedures involving human participants were conducted in accordance with the ethical standards specified by the institutional and national research committee and with the Declaration of Helsinki and its later amendments or comparable ethical standards.

In the study group 118 (52.44%) of the participants were married, 7 (3.11%) were in a common law marriage, 79 (35.11%) were bachelors, 20 (8.89%) were divorced, and 1 (0.44%) were widowers. According to military rank, 161 (71.56%) were privates, 58 (25.78%) were sergeants, and 6 (2.67%) were officers. The mean military service for 60 (26.67%) of the participants was 1.35±0.66 years: they were mobilized at the beginning of large-scale military operations without military service experience. The mean military service for 165 (73.33%) of the participants was 3.47±3.66 years: They served as conscripts or under contract before the beginning of large-scale military operations.

Participants were identified: with various manifestations of acute stress reactions; significant negative experiences, including signs of depression and suicidal ideation; the presence of PTS symptoms; sleep problems 123 (54.67%); somatic complaints 201 (89.33%),

wounds and contusions more than 169 (75%); difficulties in returning to combat missions due to the consequences of illness, injury, and wounds. Participants were randomly selected for the study. Female military personnel were not included in this study because, over the entire program period, less than 0.5% of female combatants participated.

***Instruments***

The study used psychodiagnostic tools to study the impact of traumatic experiences on the mental state of military personnel after participating in intense combat operations and to identify the characteristics of their attitude toward danger in conditions of a real threat of death.

The Mississippi Scale for Combat-Related Posttraumatic Stress Disorder (MSCRPTSD)[19] was used to diagnose posttraumatic stress disorder (PTSD) in military personnel on missions in the war zone, translated into Ukrainian.[20] The scale consists of 35 statements (4 subsets), the answers to which were given on a 5-point Likert scale (Cronbach's α 0.887). Subset 1 (11 statements) describes the symptoms of the 'intrusion' group when the traumatic event is repeatedly repeated in the experience in one (or more) ways. Subset 2 (11 statements) relates the symptoms of the group when there is a constant avoidance of stimuli associated with trauma, blocking of emotional reactions, and numbness, which was not observed before trauma. Subset 3 (8 statements) describes the symptoms of 'excitability' when persistent symptoms increased that were not observed before the injury. Subset 4 (5 statements) describes symptoms associated with guilt and suicidal tendencies. Despite the grouping of statements into four subsets, one general indicator was calculated taking into account the conversion of the answer into a score for direct and inverse statements, reflecting the severity of PTSD symptoms (range from 35 to 175 points, where 35–80 points is a variant of the norm; 81–114 points – separate symptoms of PTSD; 115–175 points – clinical manifestations of PTSD, a psychiatric examination and inpatient examination were recommended).

The "Disadaptation Express Questionnaire" (DEQ) is an abbreviated modified version of the Multilevel Personality Questionnaire 'Adaptation'[20] The DEQ made it possible to identify signs of a violation of the adaptability of the soldier's personality: violation of the regulatory function of the emotional-volitional sphere and self-esteem; lack of prospects for continuing life and the ability to overcome life's difficulties (probability of committing suicidal attempts); loss of moral convictions, the likelihood of committing addictive and delinquent acts; loss of communicative potential (comrade support, reduced ability to accept the help of one's team). DEQ consists of 45 statements included in 5 subscales (Cronbach's α=0.848): "Sincerity of answers', 'Violation of behavioral regulation", and 'Probability of committing suicide attempts", "Violation of moral normativity and "Loss of communicative potential'. Each positive response was worth 1 point, and negative 0 points. The general DEQ scale was calculated as the sum of scores on 4 scales (values on the "Sincerity of answers" scale were not included). The results of the overall DEQ scale were evaluated as follows: 1–10 points – high adaptation to combat operations, sufficient tolerance to adverse mental and physical stress, including under conditions of severe combat stress; 11–14 points – average adaptation, unstable level of performance, especially in combat conditions; 15 points or more – low adaptation (distress and adjustment disorders) that does not meet the requirements for soldiers in combat conditions.

The 'Assessment of Negative Mental Reactions and Conditions in Military Personnel Questionnaire' (ANMRCMPQ) was developed to determine negative emotional experiences in military personnel after participation in hostilities.[21] The ANMRCMPQ contained 16 items: 'Irritability', 'Anger', 'Anger', 'Inattention', 'Self-doubt', 'Devastation', 'Apathy, 'Concern', 'Concern', 'Sense of guilt", "Sense of powerlessness", "Lack of concentration, 'Unwillingness to communicate", "Lack of trust in comrades in the service, 'Lack of trust in commanders", "Inability to perform the assigned tasks" (Cronbach's α=0.944). Self-assessment of the psychological state of the participants rated on a 10-point Likert scale, where 0 is the state is not expressed at all and 10 is expressed to the maximum extent. The results were evaluated separately for each feature as follows: 1–3 points – the condition is not expressed; 4–6 points – the condition is expressed moderately; 7–10 points – the condition is expressed at a high level, and it is necessary to conduct an individual consultation.

The 'Resilience to Combat Mental Trauma Questionnaire" (RCMTQ) was used to assess the impact of combat stress on the mental health of military personnel.[20] RCMTQ is the modified Combat Experience Scale (CES).[22] CES is a 33-item measure that assesses deployment-related experiences. RCMTQ is a 45-item measure combined into 3 scales, answered on a 6-point Likert scale that assesses resilience to combat mental trauma based on combat experience gained (Cronbach's α=0.887). 'The expectation of participating in hostilities scale' allowed us to assess the professional potential of military personnel in possible combat situations. 'The scale to overcome a stressful situation' made it possible to assess the mechanisms to overcome stressful (combat) situations. The scale 'Realization of the acquired combat experience" made it possible to assess the ability to process the acquired combat experience. The general indicator of resilience to combat mental trauma (RCMT) was calculated as the sum of points on 3 scales, taking into account the conversion of the answer into a score for direct and inverse statements. The ob-

tained results of the RCMT indicator were evaluated as follows: 193–225 points – a high level of RCMT, even with a significant complication of the combat situation, such military personnel are able to cooperate and provide assistance to colleagues; 144–192 points – the average level of RCMT reflected a reduced ability to provide support to colleagues; do not always maintain the effectiveness of their activities and control over their mental state; 0–143 points – low level of RCMT reflected psychological unpreparedness to participate in hostilities.

The 'Perspectives assessment of professional motivation in Military Personnel Questionnaire" (APPMMPQ) was used for a comprehensive assessment of military personnel, including the attitude to danger in conditions of a real threat of death.[23] The APPMMPQ contains 180 statements combined into 7 scales: 'Altruistic orientation of a military man", "Professional orientation of a military man", "Self-efficacy of a military man", "Localization of the meaning of life and energy resources of a military man", "Conscious attitude to danger", "Conscious desire for professional communication of a military man", "Satisfaction of a military man with professional activity" (Cronbach's α=0.934). The 'Conscious attitude to danger" (CAD) scale contains 24 statements combined into 4 subscales: 'Desire for safety", "Awareness of mortality', 'Resistance to non-lethal stressors", and "Tolerance of uncertainty" (Cronbach's α=0.786). The responses were evaluated using a six-point Likert scale (0 to 5 points) for each subscale, and the overall indicator of a serviceman's conscious attitude to danger was determined (the results for all subscales were summarized). The results were assessed as follows: 0–15 points – low level of a serviceman's conscious attitude to danger, 16–24 points – average level, 25–30 – high level. The CAD scale was developed taking into account the provisions of TMT.[12,14,15] The scale made it possible to determine the characteristics of the professional motivation of a serviceman in dangerous conditions with or without a real threat of death, as well as in a situation of uncertainty.

The study was carried out in two stages. In the first stage, correlation analysis was used, allowing us to determine the relationships between the motivational characteristics of a conscious attitude to danger and PTS, maladaptation, negative emotional experiences, and RCMT indicators in the general sample of study participants. Using cluster analysis in the second stage of the study, the main groups of participants with different types of conscious attitudes to danger under conditions of a real threat of death were identified. For the data presented, basic descriptive statistics (arithmetical mean M, standard deviation SD) were used. The reliability of the differences in the results of the mean values was determined using the Student's t test. For the assessment of the statistical significance of the differences, we used the level of significance from $p<0.01$ to $p<0.001$. The statistical analysis of the study results was carried out using the SPSS 20.0 (IBM, Armonk, NY, USA).

**Table 1.** The relationships between motivation for a conscious attitude to danger and PTS, maladaptation, and RCMT indicators in the general sample of study participants

| Scale name | Conscious attitude to danger | | | | |
|---|---|---|---|---|---|
| | Desire for safety | Awareness of one's death | Resistance to non-lethal stressors | Tolerance for uncertainty | General indicator of CAD |
| MSCRPTSD | | | | | |
| General indicator of PTSD | -0.16* | -0.21** | -0.33*** | -0.16* | -0.33*** |
| DEQ | | | | | |
| Disruption of behavioral regulation | -0.22** | -0.28*** | -0.42*** | -0.21** | -0.43*** |
| The likelihood of committing suicide attempts | -0.03 | -0.18** | -0.32*** | -0.23** | -0.30*** |
| Violation of moral norms | -0.24*** | -0.09 | -0.25*** | -0.01 | -0.22** |
| Loss of communicative potential | -0.25*** | -0.10 | -0.29*** | -0.18** | -0.30*** |
| General indicator of maladaptation | -0.22** | -0.21** | -0.41*** | -0.21** | -0.40*** |
| ANMRCMPQ | | | | | |
| Irritability | -0.14* | -0.20** | -0.26*** | -0.17* | -0.28*** |
| Anxiety | -0.06 | -0.25*** | -0.33*** | -0.19** | -0.33*** |
| Aggressiveness | -0.14* | -0.10 | -0.17* | -0.19** | -0.20** |
| Anger | -0.16* | -0.13* | -0.13* | -0.14* | -0.18** |
| Inattention | -0.05 | -0.19** | -0.23*** | -0.13* | -0.23*** |
| Self-doubt | -0.07 | -0.18** | -0.27*** | -0.17* | -0.26*** |
| Devastation | -0.16* | -0.19** | -0.29*** | -0.11 | -0.29*** |
| Apathy | -0.12 | -0.21** | -0.15* | -0.12 | -0.20** |
| Concern | -0.09 | -0.23*** | -0.33*** | -0.21** | -0.33*** |
| Guilt | -0.02 | -0.19** | -0.17* | -0.27*** | -0.23*** |
| Powerlessness | -0.02 | -0.22** | -0.21** | -0.33*** | -0.27*** |
| Lack of concentration | -0.09 | -0.21** | -0.23*** | -0.18** | -0.25*** |
| Unwillingness to communicate | -0.07 | -0.20** | -0.21** | -0.19** | -0.24*** |
| Distrust of comrades in the service | -0.10 | -0.21** | -0.14* | -0.10 | -0.19** |
| Distrust of commanders | -0.12 | -0.10 | -0.20** | -0.04 | -0.18** |
| Inability to perform the assigned tasks | -0.10 | -0.29*** | -0.25*** | -0.08 | -0.27*** |
| RCMTQ | | | | | |
| Expectation from participating in hostilities | 0.08 | 0.37*** | 0.36*** | 0.15* | 0.37*** |
| Overcoming a stressful situation | 0.17* | 0.36*** | 0.32*** | 0.21** | 0.38*** |
| Realization of the acquired combat experience | 0.12 | 0.28*** | 0.32*** | 0.16* | 0.33*** |
| Overall indicator of RCMT | 0.14* | 0.39*** | 0.38*** | 0.20** | 0.41*** |

[a] *p≤0.05, **p≤0.01, ***p≤0.001

## Results

The results of the relationships between the characteristics of motivation for a conscious attitude towards danger and the PTS, maladaptation, negative emotional experiences and RCMT indicators in the general sample of study participants are presented in Table 1.

In the second stage of the study, based on the results of the cluster analysis, two groups (motivational types) of CAD were identified, to which 214 (95.11%) of the participants were assigned (Table 2): group 1 with low profile 191 (84.89%) and group 2 with a medium-high profile 23 (10.22%). Another 11 (4.89%) of the study

participants were assigned to different groups of 12 people in each, but due to the small number of these groups, their data were not used in a further analysis.

**Table 2.** Indicators of the motivation of CAD in the two most typical groups of military personnel who were in conditions of a real threat of death (points)

| CAD subscales | Groups of participants | | Differences between groups | |
|---|---|---|---|---|
| | Group 1 191 (84.89%) | Group 2 23 (10.22%) | t | p |
| Desire for safety | 18.79±3.65 | 26.39±2.06 | 15.08 | 0.001 |
| Awareness of one's death | 17.92±4.22 | 24.30±2.60 | 10.27 | 0.001 |
| Resistance to non-lethal stressors | 16.68±4.33 | 27.13±2.05 | 19.71 | 0.001 |
| Tolerance for uncertainty | 16.81±3.55 | 20.74±3.89 | 4.62 | 0.001 |
| General indicator of CAD | 86.87±14.3 | 125.70±8.12 | 19.57 | 0.001 |

Then, group 1 was divided into 3 subgroups. 101 (52.87%) participants in subgroup 1.1 had low scores on all subscales of motivation for CAD. 79 (41.36%) participants in subgroup 1.2 had average points on all subscales of motivation for CAD. 11 (5.76%) participants in subgroup 1.3 had predominantly low points on all subscales of motivation except for the "Desire for safety" subscale (high points). The main motivational types (profiles) of study participants who were under real threat of death are presented in Figure 1.

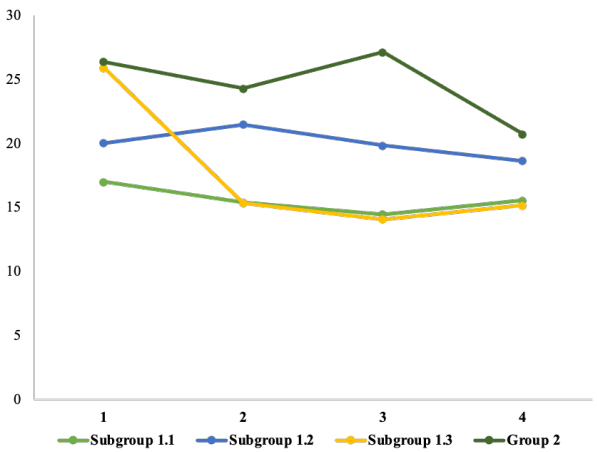


**Fig. 1.** Main motivational types (profiles) of the study participants who were under conditions of a real threat of death (points): 1) desire for safety; 2) Awareness of one's mortality; 3) resistance to non-lethal stressors; 4) Tolerance of uncertainty

The intensity of PTS symptoms of study participants with different CAD motivational types of CAD who were under real threat of death is presented in Table 3.

Maladaptation indicators and reliability of differences between groups of participants with different types of motivation CAD who were in conditions of a real threat of death are presented in Tables 4 and 5.

**Table 3.** Intensity of PTS symptoms of the study participants with different motivational types who were under conditions of a real threat of death

| Intensity of PTS symptoms | Subgroup 1.1 | Subgroup 1.2 | Subgroup 1.3 | Group 2 |
|---|---|---|---|---|
| Norm | 33 (17.24%) | 67 (35.08%) | 35 (18.32%) | 10 (43.48%) |
| Separate symptoms of PTSD | 90 (47.13%) | 83 (43.45%) | 52 (27.23%) | 8 (34.78%) |
| Clinical manifestations of PTSD | 68 (35.63%) | 41 (21.47%) | 104 (54.45%) | 5 (21.74%) |

**Table 4.** The maladaptation indicators of the participants with different motivational types who were in conditions of a real threat of death (points)

| Scale name | Subgroup 1.1 | Subgroup 1.2 | Subgroup 1.3 | Group 2 |
|---|---|---|---|---|
| Disruption of behavioral regulation | 4.85±2.28 | 3.52±2.32 | 4.55±2.62 | 1.94±1.95 |
| The likelihood of committing suicide attempts | 3.15±2.48 | 2.11±2.74 | 3.91±3.27 | 1.13±1.67 |
| Violation of moral norms | 4.05±1.95 | 3.36±1.84 | 4.09±2.47 | 1.69±1.40 |
| Loss of communicative potential | 4.18±2.01 | 3.14±2.03 | 4.00±3.03 | 2.31±1.99 |
| General indicator of maladaptation | 16.23±6.84 | 12.13±6.53 | 16.55±10.23 | 7.06±5.51 |

**Table 5.** Reliability of differences in indicators of maladaptation of participants with different types of motivational CAD who were in conditions of a real threat of death (Student's t test) a

| Scale name | Differences between groups | | | | | |
|---|---|---|---|---|---|---|
| | $t_{1.1\text{-}1.2}$ | $t_{1.1\text{-}1.3}$ | $t_{1.2\text{-}1.3}$ | $t_{1.1\text{-}2}$ | $t_{1.2\text{-}2}$ | $t_{1.3\text{-}2}$ |
| Disruption of behavioral regulation | 3.86*** | 0.37 | 1.24 | 6.27*** | 3.27** | 2.93** |
| The likelihood of committing suicide attempts | 2.63** | 0.75 | 1.74 | 4.74*** | 2.12* | 2.66* |
| Violation of moral norms | 2.42* | 0.06 | 0.95 | 6.72*** | 4.67*** | 3.01** |
| Loss of communicative potential | 3.43*** | 0.19 | 0.91 | 4.06*** | 1.75 | 1.68 |
| General indicator of maladaptation | 4.09*** | 0.10 | 1.39 | 6.86*** | 3.71*** | 2.88** |

[a] *p≤0.05, **p≤0.01, *** p≤0.001

All the maladaptation indicators of subgroups 1.1–1.3 were in the average range, and the indices of group 2 were low.

The profiles of negative emotional reactions and states in participants with different motivational types of conscious attitude toward danger, who were under the conditions of a real threat of death, are shown in Figure 2.

The profile of negative emotional reactions and states of group 2 was located mainly in the range of 1.5–3.5 points with peaks on the scales of "Anxiety", "Unwillingness to communicate" and "Irritability". The profile of subgroup 1.2 was located in the range of 2-4 points and was quite close to the profile of group 2 with peaks on the scales of 'Anxiety' and "Distrust of commanders". This could indicate a relatively low number of symptoms of PTS and manifestations of maladjustment. The profile of subgroup 1.1 was located mainly

in the range of 3-5 points of the group and differed significantly from the profiles of group 2 and subgroup 1.2 with peaks on the scales of 'Anxiety', 'Irritability', 'Unwillingness to communicate" and "Concern". This could indicate a medium-high level of PTS symptoms and maladjustment. The profile of subgroup 1.3 was located mainly in the 5-6 point range and was characterized by high points with peaks of the scales 'Powerlessness', 'Anxiety', 'Concern' and "Inattention". This could indicate certain signs of mental and physical trauma and maladaptation during combat.

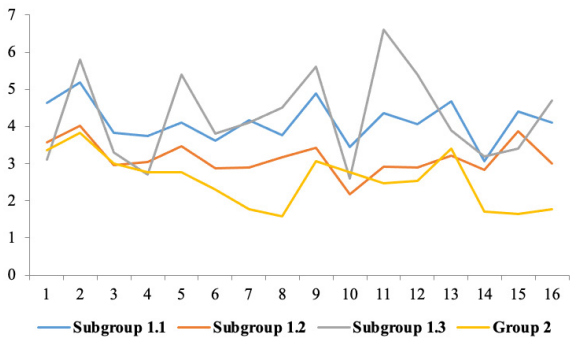


**Fig. 2.** Profiles of negative emotional reactions and states in participants with different motivational types of conscious attitude to danger, who were in conditions of a real threat of death (points): 1) Irritability; 2) Anxiety; 3) Aggressiveness; 4) Anger; 5) Inattention; 6) Self-doubt; 7) Devastation; 8) Apathy; 9) Concern; 10) Guilt; 11) Powerlessness; 12) Lack of concentration; 13) Unwillingness to communicate; 14) Distrust of comrades in the service; 15) Distrust of commanders; 16) Inability to perform the assigned tasks assigned

Tables 6 and 7 present the resilience indicators to combat mental trauma and the differences between groups of participants with different motivational types of conscious attitudes toward danger, who were in conditions of real death threat.

**Table 6.** Indicators of resilience to combat mental trauma in groups of participants with different types of conscious attitudes toward danger, who were in conditions of a real threat to death threat (points)

| Scale name | Subgroup 1.1 | Subgroup 1.2 | Subgroup 1.3 | Group 2 |
|---|---|---|---|---|
| Expectation from participating in hostilities | 41.80±10.8 | 47.56±9.07 | 44.77±9.03 | 53.75±13.77 |
| Overcoming a stressful situation | 46.04±11.13 | 51.51±9.52 | 48.00±12.23 | 59.06±11.95 |
| Realization of the acquired combat experience | 40.85±9.82 | 43.98±7.88 | 43.77±7.93 | 51.19±9.25 |
| Overall indicator of RCMT | 128.68±28.13 | 143.05±21.85 | 136.56±26.48 | 164.00±30.69 |

**Table 7.** Differences between groups in indicators of resilience to combat mental trauma of participants with different types of conscious attitude to danger, who were in conditions of a real death threat (student's t-test) a

| Scale name | Differences between groups | | | | | |
|---|---|---|---|---|---|---|
| | $t_{1.1\text{-}1.2}$ | $t_{1.1\text{-}1.3}$ | $t_{1.2\text{-}1.3}$ | $t_{1.1\text{-}2}$ | $t_{1.2\text{-}2}$ | $t_{1.3\text{-}2}$ |
| Expectation from participating in hostilities | 3.89*** | 1.02 | 0.96 | 3.90*** | 2.03* | 2.27* |
| Overcoming a stressful situation | 3.55*** | 0.51 | 0.91 | 4.78*** | 2.78** | 2.49* |
| Realization of the acquired combat experience | 2.37* | 1.13 | 0.08 | 4.78*** | 3.39*** | 2.41* |
| Overall indicator of RCMT | 3.86*** | 0.93 | 0.78 | 5.06*** | 3.06** | 2.68* |

[a] *p≤0.05, **p≤0.01, *** p≤0.001

## Discussion

At the first stage of the study, the use of correlation analysis allowed us to determine the relationships between the characteristics of motivation of a conscious attitude to danger and PTS, maladaptation, negative emotional experiences, and indicators of RCMT in the general sample of study participants. It was found that in the general sample of study participants, the PTS symptom intensity indicator was statistically significantly associated with all subscales of a conscious attitude towards danger. Our results also intersect with other data, where it was found that modern servicemen prepare too carefully for a meeting with a threat in conditions of intense combat operations[24]. However, for most servicemen who participated in combat operations, everyday problems were more pronounced in their service experience than the potential impact of lethal threats. Furthermore, family support acted as a compensatory factor for both the situation of intense lethal threats and the situation, when instead of combat operations, servicemen immersed themselves in the daily routine.[24]

The cluster analysis in the second stage of the study allowed us to identify two main groups of participants with different types of conscious attitudes toward danger in conditions of a real threat of death. Due to the specificity of the study sample (all servicemen participated in intense and long-term combat operations), motivational types were identified mainly with reduced (depleted or insufficiently formed) motivation for a conscious attitude to danger. Group 1 was then divided into 3 subgroups based on the motivation indicators for a conscious attitude towards danger. Almost all participants in group 1 (subgroups 1.1, 1.2, 1.3) had low and moderate-low scores on all subscales of conscious attitude to danger motivation.

Participants in group 2 had moderate-high scores on all subscales of conscious attitude towards danger motivation. These participants more often than others preferred professional self-realization: They had higher rates of resistance to combat mental trauma and a low level of maladaptation. They had the lowest rates of apa-

thy, emptiness, mistrust of commanders and comrades, and inability to perform assigned tasks. Our results, in particular the motivational characteristics of the group 2 participants, to a certain extent find support in the TMT, proving that high self-esteem allows a serviceman to act rationally in conditions of a real threat of death.[12-15] And also with the theory of appeasement, according to which the ability to control your emotions increases the likelihood of survival.[16] The indicators of group 2 participants largely coincide with the results of a field study, which found that in situations of threat, higher identification with the Royal Netherlands Army has a positive relationship with higher acceptance of the risk of death, higher self-efficacy, and self-assessment of operational readiness.[13]

Participants in subgroup 1.1 showed the greatest depletion of motivation to perform tasks in dangerous (combat) conditions. For the most part, they had been in military service before the large-scale Russian invasion. Exhaustion and fatigue in this group negatively affected their ability to withstand the effects of combat stress factors. They lacked the energy to maintain the necessary level of control over their lives. It was the lack of strength in this group that was the basis for the loss of control and violation of moral normativity, behavioral regulation, and insufficient personal potential to maintain the usual level of communication.

Participants in subgroup 1.2 showed average indicators for a conscious attitude towards danger, fairly high indicators of RCMT, and high indicators of symptoms of PTS. They were also physically and mentally exhausted and did not have the resources for additional self-control, which was reflected in the indicators of maladaptation.

The small subgroup 1.3 consisted mainly of ordinary military personnel, and young people, most of whom were faced with the need to master military service only during a large-scale invasion. They had a pronounced desire for safety. It was combined with an unformed attitude towards their mortality, an inability to overcome stress factors and adequately act in an uncertain situation. Such personality traits of the participants of this group did not allow the formation of RCMT. They felt powerless in the face of life circumstances: the need to be in a combat zone and were often unable to concentrate and complete assigned tasks. Their mental state could probably be further deteriorated by the inability to rest due to sleep problems. However, they were not subject to sthenic reactions: aggression, anger, irritability, and negative attitudes toward commanders and colleagues. Participants in subgroup 1.3 had an uneven profile of negative emotional reactions and states. They can probably be classified as individuals with pronounced neuroticism, which affects motivation for activity related to goal setting, expectation of goal achievement, and a general sense of self-efficacy.[25,26]

We also have reason to believe that everyday stressors, such as bureaucracy, everyday difficulties, insufficient weapons, equipment, and ammunition, could act as the main source of negative experiences even in the face of a real and intense threat to life. Most servicemen who decided to defend their family and homeland resigned themselves to their possible death. However, bureaucracy, inability to organize daily service conditions, unprofessional orders of commanders, and other factors were often the main factors in the accumulation of combat stress.[27] Family was also an additional source of worry for servicemen who valued their family life.[28]

The results obtained revealed the presence of several types of motivational personality types in servicemen with low self-efficacy, who perceive social support from colleagues and commanders as a threat and confrontation. Similar conclusions related to the importance of self-efficacy in the moderating effects of social support in stressor-strain relationships were also made in another study.[29]

To mitigate the effects of depleted motivation and reduce PTS in military personnel, it is necessary to develop and adjust military and psychological training measures before deployment and participation in combat operations. The US military, for example, developed a combat and Operational Stress Control.[30] This is a coordinated program for the prevention of and actions taken by the military leadership to prevent, identify and manage adverse combat and operational stress reactions in units.[31] The UK has a peer support program for Trauma Risk Management that aims to promote help-seeking military personnel in the aftermath of traumatic events.[32] The Israel Defense Forces developed YaHaLOM training to teach service members how to manage acute stress reactions in team members. This is a novel, rapid, peer-based intervention specifically designed for use in a high-stress event.[10]

***Study limitations***

Of course, this study had some limitations. First, the study participants were extremely exhausted after participating in intense, prolonged combat operations. Therefore, we were forced to use questionnaires with a small number of questions/statements or express versions of psychodiagnostic methods. The use of questionnaires with a large number of questions/statements caused negative emotional reactions in study participants and further worsened their mental state. Second, women did not participate in the study, since less than 0.5% of female servicemen were sent for psychological recovery. Third, most of the participants had depleted motivation, so it was not possible to establish patterns characteristic of a high level of motivation and describe the corresponding types (the study identified only one small type of highly motivated servicemen). Fourth, the

correlation links and the structure of the study itself did not allow us to state unequivocally: either the underdeveloped motivation for a conscious attitude to danger in conditions of a real threat of death did not allow the formation of RCMT, or the unformed resistance and intense PTS symptoms led to the exhaustion of motivation to perform tasks in combat conditions. Fifthly, certain limitations in the analysis arose due to the small number of subgroup 1.3 and group 2. Therefore, the correlation analysis was used only for the general sample and a certain disproportionality arose when presenting information on subgroups as percentages.

## Conclusion

The authors presented the influence of prolonged intensive traumatic combat experience on the formation of motivational types of servicemen with different attitudes to danger in conditions of real threat of death. It has been established that a high level of motivation for a conscious attitude toward danger in conditions of a real threat of death can be a factor in reducing the risk of physical and psychological injury in military personnel, but it does not eliminate this risk. The depleted motivation of a conscious attitude to danger in conditions of a real threat of death increases the risk of psychological and physical trauma. Probably, with unformed motivation, this risk is the highest. In servicemen who have formed a certain attitude to death, the threat to life did not cause an acute emotional reaction. However, they also developed symptoms of PTS, which did not decrease in a short time working with a specific trauma, which required searching for new ways of secondary prevention of PTSD and its chronic course. This partly explains the need for rapid psychological first aid to military personnel in combat operations. This care provides an unlimited wide range of activities and different levels of its provision compared to Critical Incident Stress Debriefing.

## Declarations

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***Author contributions***

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***Conflicts of interest***

The authors declare no competing interests.

***Data availability***

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

***Ethical approval***

The approval of the ethics committee was obtained before the initiation of the study (meeting date; 17/04/2024, decision number; 2024/7).

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**ORIGINAL PAPER**

# Comparative assessment of 2D photogrammetry versus direct anthropometry in nasal measurements

Shehzeen Afaq [1], S.K. Jain [1], Nidhi Sharma [2], Sonika Sharma [1]

[1] Department of Anatomy, Teerthanker Mahaveer Medical College and Research Center, Moradabad, Uttar Pradesh, India
[2] Department of Anatomy, Kalyan Singh Medical College Bulandshahr, Uttar Pradesh, India

**ABSTRACT**

**Introduction and aim.** The nose significantly influences facial aesthetics, necessitating a comprehensive understanding of nasal anatomy and facial aesthetics standards for successful rhinoplasty. This study investigates the reliability and precision of two-dimensional photogrammetry compared to direct measurements for nasal anthropometry.

**Material and methods.** This cross-sectional study was carried out at Teerthanker Mahaveer Medical College and Research Center, Moradabad which included 640 volunteers from six zones of the Indian population. Direct anthropometry and 2D photogrammetry were the two methods used to evaluate each parameter. A caliper was employed for direct anthropometry, and software analysis of nasal characteristics was used for the indirect 2D technique.

**Results.** The result revealed significant gender-based differences in all nasal dimensions assessed by direct measurements, with the exception of the length of the nasal bridge. In contrast, 2D photogrammetry demonstrated no significant variation in anatomical nasal width and nasal bridge length across the genders. Of the eight nasal parameters analyzed, five parameters (nose height, morphological nose width, alar length, nasal tip protrusion, and nasal index) exhibited excellent reliability. Nasal bridge length and columellar length showed moderate reliability of 2D photogrammetry.

**Conclusion.** This research reveals gender-based differences in nasal dimensions, with males generally having larger dimensions. It suggests 2D photogrammetry as a reliable alternative to direct anthropometry for specific nasal measurements, offering practical benefits in clinical and aesthetic applications. However, it has limitations, such as moderate reliability in measuring nasal bridge length and Columellar length, underscoring the necessity for cautious interpretations for specific parameters. Further research is recommended to improve precision and applicability, while addressing limitations like sensitivity to imaging distortion and also incorporating the 3D technique to enhance the robustness of the methodology; the absence of 3D is a benchmark.

**Keywords.** facial analysis, nasal anthropometry, photogrammetry, rhinoplasty, two-dimensional anthropometry

## Introduction

In the world of plastic surgery, creativity plays a pivotal role.[1] Plastic and reconstructive surgery thrives on a diverse range of technologies, pioneering techniques and meticulous planning to achieve spectacular results in facial anthropometry for enhancing patients aesthetics as well as their quality of life.[2] Rhinoplasty is still considered one of the most prevalent cosmetic surgical procedures throughout the world, with its favorable results reliant on careful preparation prior to surgery and intraoperative execution.[3]

2D photogrammetry has recently evolved as a non-invasive technique, particularly in the realm of facial anthropometry.[4] In the context of faces, this non-invasive method offers an efficient way to obtain multiple

**Corresponding author:** Shehzeen Afaq, e-mail: shehzeenafaq@gmail.com

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facial images from different angles and then, using specialized software (Digimizer and Crisalix) to reconstruct a 3D model, analyze various features such as symmetry, distances, angles, and proportions to extract specific measurements and data.[4] The 2D approach offers several substantial advantages in the healthcare and medical domains such as:[4]

- Increased patient comfort: This approach provides a non-invasive and pleasant means of retrieving data, which may enhance the entire experience without requiring patients to endure intrusive treatments.
- Reduced measurement variability: It can help reduce the variability in measurements compared to manual methods. Human error can lead to inconsistencies in manual methods, while proper calibration and execution of photogrammetry ensures more precise and consistent results.
- Digital record-keeping: Photogrammetry makes patient data easily accessible for future reference.

The accuracy of any method in the acquisition of anthropometric data is of prime importance to the researcher. Although, direct anthropometry is already established as a gold standard technique, it is also an established fact that it is a time consuming approach along with that it cannot be measured various angles of the face. So, for this reason we have to go for higher modalities like 2D and 3D analysis. But, the disadvantage of the newer techniques, as already stated by many studies, is that it is very expensive and can't be applied in all type of settings. On the other hand, 2D photography is still not widely explored in the literature as there has not yet been extensive research on the accuracy of 2D photography in relation to rhinoplasty.

## Aim

To close this gap, this study will compare the accuracy and reliability of 2D photogrammetry against direct methods for determining nasal measurements for rhinoplasty. We want to explore the 2D approach for the facial analysis and its use in the era of technology which can be helpful to the society. It will be a convenient method for analysis in epidemiological studies and may be an alternative of direct and 3D analysis.

## Material and methods

This descriptive cross-sectional study took place at the Department of Anatomy with in the Teerthanker Mahaveer Medical College and Research Center in Moradabad, Uttar Pradesh, India. This study, conducted from January 2023 to January 2024 involved 640 participants from six different Indian zones, with 320 males and 320 females, using a cross-sectional research formula (n=Z P (100-P)/E) to ensure a representative sample size. The assumptions used for the calculation of the sample size was confidence interval (95%), standard deviation (0.456), allowable error (0.05) and standard normal variate for a 95% confidence interval was 1.96. The study involved individuals from six distinct Indian zones: East, West, North, South, North-East, and Central. The study included healthy Indian subjects aged 18-25.[3] Those having a history of facial plastic surgery, intellectual disability, congenital craniofacial abnormality, significant damage, and mixed-ethnic background were excluded from the reserach.[3-6] The study was approved by the Institutional Ethical Committee (Ref. no. TMU/IEC/20-21/103) at Teerthanker Mahaveer Medical College and Research Center, with informed consent from each subject, following the 1975 Helsinki Declaration's ethical guidelines. Participant anonymity during photography was maintained through the following measures: informed consent, masking identifiable features, no personal identifiers (names and contact details), and restricted access (images were stored on password protected system), non-disclosure (images were exclusively used for research purpose. Eight nasal dimensions (nose height, anatomical nose width, morphological nose width, columella length, nasal bridge length, alar length, nasal tip protrusion and nasal index) were measured and calculated by two different approaches: direct and 2D indirect.

### *Direct method*

We acquired written agreement before measurements, and allayed any lingering doubts by thoroughly explaining the measuring procedure to the participants. Participants were required to maintain neutral head posture (Frankfurt plane) and facial expressions while measuring anatomical landmarks using a surgical skin pencil and caliper, with average readings considered to be accurate (Fig. 1).[7]

**Fig. 1.** Direct anthropometry

### *2D indirect photogrammetry*

A 24.2-megapixel DSLR (digital single-lens reflex) camera was utilized by the department's photography section to take pictures in well-lit conditions. We captured

the photos with the head positioned in the Frank Furt plane and aligned with the reference scale. We scrutinized the images for flaws such as misorientation, distortion, or imaging artefacts. We saved the JPEG files and sent them to the Digimizer Image Analysis program (Version 5.7.5). This software is incredibly user-friendly and versatile for facial image analysis (Fig. 2 and 3).[8]

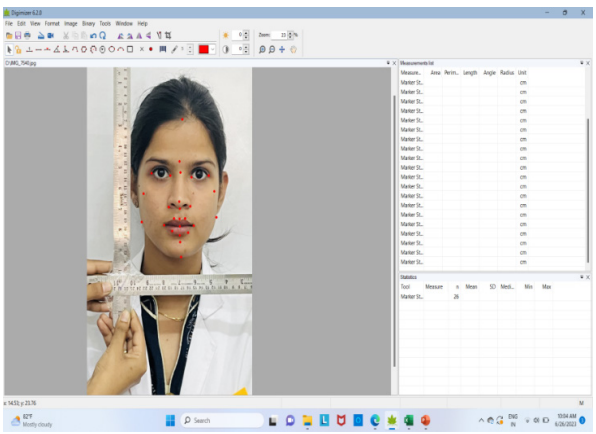

**Fig. 2**. Indirect anthropometry (frontal view)

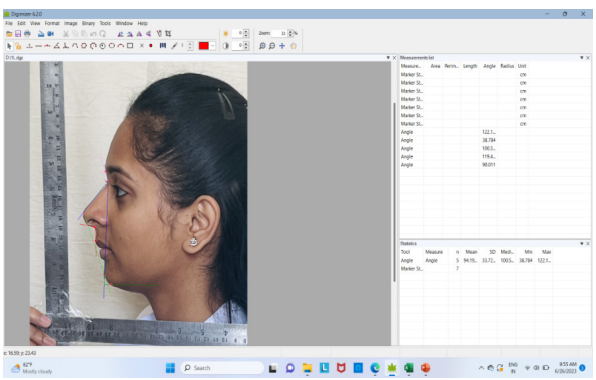

**Fig. 3**. Indirect anthropometry (lateral view)

***Statistical analysis***

We used SPSS version 25 for analysis (IBM, Armonk, NY, USA), evaluating data homogeneity using Shapiro-Wilk and Kolmogorov-Smirnov tests. A Normal distribution implies no variables deviating from the normalcy assumption. We assessed the descriptive statistics of facial parameters using mean and standard deviation, and an unpaired t-test for sexual dimorphism. We used Bland Altman (graphical presentation), regression analysis and intra-class correlation coefficient (ICC) statistical test to evaluate the reliability of the 2D method against the direct method.[4] For every statistical analysis, the threshold of significance for statistical purposes was established at $p<0.05$.

*Bland Altman analysis (BAA)*

This statistical approach is primarily used to assess the agreement between two different methods.[9] We plotted the difference of two methods on the Y-axis and average of two methods on the X-axis. After that we applied one-sample t-test on the basis of difference values of two methods and got the mean and standard deviation. After that plot generate three horizontal reference lines along the Y-axis. The middle line represent the mean values. Upper or lower limit comes when we add or subtract 1.96 in mean and multiply by the standard deviation.

In a BAA, two criteria need to be met to establish that two methods are comparable.

- The mean difference should be small and close to zero
- Standard deviation of this difference should be small

*Regression*

In the context of method comparison, the r-value indicates the strength and direction of the linear relationship between two methods, the p-value determines the significance of the comparison.[9] The level of significance, usually <0.05, at which the null hypothesis is rejected indicated by the p-value. It shows a significant differences between two approaches if it is less than or equal to this level, and no significant difference if it is larger than this level.

*Intra-class correlation coefficient (ICC)*

ICC measures the reliability of methods, with a range of 0–1 ICC coefficient.[4] If we get ICC values less than 0.4 with wider confidence interval (CI) that means poor reliability. Moderate reliability is indicated by ICC values between 0.4–0.75 with narrow CI; Excellent reliability of the method is shown by ICC values more than 0.75 with the narrowest CI.

## Results

Table 1 shows the mean and SD of nasal parameters across both approaches. Males exhibited generally higher mean values whereas females showed greater alar length and nasal tip protrusion. Both methods showed slight variation which demonstrating the reliable approximations for most of the parameters according to their mean values.

**Table 1.** Descriptive statistics of nasal parameters for each gender by both approaches

| Parameters | Measurements of nasal parameters (Mean±SD) | | | |
|---|---|---|---|---|
| | Male | | Female | |
| | Direct method | Indirect method | Direct method | Indirect method |
| Nose height (cm) | 4.5±0.43 | 4.16±0.37 | 4.1±0.54 | 4.06 ±0.55 |
| Anatomical nose width (cm) | 3.01±0.29 | 3.1±0.24 | 2.87±0.19 | 2.79±0.23 |
| Morphological nose width (cm) | 3.2±0.3 | 3.1±0.24 | 3±0.23 | 3.09±0.23 |
| Columellar length (cm) | 1.2±0.21 | 1.10±0.17 | 0.98±0.21 | 1±0.2 |
| Nasal bridge length (cm) | 4.06±0.4 | 3.6±0.38 | 4.07±0.38 | 4.12±0.39 |
| Alar length (cm) | 2.9±0.37 | 2.8±0.39 | 3.2±0.7 | 3.20±0.72 |
| Nasal tip protrusion (cm) | 0.7±0.08 | 0.69±0.11 | 0.79±0.17 | 0.79±0.18 |
| Nasal index (cm) | 79±11.3 | 76.05±8.8 | 69.9±10.2 | 70.59±10.8 |

**Table 2.** Depiction of the sexual dimorphism in nasal parameters by both approaches

| Parameters | Direct method | | 2D-indirect method | |
|---|---|---|---|---|
| | T | p | T | p |
| Nose height | -2.7 | <0.05 | -3.3 | <0.05 |
| Anatomical nose width | 2.5 | <0.05 | 0.4 | >0.05 |
| Morphological nose width | 4.27 | <0.05 | 1.17 | <0.05 |
| Columellar length | 2.35 | <0.05 | -0.58 | <0.05 |
| Nasal bridge length | -0.046 | >0.05 | -6.9 | >0.05 |
| Alar length | -3.02 | <0.05 | -3.3 | <0.05 |
| Nasal tip protrusion | -3.13 | <0.05 | 0.28 | <0.05 |
| Nasal index | 4.6 | <0.05 | 3.01 | <0.05 |

Table 2 reveals significant gender-based differences in all nasal dimensions assessed via direct measurements, with the exception of nasal bridge length. In contrast, 2D photogrammetry demonstrated no significant variation in anatomical nasal width and nasal bridge length across the genders.

Figure 4 shows the graphical representation of the male nasal parameters, for the good limit of agreement, most of the data points clustered around the mean and falling under the upper and lower limits of the scatter plot. All the parameters showing the good limit of agreement because all the parameters followed the two criteria of the BAA: The mean difference is small and close to zero and the standard deviation of this difference is also small.

Figure 5 shows a graphical representation of the female nasal parameters. For a good limit of agreement, most of the data points clustered around the mean and falling under the upper and lower limits of the scatter plot. All the parameters showing the good limit of agreement because all the parameters followed the two criteria of the BAA: The mean difference is small and close to zero and standard deviation of this difference is also small.

**Table 3.** Regression analysis for methods accuracy in both gender

| Nasal parameters | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|
| | R | $R^2$ | F | p | R | $R^2$ | F | p |
| Nose height | 0.24 | 0.059 | 3.61 | 0.062 | 0.26 | 0.046 | 3.64 | 0.064 |
| Anatomical nose width | 0.25 | 0.064 | 3.95 | 0.05 | 0.29 | 0.078 | 3.2 | 0.4 |
| Morphological nose width | 0.43 | 0.188 | 2.36 | 0.13 | 0.38 | 0.178 | 3.16 | 0.13 |
| Columellar length | 0.198 | 0.039 | 13.39 | 0.001 | 0.19 | 0.029 | 14.39 | 0.007 |
| Nasal bridge length | 0.054 | 0.003 | 0.172 | 0.68 | 0.064 | 0.001 | 0.182 | 0.59 |
| Alar length | 0.051 | 0.003 | 1.49 | 0.701 | 0.059 | 0.006 | 1.5 | 0.601 |
| Nasal tip protrusion | 0.28 | 0.08 | 5.15 | 0.27 | 0.35 | 0.09 | 5.0 | 0.17 |
| Nasal index | 0.41 | 0.16 | 11.7 | 0.001 | 0.40 | 0.15 | 12.9 | 0.009 |

Table 4 shows the linear regression for the nasal parameters, in terms of R-value, R-square value, f-value and p-value. Columellar length and nasal index are the two parameters that are showing the $p<0.05$, which expects that there could be differences between the measurements of two methods.

**Table 4.** Intra-class correlation coefficient values for accuracy of 2D method against direct method in both genders

| Parameters | Male | | Female | |
|---|---|---|---|---|
| | ICC coefficient | 95% CI (Lower to upper bound) | ICC coefficient | 95% CI (Lower to upper bound) |
| Nose height | 0.99 | 0.98–0.99 | 0.97 | 0.92–0.98 |
| Anatomical nose width | 0.85 | 0.81–0.92 | 0.94 | 0.92–0.96 |
| Morphological nose width | 0.91 | 0.86–0.96 | 0.93 | 0.82–0.97 |
| Columellar length | 0.69 | 0.56–0.89 | 0.66 | 0.62–0.96 |
| Nasal bridge length | 0.69 | 0.62–0.86 | 0.63 | 0.58–0.95 |
| Alar length | 0.99 | 0.98–0.99 | 0.96 | 0.82–0.88 |
| Nasal tip protrusion | 0.97 | 0.93–0.99 | 0.96 | 0.96–0.99 |
| Nasal index | 0.97 | 0.95–0.98 | 0.96 | 0.94–0.97 |

Table 4 presents the Intra-class correlation coefficient in both genders. Only the columellar length and nasal bridge length were represented the ICC values below the 0.75 with narrow confidence interval. While the rest of the parameters represented the ICC values >0.75 with narrowest CI.

## Discussion

A descriptive cross-sectional study was conducted among 640 normal healthy subjects from six demographic zones of the Indian population for the screening of nasal parameters. As everyone knows, India has a diverse population of many ethnicities and cultures, thus we included representatives from each of the six zones. Rhinoplasty is a type of face surgery where the intention is to preserve or restore nasal function and support while achieving aesthetic harmony with the adjacent facial characteristics.[10] By one's personality and lifestyle, the nose has varying degrees of significance in terms of facial aesthetics.[10] Face analysis is the initial stage in planning a surgical procedure and is essential to the formulation of an appropriate treatment strategy for operations involving cosmetic or reconstructive surgical procedures.[11]

So for this is the largest research study, to the best of our knowledge, which includes subjects from various zones of India and measurements taken by two methods to check the accuracy and reliability of 2D indirect photogrammetry against the direct anthropometry.

In the present study we found mean nasal length in males was 4.5±0.43 and 4.16±0.37 cm by direct and indirect method respectively. The mean nasal length in females was 4.1±0.54 and 4.06 ±0.55 cm by both methods. Parab et al. conducted a research on nasal parameters of the Indian population by the indirect method and they found mean values of nasal length in males 4.43±0.45 cm and in females 4.13±0.45 cm.[3] Patil et al. conducted an anthropometric study on a south Indian population and reported 5.8 cm mean value in males and 5.6 cm of nasal length in females.[12] After comparison, we measured slightly lower values of nasal length.

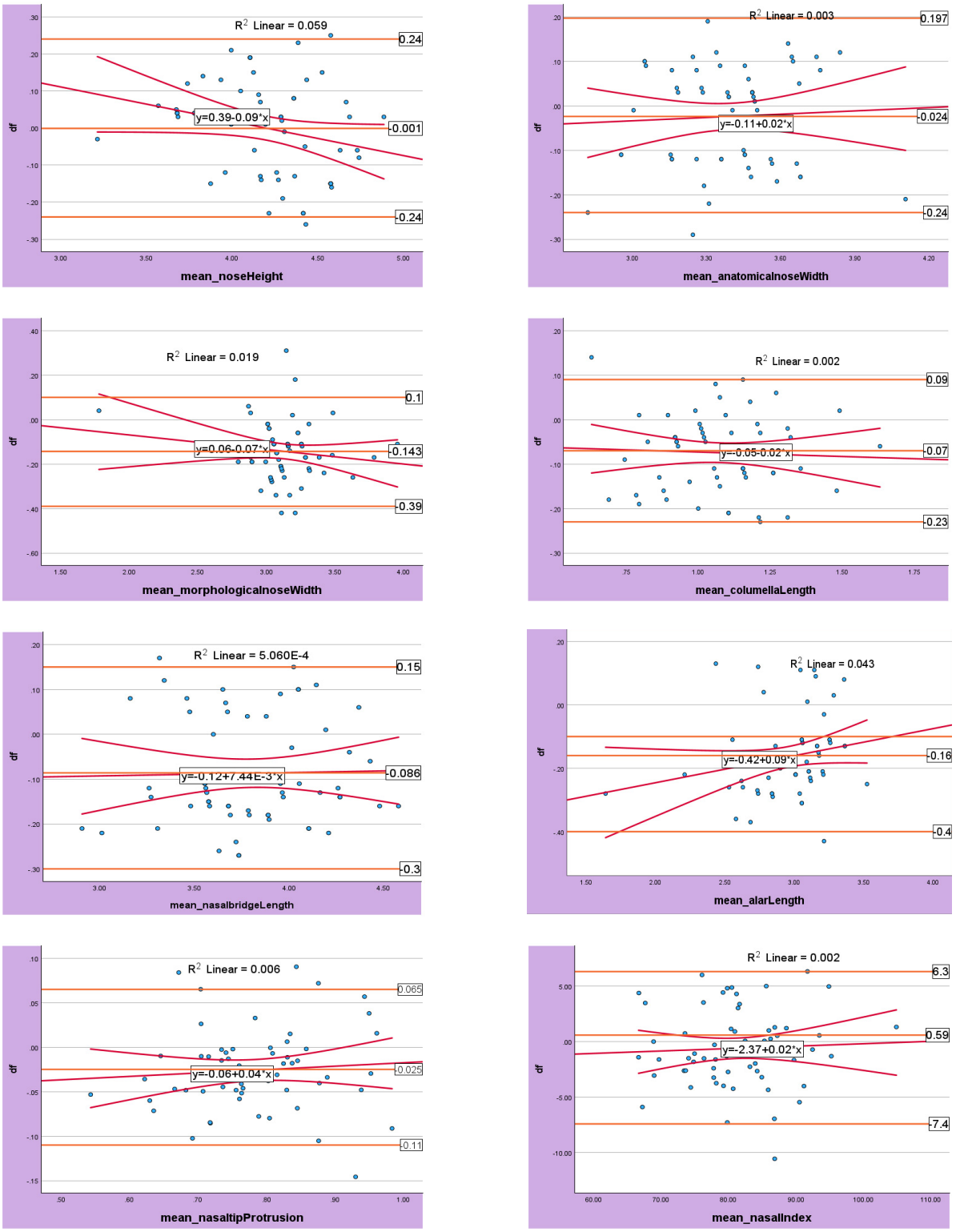


**Fig. 4**. Bland Altman graphical representation for males

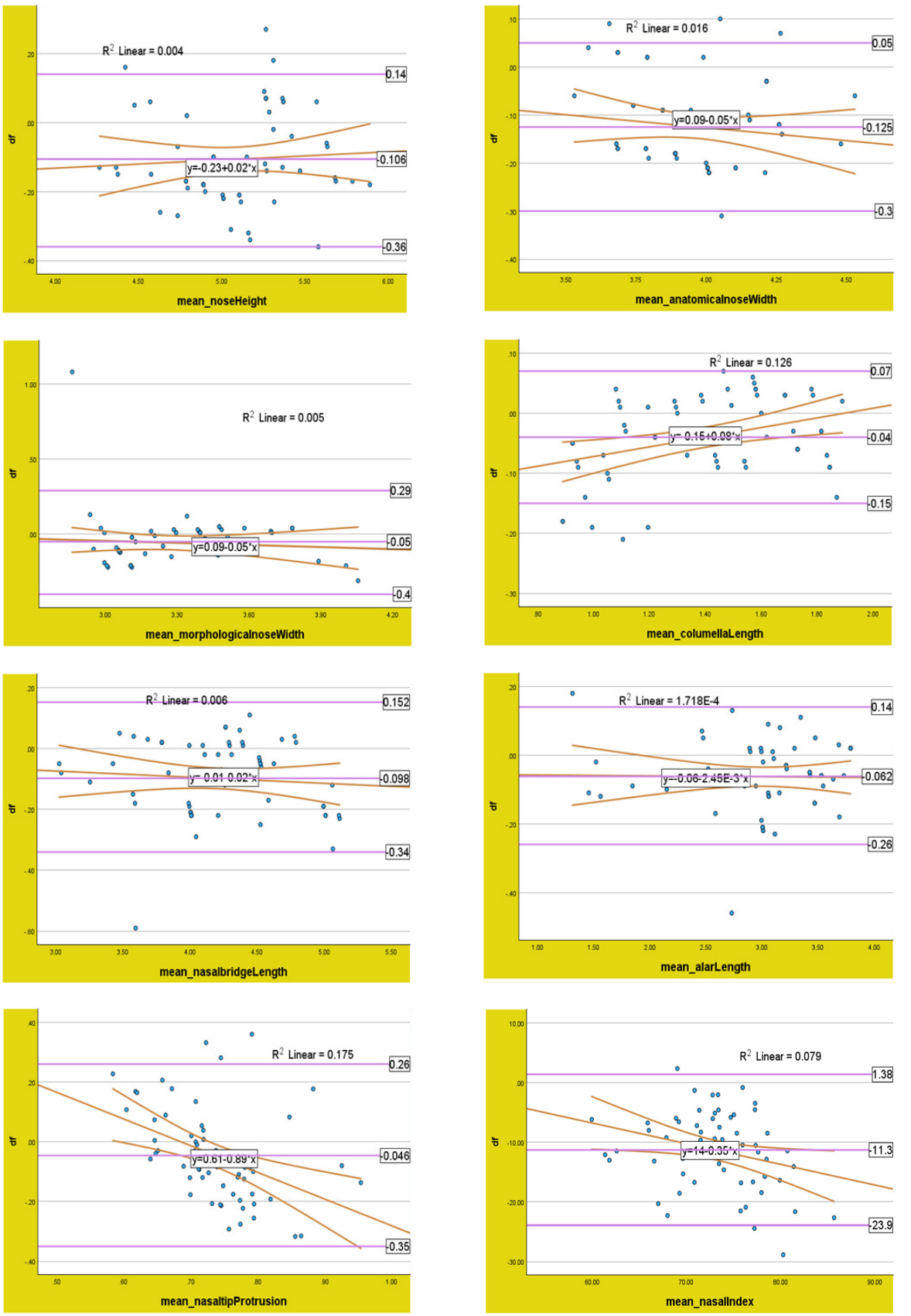


**Fig. 5**. Bland Altman Graphical Representation for females

In the current study, nose width in males was 3.2±0.3 and 3.1±0.24 cm, and in females it was 3±0.23 and 3.09±0.23 cm, by both the methods. Parab et al. showed the mean nasal width in males was 3.79±0.29 and in females 3.57±0.41.[3] Findings of our study coincide with the findings of the Parab et al study. According to our study, collumellar length in males was 1.2±0.21 and 1.10±0.17 cm, and in females 0.98±0.21 and 1±0.2 cm by both the approaches. Mehta et al. performed a study on the Indian population of different zones and they found 0.94±0.2 cm mean values of Columellar length in both sexes of the Indian population.[10] Our study coincides with the mean values of their study.

In this current study, nasal bridge length in males was 4.06±0.4 and 3.6±0.38 cm, and in females 4.07±0.38 and 4.12±0.39 cm. Mehta et al. found the 4.39±0.5 cm mean values of both genders.[10] We got slightly lower values of nasal bridge length as compared to the Mehta et al. study.

The mean values of alar length in males was 2.9±0.37 and 2.8±0.39 and in females 3.2±0.7 and 3.20±0.72 cm by both the approaches, respectively. Bhandari et al. conducted research on alar length in a New Delhi population and they report a mean of 3.48±0.21 cm in males and 3.14±0.25 in females.[13] After comparison, we got lower values of males and slightly higher values in females.

The mean values of nasal tip protrusion in males was 0.7±0.08 and 0.69±0.11 and in females 0.79±0.17 and 0.79±0.18 cm by both the approaches, respectively. The nasal tip protrusion was found to be 2.07±0.27 in males and 1.94±0.26 in females in research conducted by Parab et al. on an Indian population.[3] After comparison, we found much lower values of nasal tip protrusion.

The nasal index in males was 79±11.3 and 76.05±8.8 and in females 69.9±10.2 and 70.59±10.8. Mehta et al. found 73.27 in males and 72.35 in females.[10] Patil et al. conducted research on a south Indian population and found the values of nasal index in males was 84.91 and in females 67.75.[14] Chabra et al. conducted research on the north Indian population and they reported values of 77.39 in males and 72.28 in females.[15] After comparison, we found similar values. Our values of nasal index lies between 70–84.9, which comes under the category of mesorrhine.[20]

On the other hand, this research also assessed the reliability and accuracy of 2D indirect method against the direct method which is considered the gold standard.

The mean values for males and females of the nose height were -0.001 and -0.1, respectively. Scatter plot of both sexes showed that most of the data points fell under the limit of agreement. The regression analysis of the nose height showed the R-value 0.24 in males and 0.26 in females with the p>0.05, with expectation that there could be no difference between the measurements of methods. Nose height in both sexes have more than 0.75 ICC values with narrowest CI. Lim et al. conducted study for the reliability assessment of 2D photogrammetry with a BAA and ICC statistical approach.[4] According to their research, BAA scatter plots represented the agreement between approaches, and their ICC values were 0.99 and CIs limit 0.98-0.99. The same results were found by the current research.

The mean values for males and females of the ANW were -0.024 and -0.12, respectively. Mean values represented the agreement between the approaches. The R-value was 0.25 and 0.29 in males and females, respectively, and their p value was 0.05, which indicates no difference between measurements. The ICC values for males of 0.85 and 0.94 for females had the narrowest CI. Negi et al. conducted research to check the relation between direct anthropometry and indirect anthropometry by using BAA and regression analysis, and they report strong agreement between the approaches.[5] The present study showed the same result.

The mean values for males and females of the MNW were -0.143 and -0.05, respectively. R-value for the MNW was 0.43 in males and 0.38 in females with an insignificant p-value, which suggests no difference between the method measurements. On the other hand, ICC values for the both sexes were 0.91 and 0.93 respectively. Lim et al. reported 0.92 ICC with narrowest CI for the nose width; they suggest an excellent reliability of the method.[4] Similar results were provided by the current research.

The mean values for males and females of the columellar length were -0.07 and -0.04, respectively. R-value showed by the CL parameter was 0.198 in males and 0.19 in females with significant p value, which indicates differences between the methods. Their ICC values for males 0.69 and 0.66 for females with a wide CI. Farkas et al. verified the accuracy of photogrammetry by using 104 facial parameters. After analysis, they stated that columellar length was quite reliable but there was technique measurement differences.[16] Similar results are shown by the present study.

The mean values for males and females of the nasal bridge length were -0.098 and -0.086, respectively. Nasal bridge length represented the R-value 0.54 in males and 0.064 in females with >0.05 p-value. Their ICC values for males 0.69 and 0.63 for females with a wider CI. Aksu et al. used BAA and ICC methods, and they concluded that NBL demonstrated consistency along the reference plane of the measurements.[17] Our study only showed the moderate reliability according to the ICC, and level of agreement.

The mean values for males and females of the alar length were -0.16 and 0.06, respectively. Alar length represented an R-value 0.51 in males and 0.059 in females with >0.05 p-value. Their ICC values for males 0.99 and 0.96 for females with a narrow CI. Farkas et al.

also suggested a strong agreement and reliability of the alar length.[16] After comparison, we also found the same results.

The mean values for males and females of the nasal tip protrusion were -0.046 and -0.025, respectively. Nasal tip protrusion represented the R-value 0.28 in males and 0.35 in females with >0.05 p-value. Their ICC values for male 0.97 and 0.96 for females had a narrow CI. The nasal tip protrusion had an ICC of 0.81 with 95% confidence intervals ranging from 0.72 to 0.88 according to the Lim et al. study.[4] We also report higher values of ICC with a narrow CI, that represented strong reliability.

The mean values for males and females of the nasal index were 0.59 and -1, respectively. Nasal index represented the R-value 0.41 in males and 0.4 in females with <0.05 p-value. Their ICC values for male 0.97 and 0.96 for females with a narrow CI. The mean values of nasal index BAA scatter plot showed strong association between the approaches while the significant p-value of the regression indicates differences between the measurements of the methods. On the other hand, values of ICC and CIs suggest strong reliability of the 2D method.

Our research introduces novelty by employing three methods to assess the reliability of the 2D method: Bland-Altman, regression and ICC analysis. Both agreement and reliability parameters are pivotal in evaluating the quality of the method employed, and our study uniquely combines these three statistical parameters, which are not taken in consideration in previous studies.

The two-dimensional photogrammetric method has been widely applied in research both nationally and internationally, as documented in references 18 and 19. However, there is limited research and certain constraints in studies that compare 2D photogrammetry with direct measurement. Furthermore, current research exhibits conflicting findings on this matter. Some researchers showed that, for numerous facial dimensions, 2D photogrammetry is less precise compared to direct and 3D measurement methods, while the other two studies concluded that 2D photogrammetry aligns closely with direct measurement.[20,21]

In the present study, eight parameters were examined, all the parameters showed mean value towards the zero and minimal SD for the BAA statistical approach. All the scatter plot represented the good agreement between the methods. According to the regression analysis, most of the measurement showed insignificant values (p>0.05), which indicates that there is no difference between measurements of two methods. It's worth noting that a method's reliability is only beneficial if it also demonstrates good agreement, and conversely, good agreement is not useful if the method lacks reliability.

That is why another method used for the reliability assessment- the ICC coefficient. Most of the parameters showed excellent reliability except the columellar length and nasal bridge length. Therefore according to our research, only six out of the eight nasal dimensions can be reliably and accurately measured using 2D photogrammetry. These parameters warrant further research due to technical challenges such as image distortion, resolution of image, head orientation, magnification discrepancies, parallax effects, lighting inconsistencies and measurements difficulty from the lateral aspects. The recognition of the above aspects will help us to interpret the findings in an intricate manner. Researchers will go to more detail on this subject in future articles, stressing the measures used to accuracy while recognizing that other studies using bigger data sets and stratified sampling are required to validate the generality of the result. Future research also incorporating the three-dimensional technique to enhance the robustness of the methodology, the absence of 3D is a benchmark.

We recognize certain limitations in our study. Firstly, both the approaches were conducted by a single observer and secondly, research is limited by exclusion of 3D photogrammetry, a method known for its enhanced accuracy in facial dimension measurement. Finally, future research endeavors should aim to integrate various measurement approaches, including direct, 2D indirect, and 3D technologies.

## Conclusion

This study concludes gender-based differences in nasal dimensions, with males generally having larger dimensions. This study highlight the need for tailored approaches in cosmetic procedures to ensure aesthetically sensitive appropriate outcomes. The findings of this study indicate that 2D Photogrammetry emerges as a dependable alternative to the direct method. It offers cost-effectiveness, non-invasiveness, time efficiency, reduced operator dependence, and avoids direct patient contact. It suggests 2D photogrammetry as a reliable method for specific nasal measurements, offering practical benefits in clinical and aesthetic applications.

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## Declarations

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***Author contributions***

Conceptualization, S.K.J. and N.S.; Methodology, S.A.; Software, S.A.; Validation, S.A., N.S. and S.S.; Formal Analysis, S.A.; Investigation, S.A; Resources, S.A.; Data Curation, S.A.; Writing – Original Draft Preparation,

S.A.; Writing – Review & Editing, S.A; Visualization, N.S.; Supervision, S.K.J.; Project Administration, S.K.J.

***Conflicts of interest***

The author(s) declare no competing interests.

***Data availability***

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

***Ethics approval***

The study was approved by the Institutional Ethical Committee (Ref. no. TMU/IEC/20-21/103) at Teerthanker Mahaveer Medical College and Research Centre.

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**ORIGINAL PAPER**

# A comparative study of neuropharmacological properties of *Tabernaemontana divaricata* (Apocynaceae) leaves extracts in a Swiss albino mouse model

Konatham Teja Kumar Reddy [1], Jeetendra Kumar Gupta [2], Prabitha P. [3], M. Mubeen [4], Sabeena Hussain Syed [5], K. Dhunmati [6], Anoop Bodapati [7], Poonam Popatrao Taru [8], Pavankumar Krosuri [9], P. Dharani Prasad [10]

[1] Department of Pharmacy, University College of Technology, Osmania University, Amberpet, Hyderabad, Telangana, India
[2] Department of Pharmacology, Institute of Pharmaceutical Research, GLA University, Chaumuhan, Mathura, Uttar Pradesh, India
[3] Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Mysuru, India
[4] Department of Pharmacognosy, Sri Ramachandra Faculty of Pharmacy, Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai, India
[5] School of Pharmacy, Vishwakarma University, Kondhwa, Pune, Maharashtra, India
[6] Department of Pharmaceutical Chemistry, C.L Baid Metha College of Pharmacy, Thoraipakkam, Chennai, Tamilnadu, India
[7] Department of Pharmacy, Vignan's Foundation for Science, Technology and Research (Deemed to be University), Vadlamudi, Guntur, India
[8] Department of Pharmacognosy, School of Pharmacy, Vishwakarma University, Kondhwa Pune, Maharashtra, India
[9] Department of Pharmaceutics, Santhiram College of Pharmacy, Nandyal, Andhra Pradesh, India
[10] Department of Pharmacology, Mohan Babu University, MB School of Pharmaceutical Sciences, (Erstwhile Sree Vidyaniketan College of Pharmacy), Tirupati, India

**ABSTRACT**

**Introduction and aim.** Interest in natural products and nutraceuticals for the treatment of mental diseases such as anxiety, stress, sadness, and psychosis has increased due to their high safety index and cost. The primary objective of this work was to analyze the neuropharmacological attributes of leaf extracts of *Tabernaemontana divaricata* using models from Swiss albino mice.

**Method and materials.** Methanol, acetone, and ethyl acetate extracts were prepared from authenticated *T. divaricata* leaves. Experiments were conducted on 170 mice to evaluate the effects of thiopental sodium on sleeping time, hole cross, hole board, and open field behaviors. The duration of sleep caused by thiopental sodium was assessed at several doses, including 50 mg/kg, 100 mg/kg, and 200 mg/kg of body weight. Additionally, doses of 100 mg/kg and 200 mg/kg of body weight were used in the remaining tests.

**Results.** All extracts significantly increased thiopental-induced sleeping time in a dose-dependent manner, with maximum effects observed at 200 mg/kg (methanol: 684.77%, acetone: 655.63%, ethyl acetate: 666.89%). Locomotor and exploratory

**Corresponding author:** Konatham Teja Kumar Reddy, e-mail: teja.konatham1704@gmail.com
or P. Dharani Prasad, e-mail: dharaniprasad.p@vidyanikethan.edu

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behaviors were reduced in all behavioral models, including a significant decrease in head dips and square crossings ($p<0.01$), supporting central nervous system depressant activity.

**Conclusion.** The study reveals that extracts of *T. divaricata* exhibit depressive and hypnotic effects on the central nervous system, indicating the need for further research.

**Keywords.** diazepam, GABA, locomotive behavior, neuropharmacological properties, *Tabernaemontana divaricata*, thiopental sodium

## Introduction

In today's world, neuroprotective and neuropharmacological agents are crucial to improving attention and brain memory in people with depression and stress.[1] Depression is a prevalent medical or mental illness that affects 10%-30% of the global population.[2] Psychiatric conditions, also known as psychiatric diseases, are mental health issues affecting various aspects of life. Stress, a complex mental disorder, can lead to depression, anxiety, and cognitive dysfunction, affecting cognitive processes and slowing learning.[3–6] Sedative hypnotics, which suppress the central nervous system and affect gamma-aminobutyric acid (GABA) receptors, relieve anxiety and maintain relaxation and sleep by causing neurotransmitters in the brain.[7] Antidepressants are drugs that mitigate the effect of depression conditions by altering the chemical imbalanced mood and brain neurotransmitters. Common adverse drug reactions include dry throat, fatigue, nervous anxiety, drowsiness, vertigo, stomach problems, and cardiac arrhythmias (heart and apostasy disorders).[8] The most common antidepressants are selective serotonin reuptake inhibitors (SSRI). SSRIs reduce stress by increasing serotonin levels in the brain. As one chemical messenger, serotonine may transport impulses between brain cells. Synthetic medications, now widely available, can cause cognitive decline, physical dependence, and immunity issues due to serious side effects such as respiratory, digestive, and immune dysfunction.[8]

Plants serve as a fundamental reservoir of important medicinal compounds in worldwide healthcare. Throughout history, humans have employed many plant species for an extended period of time to address and alleviate a wide range of ailments.[9,10] The World Health Organization reports that over 75% of those usually use herbal medicines to meet their daily health needs.[11,12] *Tabernaemontana divaricata* is an arboreal plant that belongs to the family apocynaceae popularly known as "jasmine-vane," "dairy-two brothers," "jasmine", "forquilheira and snake bark".[13,14] This species is currently distributed in the north, north-east, southeast, central-west, and south areas.[15] The folk medicine used in this herb is antiophytic, vermifugal, dental antidota, anti-inflammatory, and analgesic extracts; injections and alcohol extracts of that plant.[14,16–18] A variety of chemical elements such as alkaloids, triterpenoids, hormones, flavonoids, phenylpropanoids, and phenolic acids have been thoroughly studied and isolated from leaves, roots, stems, and whole plant.[19,20]

## Aim

This study evaluates the role of Indian plants in neuropharmacological mouse regulation, highlighting the benefits of sedative and hypnotic medications, which can be manufactured from prescriptions despite possible side effects and allergic reactions.

## Material and methods

### *Chemicals and drugs*

Thiopental sodium and Diazepam are utilized as standard medications. All required components were purchased from the Lab Trading Laboratory, Aurangabad, India.

### *Plant materials*

The leaves of *T. divaricata* were bought from Rise N' Shine Botanical Boutique, Pune, Maharashtra, India. Dr. K. Madhava Chetty, Director of the Botanical Department of Sri Venkateswara University, Tirupati, 517502, Andhra Pradesh, India, authenticated the herbarium. A voucher specimen no. 0972 was deposited. After collecting the authentication certificate, the extraction procedure was performed.

### *Drying and grinding*

The leaves of *T. divaricata* were separated, dried, and pulverized into a coarse powder to prevent decomposition and photochemical debasement, ensuring that the dynamic constituents remained intact. Before the study began, the powder was meticulously enclosed within a container and stored in an environment that included low temperature, lack of light, and dry conditions.

### *Preparation of methanol, acetone and ethyl acetate extracts*

300, 350 and 350 g of granulated leaves of *T. divaricata* were doused in 1000 mL, 1200 mL and 1500 mL of 95% methanol, acetone, and ethyl acetate, respectively, in separate glass compartments for 10 days going with standard shaking and mixing. The entire blend experienced rough filtration through some fine white cotton material. It is separated by Whatman filter paper and the extracts are obtained. The extracts of methanol, acetone, and ethyl acetate obtained from the leaves exhibited yield values of 2.07% w/w, 2.01% w/w, and 2.23% w/w, respectively.[21]

### *Experimental animals*

170 Swiss albino mice (22–25 g) were used in the present study and these were obtained from Flair Labs, Gujrat, In-

dia. Both sexes of mice were between six and seven weeks old. They were kept in animal cages, adhering to standard environmental parameters, including a temperature range of 22–25°C, a humidity level of 60–70%, and a light-dark cycle of 12 hours each. The mice were provided with a standard pellet diet. The study was conducted following internationally recognized guidelines for the use and welfare of laboratory animals. Flair Labs' research ethics committee, Surat-394315, Gujarat, India, authorized our research procedures and *in vivo* studies (ethical approval number: 1250/PO/RcBi/S/23/CPCSEA).

***Phytochemical screening***

Identify functional groups as described; phytochemical screening of *T. divaricata* was performed.[20]

***Sleeping time test***

The study by Ali et al. utilized a specific methodology to investigate the effects of three extracts on the thiopental sodium-produced sleep time test produced by thiopental sodium.[22] In this case, the mice were divided into eleven distinct groups, each consisting of five. The control group, group I, received distilled water and diazepam (0.5 mg/kg, body weight, p.o.). The standard was utilized and acquired by group II. Groups III, IV, V, VI, VII, VIII, IX, X, and XI have individually administered all extracts at 50, 100 and 200 mg/kg body weight doses. After thirty minutes, thiopental sodium (20 mg/kg body weight) was administered intraperitoneally to all experimental groups to induce sleep. As a result of their lack of coordination, individual mice placed an object on a table and recorded it. The mice were seen to promptly suppress their right reflex after administering thiopental sodium, resulting in a sleep duration characterized by the period between the initial inhibition. The proportion of effect was obtained by employing the following equation:

$$\text{Effect (\%)} = \frac{\text{Average duration of loss of right reflex in the test group}}{\text{Average duration of loss of right reflex in the control group}} \times 100$$

***Hole cross test***

As stated, the research was carried out by Uddin et al.[23] A cage measuring 0.30×0.20×0.14 m was utilized. A divider was affixed to the central location of the enclosure. A circular aperture measuring 0.03 m in diameter was precisely positioned at a height of 0.075 m in the frame's center. The experimental subjects were split into three groups: control, standard and extract. They were then placed on one side of the frame. After administering the control, standard, and test samples, subsequent quantification of the mouse's passage through the hole connecting the two chambers was conducted. This quantification was performed in 3 minutes. The mice were divided into eight groups, and each group had five. Group I was designated as the control group and administered distilled water, while group II was administered diazepam, which served as standard treatment. Methanol leaf extracts were obtained for groups III and IV at doses of 100 and 200 mg/kg body weight, respectively. The experimental study involved administering acetone leaf extract to two groups, group V and group VI, at the same dosages. The experimental subjects in groups VII and VIII received leaf extract of ethyl acetate at the same doasges.

$$\text{Movements Inhibition (\%)} = \frac{\text{Mean No. of movements (control)} - \text{Mean No. of movements (test)}}{\text{Mean No. of movements (control)}} \times 100$$

***Hole board test***

The technique mentioned above was employed in a study by Sheikh et al.[24] The present investigation employed a level base of 0.9×0.9 m in diameter, featuring 16 evenly spaced holes. Furthermore, the height of this stage was 0.05 m. The mice were divided into eight groups: control, standard, and test. Each group consists of five mice (n=5). Group 1 was assigned to the control condition and administered distilled water. Diazepam was administered orally at a dose of 1 mg/kg body weight and served as the standard in group II. Groups III, IV, V, VI, VII, and VIII have individually administered all extracts at 100 and 200 mg/kg body weight doses. The study recorded the frequency of head dips made by individual mice into monitoring holes for 10 minutes.

$$\text{Inhibition (\%)} = \frac{\text{Mean No. of head dips (control)} - \text{Mean No. of head dips (test)}}{\text{Mean No. of head dips (control)}} \times 100$$

***Open field test***

The investigation described in this study was conducted by Anisuzzman et al.[25] The test apparatus consisted of a flat field measuring 0.5 $m^2$ with a square pattern. One side of the field had squares painted in alternating black and white, resembling a chessboard. The mechanical system used in the experiment had a compartment height of 0.1 m. The mice were divided into eight groups, each containing five (n=5) mice. Group I served as the control and received distilled water. Diazepam (1 mg / kg, bw, po) was administered to group II as standard treatment. Groups III, IV, V, VI, VII and VIII received different extracts (methanol, acetone, and ethyl acetate) at doses of 100 and 200 mg/kg body weight doses. The number of squares moved by the animals at various intervals after oral administration of the test substances was recorded.

$$\text{Movements inhibition (\%)} = \frac{\text{Mean No.of movements (control)}-\text{Mean No.of movements (test)}}{\text{Mean No.of movements (control)}} \times 100$$

***Statistical analysis***

Data were analyzed using SPSS statistical tools, version 20, IBM, Chicago, IL, USA. Findings were expressed as mean±standard error of the mean (SEM). Additionally, a single-way ANOVA accompanied by a post hoc Dunnett test for sleep time, hole board, hole cross and open field tests were used to compare groups.

## Results

***Phytochemical screening***

The phytochemical composition of the extracts, as depicted in Table 1, revealed the existence of various distinct groups of chemicals such as alkaloids, flavonoids, saponins, tannins, steroids, gums, glycosides, and terpenoids. The methanol extract (ME) did not include steroids, glycosides, or terpenoids, while the acetone extract (AE) lacked saponins or glycosides, and the ethyl acetate extract (ETAE) lacked tannins or gums.

**Table 1.** The phytochemical components present in leaf extracts of *T. divaricata*

| Compounds | ME | AE | ETAE |
|---|---|---|---|
| Alkaloids | + | + | + |
| Flavonoids | + | + | + |
| Saponins | + | - | + |
| Tannins | + | + | - |
| Steroids | - | + | + |
| Gums | + | + | - |
| Cardiac glycosides | - | - | + |
| Terpenoids | - | + | + |

**Table 2.** Sleeping time in mice was induced by the effect of. *divaricata* leaves extracts on thiopental-Naa

| Group | Dose (mg/kg) | Latent period | Sleeping time | Effect (%) |
|---|---|---|---|---|
| Control | 10 mL/kg | 11.8±0.37 | 30.2±4.71 | 0 |
| Standard | 0.5 | 2.5±0.316 | 199.4±7.44 | 660.26** |
| ME | 50 | 7.0±0.71 | 72.6±4.01 | 240.4* |
| ME | 100 | 4.3±0.49 | 147.8±6.74 | 489.4** |
| ME | 200 | 2.2±0.25 | 206.8±5.30 | 684.77** |
| AE | 50 | 6.8±0.73 | 66.8±3.94 | 221.19* |
| AE | 100 | 4.0±0.32 | 136.4±4.02 | 451.66z** |
| AE | 200 | 3.4±0.37 | 198.0±5.94 | 655.63** |
| ETAE | 50 | 6.6±0.87 | 76.2±4.42 | 252.32* |
| ETAE | 100 | 5.0±0.32 | 142.8±6.94 | 472.85** |
| ETAE | 200 | 3.1±0.19 | 201.4±6.19 | 666.89** |

[a] The results are presented as the mean value with the SEM for a sample size of 5, the statistical significance was determined using a one-way analysis of variance (ANOVA) followed by a Dunnett's test, the obtained were * – p<0.05, ** – p<0.01, indicating significant differences compared to the control group

***Sleeping time test***

The extracts at doses of 50, 100, and 200 mg/kg demonstrated a substantial dose-dependent reduction in the time to start sleep in the thiopental-induced hypnosis procedure, mainly in the case of leaf extracts of *T. divaricata***.** The results of the extracts at the beginning of sleep were equivalent to those of the standard drug diazepam. The study found that the leaf extracts of methanol, acetone and ethyl acetate leaf extracts had a maximum dose-dependent effect of 684.77%, 655.63%, and 666.89% during loss of right reflex, respectively (Table 2).

***Hole cross test***

The hole cross test of the treated *T. divaricata* groups indicated a reduction in activity from its rudimentary value of 0 to 120 minutes. At doses of 200 mg/kg (p<0.01), the maximum inhibition of locomotor activity was observed, which was similar to the standard diazepam (Table 3).

**Table 3.** Neuropharmacological potential test of *T.divaricata* leaf extracts by hole cross methoda

| Group | Dose | Number of movement (% of movememts imhibition) | | | | |
|---|---|---|---|---|---|---|
| | | 0 min | 30 min | 60 min | 90 min | 120 min |
| Control | 10 mL/kg | 4.8±0.58 | 5.4±1.21 | 4.2±0.58 | 4.8±0.80 | 4.4±0.51 |
| Standard | 1 | 2.0±0.55** | 1.8±0.58** | 2.6±0.24** | 2.2±0.49** | 1.2±0.37** |
| ME | 100 | 3.8±0.20 | 3.6±0.60* | 4.2±0.73 | 3.6±1.83* | 3.8±0.37 |
| ME | 200 | 3.0±1.22** | 3.6±0.68* | 2.8±0.86** | 3.2±0.86* | 2.6±0.68** |
| AE | 100 | 3.4±0.98* | 2.8±0.37** | 4.8±1.24 | 3.8±1.77 | 3.0±0.63** |
| AE | 200 | 4.0±0.77 | 3.8±0.73* | 2.6±1.03** | 2.4±0.40** | 2.0±0.45** |
| ETAE | 100 | 6.4±0.68 | 4.2±1.02 | 4.8±1.11 | 6.2±1.59 | 4.2±0.86* |
| ETAE | 200 | 3.6±0.40* | 3.8±0.66 | 3.8±0.97 | 3.0±0.63** | 2.6±0.24** |

[a] the results are presented as the mean value with the SEM for a sample size of 5, the statistical significance was determined using a one-way analysis of variance (ANOVA) followed by a Dunnett test, the obtained p values were * – p<0.05, * – p<0.01, indicating significant differences compared to the control group

***Hole board test***

At the dosage amount of 100 mg/kg and 200 mg/kg body weight (p<0.01) of extracts from *T. divaricata* leaves, the number of holes transported by mice was substantially reduced from its original amount at 0 to 120 min. The outcome demonstrated that the leaf extracts of methanol, acetone, and ethyl acetate showed 31.19, 36.14, and 30.70% inhibition at the given doses, and the 67.34% inhibition was higher for the standard diazepam (Table 4).

***Open field test***

At administered dosages of 100 mg/kg and 200 mg/kg of body weight, leaf extracts significantly reduced the number of squares moved by mice compared to the initial count at 0 to 120 minutes (p<0.01). The study found that the methanol extract of the *T. divaricata* plant inhibited locomotive activity in mice, with a maximum inhibition of 40.8%, similar to the 45.1% inhibition of the acetone extract, and for the ethyl acetate extract 39.1% inhibition. This suggests leaf neuro-modulatory properties (Table 5).

**Table 4.** Neuropharmacological potential test of extracts from *T. divaricata* leaves using the hole board method[a]

| Group | Dose (mg/kg) | Number of head dips | Inhibition (%) |
|---|---|---|---|
| Control | 10 mL/kg | 44.4±1.86 | 0 |
| Standard | 1 | 17.2±1.24 | 67.34** |
| ME | 100 | 33.8±3.43 | 23.87* |
| ME | 200 | 27.8±2.29 | 31.19** |
| AE | 100 | 29.8±2.08 | 36.14** |
| AE | 200 | 29.2±1.59 | 27.72** |
| ETAE | 100 | 32.6±1.60 | 19.31* |
| ETAE | 200 | 28.0±1.64 | 30.70** |

[a] the results are presented as the mean value with the SEM for a sample size of 5, the statistical significance was determined using a one-way analysis of variance (ANOVA) followed by a Dunnett test, the obtained p-values were * – p<0.05, ** – p<0.01, indicating significant differences compared to the control group

**Table 5.** Neuropharmacological potential test of *T. divaricata* leaf extracts by open field methoda

| Group | Dose | Number of movement (% of movememts imhibition) | | | | |
|---|---|---|---|---|---|---|
| | | 0 min | 30 min | 60 min | 90 min | 120 min |
| Control | 10 mL/kg | 44.4±2.99 | 41.2±2.06 | 45.6±1.89 | 43.0±2.59 | 43.6±1.6 |
| Standard | 1 | 15.4±3.85** | 17.2±4.29** | 19.6±3.23** | 19.6±3.74** | 20.6±2.66** |
| ME | 100 | 33.4±2.58 | 30.6±2.62* | 32.4±2.18* | 29.8±1.39** | 31.6±2.71* |
| ME | 200 | 26.6±1.94** | 28.6±1.97* | 22.2±1.24** | 25.4±1.33** | 25.8±1.66** |
| AE | 100 | 35.8±1.77 | 34.6±1.44 | 33.6±2.25 | 31.0±1.70 | 32.6±2.11 |
| AE | 200 | 28.6±0.93* | 29.2±1.69* | 25.0±1.14** | 27.8±1.07** | 28.2±1.85* |
| ETAE | 100 | 35.2±3.77 | 30.4±3.17* | 24.2±3.31** | 23.0±2.61** | 28.4±1.83* |
| ETAE | 200 | 29.0±1.38* | 28.8±1.07** | 27.4±3.04** | 29.2±2.08* | 28.4±1.70** |

[a] the results are presented as the mean value with the SEM for a sample size of 5. The statistical significance was determined using a one-way analysis of variance (ANOVA) followed by a Dunnett test, the obtained p-values were * – p<0.05, ** – p< 0.01, indicating significant differences compared to the control group

## Discussion

Plant-derived natural medicines have historically shown therapeutic potential, with natural chemicals commonly used in herbal medicines, minerals, nutritional supplements, and therapeutic interventions in various sectors. The study tested the sedative effects of *T. divaricata* on mice's spontaneous locomotor activity. The results showed that the extracts reduced the duration and frequency, suggesting a soothing effect. The study also found that head tilt is correlated with cognitive condition. The findings show a statistically significant reduction in hole crossings (p<0.05, p<0.01) following the oral administration of leaf extracts, including experimental methanol, acetone and ethyl acetate at 200 mg/kg of body weight (Table 3). When administering leaf extracts, two dose amounts were used: 100 mg/kg and 200 mg/kg body weight. Repressive activity was observed 120 minutes before extract administration was extended for 120 minutes. However, the experimental extracts caused significant inhibition (p<0.05, p<0.01,), which was expanded in the observation period from 0 minutes to 120 minutes in the doses measured (Table 5). Tables 3 and 5 show that the locomotive operation condensed with the extract supports the CNS-depressant results (Table 3 and 5). Both experiments significantly decreased mouse locomotion. GABA is the most important inhibitory intravenous neurotransmitter of the central nervous system implicated in physiological and emotional processes.[26] By modifying the alteration of the GABA receptor in the synthesis, eclectic medicine could modify the GABA system by potentiating post-synaptic induced GABA inhibition.[27–29] The conductivity of chloride or GABA performance can be improved by simultaneous voltage depression of the $Ca^{2+}$ channel.[29] The study reveals that CNS GABAergic neurons can be inhibited or activated by brain neurons, enhancing GABA affinity and potentially increasing head dip in animals, indicating anxiety activity. However, the frequency of head dips related to depressing properties was reduced.[31,32]

Dose-dependent sleep extends the sleep cycle, suggesting a deep sedating effect in sleep induced by Thiopental Sodium. Thiobarbiturate sodium is part of a thiopental pathway that contributes to sleep in humans and mice. It has an affinity for the GABA receptor complex and induces hyperpolarization of the post-synaptic neuron through GABA-mediated mechanisms.[33–35] It promotes GABA activity and can also hinder glutamate excitatory receptors. This molecular action leads to a reduction in neuronal function. A mixture of components could depend on the therapeutic benefits of conventional remedies. Several studies have reported the anxiolytic and sedative properties of alkaloids, glycosides, terpenoids, and flavonoids. Additionally, tannin can also be attributed to nonspecific CNS depression.[36–41] By activating protein kinase C and inducing cell survival genes to produce transcriptional factors, flavonoids and steroids are psychocinomatic.[42] The phytochemical investigations conducted in the extracts of *T. divaricata* indicated the presence of alkaloids, flavonoids, saponins, tannins, steroids, gums and glycosides. Bioactive compounds from nature and human nutrition are potential pharmaceutical candidates for the prevention of chronic diseases, the mitigation of stress-induced depression and neuro-pharmacological properties in antidepressant and anxiolytic medicines.[43,44]

### *Study limitations*

The study on the neuropharmacological action of the *T. divaricata* plant extract in a mice model has limitations, as its results may not accurately represent human neurological systems. This investigation faces limitations in dosage optimization, species-specific metabolic pathways, and detailed mechanistic investigations. The neurochemical and behavioral results are encouraging,

but the precise molecular processes are not fully understood. The study may not consider potential toxicity or long-term effects, and its wider use may be restricted by a lack of research on extract standardization and interactions.

## Conclusion

The study found a significant correlation between the dose administered and observed results, indicating that the crude extracts of *T. divaricata* have significant neuropharmacological activity. *The extracts of T. divaricata* leaves show potential for sedative, anxiolytic, and anticonvulsant properties, potentially reducing mouse locomotive function and avoiding the tranquilizing side effects of non-selective GABA agonists. Sedative components in methanol, acetone, and ethyl acetate extracts could be used to create insomnia treatments, but more research is needed to understand their neuropharmacological activity.

## Declarations

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***Authors' contributions***

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

***Conflicts of interest***

There are no conflicts of interest involved in the study.

***Data availability***

Due to privacy concerns, the data are not publicly available, but can be accessed upon reasonable request from the corresponding author with a signed data access agreement.

***Ethics approval***

Flair Labs' research ethics committee, Surat-394315, Gujarat, India, authorized our research procedures and the *in vivo* studies (ethical approval number: 1250/PO/RcBi/S/23/CPCSEA).

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**ORIGINAL PAPER**

# Evaluation of hormonal and adipokine biomarkers in the diagnosis of polycystic ovary syndrome – a case-control study

Redha Dawud Abd Alredha [1], Krarr Haider Haddawi [2], Wisal Raoof Al-Hayali [3], Bushra Raouf Yaseen [4]

[1] Department of Medical Laboratory Techniques, College of Health and Medical Techniques, Al-Furat Al-Awsat Technical University, Al-Kufa, Iraq
[2] Department of Clinical Biochemistry, College of Medicine, University of Al-Qadisiyah, Iraq
[3] Department of Microbiology, College of Medicine, University of Tikrit, Iraq
[4] Natural Resources Research Center, University of Tikrit, Iraq

**ABSTRACT**

**Introduction and aim.** Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder that significantly affects women of reproductive ages. This study evaluated the diagnostic potential of hormonal biomarkers such as anti-Müllerian hormone (AMH), total and free testosterone, ratio of luteinizing hormone (LH/FSH) ratio, and adipokines, including visfatin and kisspeptin, in distinguishing PCOS patients from healthy controls.

**Material and methods.** In this case-control study, 50 women diagnosed with PCOS were compared with 50 controls of the same age. Demographic and clinical data were collected through structured interviews and physical examinations. Physical activity levels was assessed using the International Physical Activity Questionnaire short form as well as anthropometric measurements were performed using a calibrated digital scale Seca 803. Blood samples were analyzed for AMH, total and free testosterone, LH/FSH ratio, visfatin, and kisspeptin levels using enzyme-linked immunosorbent assay.

**Results.** PCOS patients exhibited significantly higher levels of AMH (8.1±2.3 ng/mL vs. 4.07±1.1 ng/mL, p<0.001), Free testosterone (4.55±0.95 pg/mL vs. 2.47±0.46 pg/mL, p<0.001), visfatin (86.6±11.02 ng/mL vs. 49.53±10.25 ng/mL, p<0.001), and kisspeptin (9.88±1.96 ng/mL vs. 4.84±1.07 ng/mL, p<0.001) compared to controls. Logistic regression showed that elevated levels of AMH (odds ratio [OR]=2.95, p=0.0056), visfatin (OR=1.7, p=0.0043) and kisspeptin (OR=18.3, p=0.0015) were strongly associated with PCOS.

**Conclusion.** These findings confirmed the significant role of AMH, testosterone, visfatin, and kisspeptin in the diagnosis of PCOS. Integration of adipokine markers, particularly visfatin and kisspeptin, with traditional hormonal markers enhances diagnostic accuracy.

**Keywords.** anti-Müllerian hormone, diagnosis, kisspeptin, LH/FSH ratio, PCOS, testosterone, visfatin

## Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common endocrine disorders affecting women of reproductive age, with an estimated prevalence ranging from 6% to 20%, depending on the diagnostic criteria used.[1] PCOS is a multifaceted condition with various clinical presentations, commonly involving hyperandrogenism, ovulatory irregularities, and polycystic ovarian

**Corresponding author:** Krarr Haider Haddawi, e-mail: krarr.haidar@yahoo.com

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morphology.[2] These symptoms not only affect reproductive health, but are also associated with metabolic disturbances such as insulin resistance, obesity and an increased risk of developing type 2 diabetes and cardiovascular disease.[3]

The pathophysiology of PCOS involves systemic changes, including metabolic and hormonal dysfunction, as changes in the well as central nervous system (CNS), such as dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis and abnormal secretion of gonadotropin-releasing hormone (GnRH) secretion.[4] Evidence suggests that the CNS plays a role in PCOS pathophysiology through the dysregulation of GnRH secretion, which disrupts the pulsatile release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). This dysregulation contributes to hyperandrogenism and anovulation observed in PCOS patients.[5] Additionally, chronic anovulation results in irregular menstrual cycles and infertility, while metabolic complications contribute to the development of insulin resistance and obesity in many PCOS patients.[6]

PCOS is typically diagnosed using one of three main criteria: the National Institutes of Health (NIH) 1990 criteria, the Rotterdam 2003 criteria, or the Androgen Excess and PCOS Society (AE-PCOS) 2006 criteria.[7] Rotterdam criteria are the most widely used and require the presence of two of the following three characteristics: hyperandrogenism, oligo or anovulation, and polycystic ovaries on ultrasound.[8]

Despite established diagnostic criteria, the identification of reliable biomarkers for PCOS remains a key challenge. Hormonal markers, including LH, FSH and androgens (total and fee testosterone), have long been used to assess reproductive and endocrine dysfunction in PCOS patients.[9] More recently, anti-Müllerian hormone (AMH) has emerged as a promising biomarker due to its correlation with the number of ovarian follicles, reflecting ovarian reserve and hyperandrogenism in PCOS.[10,11] Elevated AMH levels are commonly observed in PCOS patients, making it a valuable diagnostic tool, although it may not be universally applicable in all phenotypes.[12]

In addition to these hormonal markers, emerging research has focused on the role of adipokines, signaling proteins secreted by adipose tissue, and metabolic dysfunction observed in PCOS. Adipokines such as visfatin and kisspeptin have been implicated in insulin resistance, inflammation, and the regulation of reproductive hormones.[13,14] Visfatin, in particular, is associated with insulin resistance and has been shown to be elevated in PCOS patients, suggesting a link between metabolic disturbances and reproductive dysfunction.[15] Kisspeptin, on the other hand, plays a critical role in the regulation of GnRH secretion, and its dysregulation in PCOS contributes to the abnormal hormonal environment characteristic of the syndrome.[13]

Given the complexities of PCOS, a multimarker approach integrating both hormonal and adipokine profiles may improve diagnostic accuracy and provide greater insight into the pathophysiology of the syndrome. This study aimed to evaluate the diagnostic utility of key hormonal markers (AMH, testosterone, LH/FSH ratio) and adipokines (visfatin, kisspeptin) in differentiating PCOS patients from healthy controls. We hypothesized that elevated levels of AMH, visfatin, and kisspeptin, along with an increased LH/FSH ratio and hyperandrogenism, could serve as reliable markers for the diagnosis of PCOS. By incorporating adipokine profiles into the diagnostic framework, we sought to enhance the early detection of PCOS and provide a more comprehensive understanding of its metabolic and reproductive components.

## Material and methods

### *Study design and participants*

A case-control study was conducted between January 2023 and December 2024 at [Al-Sadr Teaching Hospital in Najaf, Iraq], following approval of the Institutional Review Board (Approval Number: 34328). Written informed consent was obtained from all participants prior to inclusion, in accordance with the principles of the Declaration of Helsinki.[16] The sample size (n) was determined using the following formula to compare the two proportions in case-control studies:[17]

$$n = \left\{ \left( Z_{\left\{\frac{\alpha}{2}\right\}} + Z_{\{\beta\}} \right)^2 \cdot \left[ p_{1(1-p_1)} + p_{2(1-p_2)} \right] \right\} / \{ (p_1 - p_2)^2 \}$$

- $Z_{\left\{\frac{\alpha}{2}\right\}} = 1.96$ for a 95% confidence level,
- $Z_{\{\beta\}} = 0.84$ for 80% power,
- $p_1 = 0.70$ (proportion of a specific marker in the PCOS group based on previous studies)
- $p_2 = 0.30$ (proportion in controls).

A total of 100 women aged 18 to 40 years were recruited and divided into two groups: 50 women diagnosed with PCOS according to the revised Rotterdam criteria and 50 age-matched healthy controls of the same age without PCOS.[18] The diagnostic criteria for PCOS include at least two of the following: (1) oligo or anovulation, (2) clinical and/or biochemical signs of hyperandrogenism and (3) polycystic ovarian morphology on ultrasound, excluding other endocrine disorders such as congenital adrenal hyperplasia, Cushing syndrome, thyroid dysfunction, or androgen-secreting tumors. Control participants were recruited from the general population and had regular menstrual cycles (21–35 days), no clinical or biochemical signs of hyperandrogenism, and normal ovarian morphology on transvaginal ultrasound.

Exclusion criteria for all participants included pregnancy, lactation, use of hormonal medications or insulin sensitizing agents within the last three months, smoking, alcohol abuse, and chronic systemic diseases such as diabetes mellitus, hypertension, or cardiovascular disease.

***Data collection***

Demographic and clinical data were collected through structured interviews and physical examinations performed by trained clinicians. The information gathered included age, body mass index (BMI) and physical activity levels assessed using the International Physical Activity Questionnaire (IPAQ) short form.[19]

Anthropometric measurements were performed with the participants wearing light clothing and no shoes. Weight was measured to the nearest 0.1 kg using a calibrated digital scale (Seca 803; Seca GmbH & Co. KG, Hamburg, Germany), and height was measured to the nearest 0.1 cm using a wall mounted stadiometer (Seca 217). BMI was calculated as weight in kilograms divided by height in meters squared ($kg/m^2$).

Clinical evaluations included detailed menstrual history (age at menarche, cycle duration, and regularity), reproductive history (eg pregnancies and miscarriages), and evaluation of signs of hyperandrogenism, such as hirsutism. Hirsutism was evaluated using the modified Ferriman-Gallwey scoring system, with a score ≥8 indicating hirsutism.[20]

***Hormonal and metabolic assessments***

Venous blood samples were collected from all participants between 8:00 a.m. and 9:00 a.m. after an overnight fast of at least 8 h. For women with regular menstrual cycles, samples were collected during the early follicular phase of the menstrual cycle (days 2–5). For women with oligomenorrhea or amenorrhea, samples were collected on a random day and progesterone levels were measured to confirm the absence of ovulation. Blood samples were centrifuged at 3,000 rpm for 10 min at 4°C, and the serum was separated and stored at − 80°C until analysis.

Serum levels of key hormonal markers, including AMH, total testosterone, LH, and FSH, were measured. These were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits: AMH: human AMH ELISA kit (catalog number: E-EL-H0317, Elabscience, Houston, TX, USA), total testosterone: testosterone ELISA kit (catalog number: E-EL-0072, Elabscience, Houston, TX, USA). Free testosterone levels were quantified using a commercially available free testosterone ELISA kit (Catalog Number: ab178663, Abcam, Cambridge, UK). LH: LH ELISA Kit (catalog number: ab178658, Abcam, Cambridge, UK). FSH: FSH ELISA kit (catalog number: E-EL-H1143, Elabscience, Houston, TX, USA). The assays were performed according to the manufacturer's instructions. The luteinizing hormone / follicle stimulating hormone ratio was calculated.

***Adipokine and neuropeptide measurements***

Serum levels of visfatin and kisspeptin were measured to assess their roles in the pathophysiology of PCOS. Quantification was performed using specific ELISA kits. Visfatin: human visfatin ELISA kit (catalog number: ab267658, Abcam, Cambridge, UK) and kisspeptin (catalog number: MBS165884, MyBioSource, Inc., San Diego, CA, USA).

All assays were performed according to the manufacturer's instructions. The intra- and inter-assay coefficients of variation were less than 10% and<15%, respectively, for all assays.

***Statistical analysis***

The Statistical Package for the Social Sciences (SPSS, IBM, Armonk, NY, USA) program was used to detect the effects of different groups (patients and controls) on the study parameters. A t-test was used to compare the means. The Chi-square test was used to compare the percentages (0.05 and 0.01 probability). Estimation of the correlation coefficient and multiple linear regression between variables. Sensitivity and specificity of parameters in the patient and control groups. Cutoff values for biomarkers were determined using receiver operating characteristic (ROC) curve analysis to maximize sensitivity and specificity. The Youden index is used to identify the optimal threshold for each parameter.[21]

## Results

***Demographic characteristics of study groups***

These findings highlight notable differences in metabolic, reproductive, and cardiovascular health between women with PCOS and healthy controls. Although no age differences were observed ($p=0.22$), the PCOS group had a significantly higher body mass index ($p<0.001$) and longer menstrual cycles ($p<0.001$). Analysis of pregnancies in the study population (Table 1) revealed significant differences between PCOS patients and controls ($p<0.001$). In the control group, 36% had one pregnancy, 30% had two pregnancies, and 34% had no previous pregnancies. On the contrary, 38% of PCOS patients had one pregnancy, 28% had two pregnancies, and 34% had three or more pregnancies. None of the PCOS patients was nulliparous. Elevated hirsutism scores ($p<0.001$) indicated more severe androgenic symptoms in the PCOS group. Furthermore, both Systolic and Diastolic blood pressures were significantly higher ($p<0.001$), suggesting an increased cardiovascular risk. These results highlight notable metabolic, re-

productive, and cardiovascular differences between the groups.

**Table 1.** Demographic characteristics of control subjects and patients with PCOS[a]

| Characteristic | Control n=50 | PCOS n=50 | p |
|---|---|---|---|
| Age (years) | | | |
| Mean±SD | 27.88±6.12 | 29.4±6.6 | 0.22 I NS |
| Range | 18–39 | 18–39 | |
| BMI (kg/m$^2$) | | | |
| Mean±SD | 24.4±2.1 | 31.98±3.21 | <0.001 I*** |
| Range | 20.27–29.9 | 25.46–39.9 | |
| Cycle length (days) | | | |
| Mean±SD | 30.86±2.06 | 43.4±4.02 | <0.001 I*** |
| Range | 28–34 | 35–50 | |
| Pregnancies | | | |
| Non pregnancies, n (%) | 0 (0%) | 17 (34%) | <0.001 F*** |
| Pregnancies (1), n (%) | 19 (38%) | 18 (36%) | |
| Pregnancies (2), n (%) | 14 (28 %) | 15 (30%) | |
| Pregnancies (3), n (%) | 17 (34 %) | 0 (0%) | |
| Hirsutism (FG score) | | | |
| Mean±SD | 3.06±0.9 | 8.2±1.94 | <0.001 I*** |
| Range | 1.31–5.03 | 4.13–14.2 | |
| Systolic (mmHg) | | | |
| Mean±SD | 119±9.98 | 128.5±10.25 | <0.001 I*** |
| Range | 97.5–143 | 106.8–157.8 | |
| Diastolic (mmHg) | | | |
| Mean±SD | 74.98±5.1 | 85.64±4.59 | <0.001 I*** |
| Range | 65.4–86.1 | 75.4– 96.2 | |

[a] Pregnancies were classified as follows: non-pregnancies – no previous pregnancy, pregnancies (1) one previous pregnancy, pregnancies (2) two previous pregnancies, and pregnancies (3) three or more previous pregnancies; n number of cases, SD – standard deviation, BMI body mass index, Fisher's exact test; I independent samples t-test; NS not significant (p≥0.05)

### *Comparison of mean hormonal values among control group and patients with PCOS*

Table 2 shows significant hormonal imbalances in PCOS patients compared to controls, with elevated free testosterone (p<0.001) and AMH levels (p<0.001). The LH/FSH ratio was significantly higher in PCOS patients (3.44±0.47) than in controls (1.54±0.42, p<0.001). The mean serum concentrations of LH and FSH in PCOS patients were 10.32±2.4 mIU/mL and 3.00±0.95 mIU/mL, respectively, while in the control group, LH was 5.60±1.5 mIU/mL and FSH was 3.64±0.8 mIU/mL. The FSH/LH ratio was higher in PCOS patients than in controls; however, this finding is consistent with historical observations and has limited diagnostic significance compared to AMH and adipokines. PCOS patients also exhibited lower total testosterone levels (p<0.001). These findings underscore key hormonal disruptions in PCOS, as shown in Figure 1.

**Table 2.** Comparison of hormonal markers between control and PCOS patients[a]

| Characteristic | Control (n=50) | PCOS (n=50) | p |
|---|---|---|---|
| LH (mIU/mL) | | | |
| Mean±SD | 5.60±1.5 | 10.32±2.4 | <0.001 I*** |
| Range | 3.5–8.2 | 7.0–15.6 | |
| FSH (mIU/mL) | | | |
| Mean±SD | 3.64±0.8 | 3.00±0.95 | <0.001 I*** |
| Range | 2.4–5.2 | 1.8–5.0 | |
| LH/FSH Ratio | | | |
| Mean±SD | 1.54±0.42 | 3.44±0.47 | <0.001 I*** |
| Range | 0.58–2.5 | 2.4–4.63 | |
| Total testosterone (ng/dL) | | | |
| Mean±SD | 45.3±9.2 | 81.4±16.5 | <0.001 I*** |
| Range | 22.6–63.08 | 26.48–118.6 | |
| Free testosterone (pg/mL) | | | |
| Mean±SD | 2.47±0.46 | 4.55±0.95 | <0.001 I*** |
| Range | 1.36–3.188 | 1.922–7.26 | |
| AMH (ng/mL) | | | |
| Mean±SD | 4.07±1.1 | 8.1±2.3 | <0.001 I*** |
| Range | 1.59–6.68 | 2.57–11.2 | |

[a] anti-Müllerian hormone, LH – luteinizing hormone, FSH follicle-stimulating hormone, statistical significance is indicated by ***p<0.001, I – independent sample t-test

### *Analysis of adipokine variations between the control and PCOS groups*

Table 3 reveals significantly elevated levels of adipokines in PCOS patients compared to controls, with visfatin (86.6±11.02 vs. 49.53±10.25 ng/mL) and kisspeptin (9.88±1.96 vs. 4.84±1.07 ng/mL) both showing higher mean values in the PCOS group (p<0.001). These findings highlight notable differences in adipokine regulation in PCOS, as shown in Figure 2.

**Table 3.** Comparative analysis of adipokine levels in control and PCOS patients[a]

| Characteristic | Control (n=50) | PCOS (n=50) | p |
|---|---|---|---|
| Visfatin (ng/mL) | | | |
| Mean±SD | 49.53±10.25 | 86.6±11.02 | <0.001 I*** |
| Range | 31.64–68.9 | 62.5–115.3 | |
| Kisspeptin (ng/mL) | | | |
| Mean±SD | 4.84±1.07 | 9.88±1.96 | <0.001 I*** |
| Range | 2.58–7.33 | 6.3–15.13 | |

[a] Statistical significance is indicated by ***p<0.001, I independent sample t-test

### *Analysis of adipokine and hormonal correlations in PCOS patients*

Table 4 shows the significant positive correlations between adipokines (Visfatin and Kisspeptin) and hormonal markers in PCOS patients. For example, visfatin was strongly correlated with kisspeptin (r=0.720, p<0.001), indicating its potential interdependence in the pathophysiology of PCOS. Similarly, both adipokines

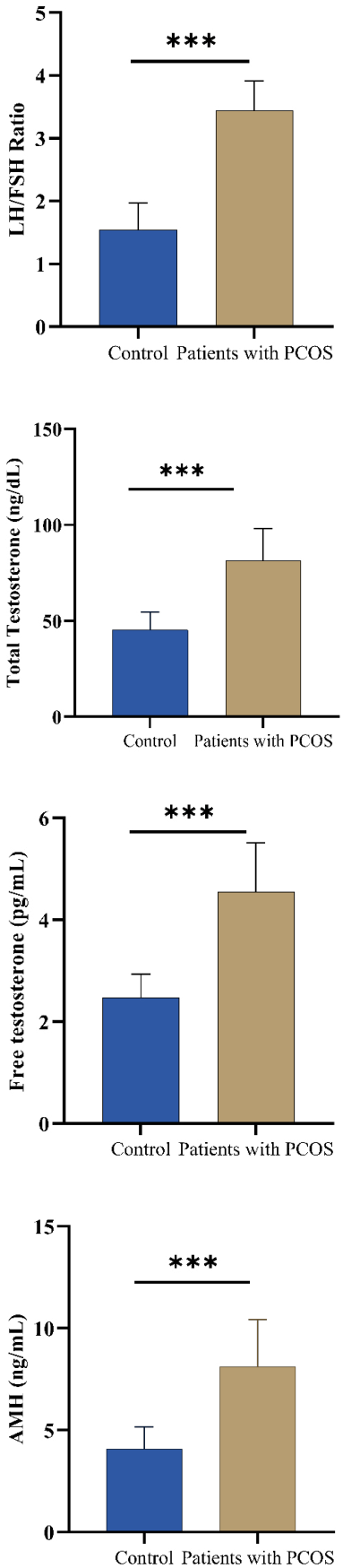


**Fig. 1.** Bar chart showing the mean comparison among control group and patients with PCOS

showed strong correlations with AMH and testosterone levels, suggesting their roles in hyperandrogenism and ovarian dysfunction.

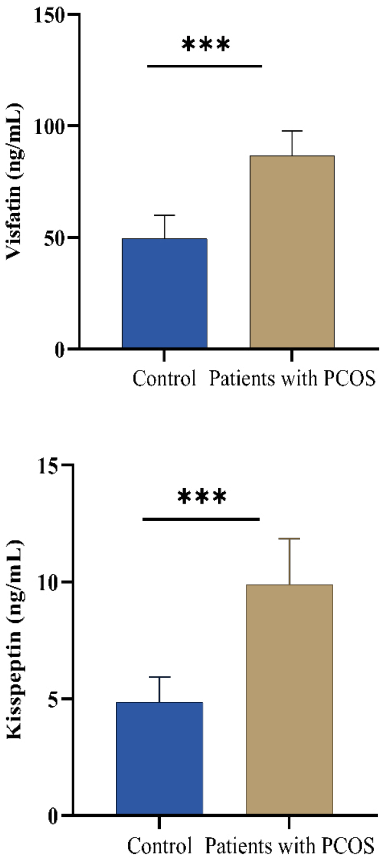


**Fig. 2.** Bar chart showing the mean comparison among control group and patients with PCOS

**Table 4.** Correlations of adipokines and hormone levels in patients with PCOS.

| | Correlation coefficient (r) and p | | | | |
|---|---|---|---|---|---|
| | Visfatin | Kisspeptin | Free testosterone | Total testosterone | AMH |
| Visfatin (ng/mL) | 1 | r= 0.720 p<0.001 | r=0.699 p<0.001 | r=0.711 p<0.001 | r=0.695 p<0.001 |
| Kisspeptin (ng/mL) | r= 0.720 p<0.001 | 1 | r= 0.692 p<0.001 | r= 0.667 p<0.001 | r= 0.647 p<0.001 |
| Free testosterone (pg/mL) | r= 0.699 p<0.001 | r= 0.692 p<0.001 | 1 | r= 0.668 p<0.001 | r= 0.551 p<0.001 |
| Total testosterone (ng/dL) | r=.711 p<0.001 | r= 0.667 p<0.001 | r= 0.668 p<0.001 | 1 | r= 0.637 p<0.001 |
| AMH (ng/mL) | r= 0.695 p<0.001 | r= 0.647 p<0.001 | r= 0.551 p<0.001 | r= 0.637 p<0.001 | 1 |

### *Diagnostic efficacy of hormonal and adipokine markers in PCOS*

Table 5 highlights various hormonal and adipokine markers for PCOS, all showing significant predictive power with p values of 0.001. For AMH, a cut-off value of >5.74 ng/mL achieved 86% sensitivity and 94% specificity, reflecting its high diagnostic utility for PCOS. Similarly, visfatin and kisspeptin demonstrated excel-

lent predictive power, with cut-off values of >68.91 ng/mL and >6.96 ng/mL, respectively, achieving sensitivities and specificities exceeding 95% (Fig. 3).

**Table 5.** Analysis of the ROC curve for hormones and adipokines in the diagnosis of PCOS a

| Variables | Cut-off value | Sens**% | Spec% | Ppv** | Npv | Accuracy | AUC% | p (AUC= 0.05) |
|---|---|---|---|---|---|---|---|---|
| LH/FSH ratio | >2.38 | 100 | 96 | 98 | 100 | 98 | 100 | 0.001** |
| Total testosterone (ng/dL) | >63.07 | 92 | 100 | 100 | 92.7 | 92 | 96 | 0.001** |
| Free testosterone (pg/ml) | > 3.09 | 96 | 98 | 98 | 96 | 94 | 97.6 | 0.001** |
| AMH (ng/ml) | >5.74 | 86 | 94 | 93.5 | 87 | 80 | 91 | 0.001** |
| Visfatin (ng/ml) | >68.91 | 98 | 100 | 100 | 98 | 98 | 99.7 | 0.001** |
| Kisspeptin (ng/ml) | >6.96 | 96 | 98 | 100 | 96 | 94 | 99.6 | 0.001** |

[a] Sens sensitivity, Spec – specificity, PPV positive predictive value, NPV negative predictive value, accuracy [(Sensitivity + Specificity) - 1], AUC area under the curve

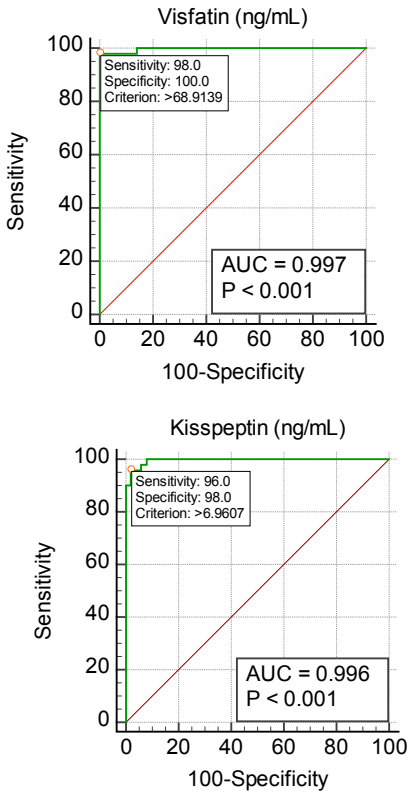


**Fig. 3.** The ROC curve for adipokines

### *Statistical Analysis of adipokines and insulin resistance in PCOS*

Table 6 presents the logistic regression analysis showing a strong model fit ($R^2$=0.87) to explain the relationship between adipokines and PCOS. Visfatin and kisspeptin were significantly associated with an increased risk of PCOS, with odds ratios of 1.7 and 18.3, respectively, both of which were statistically significant. These findings suggest that high levels of adipokines strongly predict the probability of PCOS.

**Table 6.** Logistic regression analysis of adipokines in PCOS patients (model 1, $R^2$=0.87)[a]

| Variables | B (coef) | Wald | Odds ratio | 95% CI for odds ratio | p |
|---|---|---|---|---|---|
| Visfatin | 0.53 | 1.8 | 1.7 | 1.18 to 2.45 | 0.0043** |
| Kisspeptin | 2.3 | 9.1 | 18.3 | 3.59 to 180.81 | 0.0015** |

[a] B (coef) regression coefficient, CI – confidence interval

### *Evaluation of hormonal profiles in predicting PCOS*

Table 7 presents the results of the logistic regression analysis identifying the significant hormonal predictors of PCOS. AMH was strongly associated with PCOS, with an odds ratio (OR) of 2.95 (95% CI: 1.33–5.28, p=0.0056). This underscores the utility of AMH as a reliable biomarker for the diagnosis as it reflects ovarian reserve and correlates with polycystic ovarian morphology. The total testosterone level also had a significant effect (odds ratio=1.19, p=0.0002). On the contrary, the historically significant LH / FSH ratio was not a statistically significant predictor in this model (p=0.3168). This finding aligns with the current literature, suggesting that the clinical utility of the LH/FSH ratio has decreased due to its variability across PCOS phenotypes and the influence of confounders, such as obesity and age.

**Table 7.** Hormonal predictors of PCOS a logistic regression model (model 2, $R^2$=0.9)[a]

| Variables | B (coef) | Wald | Odds ratio | 95% CI for odds ratio | p |
|---|---|---|---|---|---|
| AMH | 0.97 | 6.7 | 2.95 | 1.33 to 5.28 | 0.0056** |
| Total testosterone | 0.17 | 14 | 1.19 | 1.088 to 1.309 | 0.0002*** |
| LH/FSH ratio | 0.180 | 0.067 | 0.455 | 0.0972 to 2.1274 | 0.3168 |

[a] B (coef) regression coefficient, CI – confidence interval

## Discussion

This study provides a comprehensive evaluation of the hormonal and adipokine profiles in women with PCOS, advancing our understanding of their diagnostic utility and clinical relevance. Our findings revealed that AMH, testosterone, visfatin, and kisspeptin, when used in combination, significantly improved the accuracy of PCOS diagnosis. These results are consistent with a growing body of evidence, although some discrepancies with previous studies highlight the complex nature of PCOS and its diverse phenotypes.

Our study confirmed that AMH, testosterone (both total and free), visfatin, and kisspeptin levels were significantly elevated in patients with PCOS compared to healthy controls. These findings are consistent with well-established theories regarding the characteristics of PCOS endocrine and metabolic dysfunction. Additionally, logistic regression analysis demonstrated that these markers, particularly AMH and adipokines, were strong

predictors of PCOS with excellent diagnostic precision, as shown by ROC curve analyzes.

AMH has emerged as a crucial biomarker for PCOS due to its role in reflecting ovarian reserve and follicular activity.[22] In our study, AMH levels were significantly higher in PCOS patients (8.1±2.3 ng/mL) than in controls (4.07±1.1 ng/mL), which is consistent with previous studies that have also reported elevated levels of AMH in PCOS patients.[23,24] Begum et al. found that AMH is a surrogate marker for antral follicle count, with levels typically 2 to 3-fold higher in women with PCOS than in women without PCOS. Our findings support this and reinforce the idea that AMH is strongly correlated with polycystic ovarian morphology, making it a valuable diagnostic tool.

However, AMH remains a cornerstone in the diagnosis of PCOS due to its strong correlation with ovarian reserve and antral follicle count. Our findings, which showed a significant association between elevated AMH levels and PCOS (OR, 2.95; p=0.0056), further strengthen its diagnostic value. Similarly, total testosterone levels were significantly associated with PCOS (OR, 1.19; p=0.0002), reflecting the role of hyperandrogenism in pathophysiology. Although our study found that AMH is a reliable predictor of PCOS, other studies have raised concerns about its variability across different phenotypes of PCOS. Bahadur et al. and Alsolaiman et al. noted that AMH diagnostic performance of AMH may be lower in women with milder forms of PCOS or those without overt polycystic ovarian appearance.[25,26] Therefore, while AMH was a useful marker in our cohort, it may not be universally applicable to all PCOS subgroups.

Elevated androgen levels are a hallmark of PCOS, and our study confirmed this, with both total testosterone (81.4±16.5 ng/dL) and free testosterone (4.55±0.95 pg/mL) being significantly higher in PCOS patients. These findings are consistent with the extensive literature on hyperandrogenism in PCOS.[27,28] Hyperandrogenism contributes to clinical manifestations such as hirsutism, acne, and alopecia and is one of the key diagnostic criteria for PCOS.

In our logistic regression model, the total testosterone level had a modest but significant association with PCOS (OR=1.19, p=0.0002). This is consistent with studies by Grassi et al. and Ye et al., highlighting the role of hyperandrogenism as a critical driver of this syndrome.[29,30] However, the diagnostic utility of testosterone, particularly in milder cases of PCOS, has been questioned. Some studies, such as those of Pace and Azziz, reported that androgen levels could not be elevated in all PCOS patients, particularly those with less severe phenotypes or without clinical signs of hyperandrogenism.[31]

Although the FSH/LH ratio has historically been considered a marker for PCOS, its diagnostic utility is now considered limited compared to newer biomarkers such as AMH, visfatin, and kisspeptin. In our study, the LH/FSH ratio was significantly higher in the PCOS group (3.44±0.47) than in controls (1.54±0.42), consistent with the results of previous studies.[5,32] However, in our logistic regression analysis, the LH / FSH ratio did not emerge as a statistically significant predictor of PCOS (p=0.3168), suggesting that while it may serve as a useful clinical marker, it may not be as robust as other markers such as AMH or testosterone.

This result aligns with recent literature questioning the reliability of the LH / FSH ratio in all PCOS phenotypes, particularly in obese patients or those with insulin resistance.[33] These findings suggest that the LH/FSH Ratio may be more variable than previously thought and its diagnostic utility may be limited when used alone.

Chemokines play a crucial role in PCOS pathophysiology by mediating inflammatory responses and metabolic dysfunctions. Elevated chemokine levels in PCOS patients have been associated with increased insulin resistance and systemic inflammation, which contribute to the breakdown of reproductive and metabolic homeostasis. These findings suggest that chemokines act as intermediaries in the crosstalk between adipose tissues and the reproductive system.

Visfatin and kisspeptin have emerged as promising biomarkers in PCOS, particularly in light of their roles in metabolic and reproductive dysfunction. Visfatin, an adipokine secreted by visceral adipose tissue, is strongly associated with insulin resistance and systemic inflammation, which are the two hallmark features of PCOS. In this study, visfatin levels were nearly double in PCOS patients compared to controls, underscoring the potential role of visfatin in linking metabolic disturbances to reproductive dysfunction. This finding is supported by previous studies that reported elevated visfatin levels in women with PCOS, linking it to insulin resistance and inflammation.[15,34] Koleva-Tyutyundzhieva et al. found that visfatin levels were significantly higher in patients with insulin-resistant PCOS, which may explain the metabolic disturbances commonly observed in this syndrome.

Kisspeptin is a neuropeptide that plays a pivotal role in the regulation of the HPG axis by stimulating the release of GnRH. Dysregulation of kisspeptin signaling has been implicated in abnormal gonadotropin secretion characteristics of PCOS. The elevated levels of kisspeptin observed in this study may reflect compensatory mechanisms to counteract disrupted HPG axis signaling in PCOS. These findings highlight the dual role of kisspeptin as a metabolic and reproductive biomarker in PCOS.[35] In our study, kisspeptin emerged as a strong predictor of PCOS (OR=18.3, p=0.0015), highlighting its potential as a biomarker of reproductive dysfunction in PCOS. Studies by Yeung et al. and Gao et al. demonstrated that elevated kisspeptin levels are associated with

abnormal gonadotropin secretion in PCOS, further supporting our findings.[36,37]

Recent studies have highlighted the role of novel biomarkers such as callistatin in PCOS. Callistatin, a member of the kallikrein-related peptide family, has been shown to play a regulatory role in inflammation and metabolic dysfunction, which are key features of PCOS. Evidence suggests that callistatin exerts anti-inflammatory effects by modulating cytokine release and mitigating oxidative stress, making it a potential biomarker for identifying and managing PCOS.[38]

While our study focused on hormonal and adipokine markers, such as AMH, visfatin, and kisspeptin, the integration of emerging biomarkers, such as callistatin, could provide a more comprehensive diagnostic framework for PCOS. The inclusion of callistatin in future studies may help elucidate its interplay with established markers and its potential utility in predicting PCOS phenotypes and associated metabolic risks.

The combined use of hormonal and adipokine markers provided excellent diagnostic accuracy in our study, with ROC analysis showing AUC values of 0.95 or higher for the LH/FSH ratio, visfatin, and kisspeptin. These findings highlight the value of integrating adipokine markers into traditional diagnostic criteria for PCOS. By combining markers that reflect both reproductive and metabolic dysfunction, this multimarker approach could provide a more comprehensive diagnostic tool, particularly for identifying women with atypical or mild PCOS.

Our findings align with those of Liu et al. and Ruan et al., who suggested that novel biomarkers such as visfatin and kisspeptin could improve the early detection of PCOS, especially in women with metabolic abnormalities.[39,40] The high sensitivity and specificity of these markers in our study suggest that they could be incorporated into clinical practice to improve both diagnostic accuracy and potential for early intervention.

***Study limitations and strengths***

One of the main strengths of our study is the comprehensive analysis of hormonal and adipokine markers, which provides a more holistic view of endocrine and metabolic disturbances in PCOS. The use of logistic regression and ROC curve analyzes strengthened the statistical validity of our findings, and the relatively large sample size and inclusion of well-matched controls increased the reliability and generalizability of our results. However, this study has some limitations. This case-control design limits our ability to establish causal relationships between elevated markers and the development of PCOS. Longitudinal studies are necessary to assess whether changes in these biomarkers can predict the onset of PCOS over time. It is important to note that, while testosterone levels are elevated in many patients with PCOS, this may not be universally observed in all phenotypes. This variability underscores the need for a multimarker approach to PCOS diagnosis. Furthermore, the study population may not represent all PCOS phenotypes, particularly those with normal weight or those without insulin resistance. More research is required to evaluate the utility of these markers in diverse populations.

***Future directions***

Future studies should explore the longitudinal dynamics of these biomarkers in women at risk of developing PCOS. Furthermore, the role of adipokines, such as visfatin and kisspeptin, in the metabolic aspects of PCOS warrants further investigation, particularly with respect to their potential as therapeutic targets. Expanding the panel to include additional adipokines and inflammatory markers may also provide a more nuanced understanding of the metabolic and reproductive interactions in PCOS.

## Conclusion

These findings confirmed the significant role of AMH, testosterone, visfatin, and kisspeptin in the diagnosis of PCOS. Integrating adipokine markers, particularly visfatin and kisspeptin, with traditional hormonal markers improves diagnostic accuracy and provides a more comprehensive understanding of the pathophysiology of PCOS. These results suggest that a multimarker approach could be beneficial in clinical practice for early detection and management of PCOS. In addition to the hormonal and adipokine biomarkers evaluated in this study, recent advances have identified callistatin as a new marker with potential diagnostic and therapeutic implications in PCOS[38]. Future research should explore the integration of callistatin with other emerging biomarkers to develop a more holistic approach to diagnose and managing PCOS.

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## Declarations

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***Author contributions***

Conceptualization, R.D.A.A., K.H.H., W.R.A-H. and B.R.Y.; Methodology, R.D.A.A., K.H.H., W.R.A-H. and B.R.Y.; Investigation, R.D.A.A., K.H.H., W.R.A-H. and

B.R.Y.; Data Curation, R.D.A.A., K.H.H., W.R.A-H. and B.R.Y.; Writing – Original Draft Preparation, R.D.A.A., K.H.H., W.R.A-H. and B.R.Y.; Writing – Review & Editing, R.D.A.A., K.H.H., W.R.A-H. and B.R.Y.; Supervision, R.D.A.A., K.H.H., W.R.A-H. and B.R.Y.

***Conflicts of interest***

The author declare that they have no competing interests.

***Data availability***

The data sets used and analyzed during the current study are available from the corresponding author upon reasonable request.

***Ethics approval***

Ethical clearance was granted by the Institutional Review Board of the Medical Laboratory Techniques, College of Health and Medical Techniques at Al-Furat Al-Awsat Technical University, Al-Kufa, Iraq (Approval Number: 31003).

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**ORIGINAL PAPER**

# A study of several hematological and immunological parameters of patients with rheumatoid arthritis and their relationship with type 2 diabetes mellitus

Ghadeer Osama Abd , Baida Rihan Ali

Department of Pathological Analysis, College of Science, University of Thi-Qar, Nasiriya City, Iraq

**ABSTRACT**

**Introduction and aim.** Rheumatoid arthritis (RA) is a systemic inflammation that damages the joints and causes disability. In RA, glucocorticoids reduce inflammation and peripheral insulin resistance. This study aimed to investigate hematological and immunological parameters, including interleukin-24 (IL-24), interleukin-32 (IL-32), and rheumatoid factor (RF), in patients with RA, type 2 diabetes mellitus, or both, and to assess their interrelationships.

**Material and methods.** A case-control study on RA and type 2 diabetes mellitus was conducted at Al-Nasiriyah Education Hospital with 100 blood samples collected from patients, divided into four groups. Complete blood counts (CBC), erythrocyte sedimentation rate (ESR), RF, IL-24, and IL-32 levels were measured using automated analyzers and enzyme-linked immunosorbent assay.

**Results.** Patients with both diseases showed elevated ESR ($p<0.001$) and RF ($p<0.01$). RA patients increased significantly in ESR and RF, but there was no statistically significant difference in RF in type 2 diabetic patients. IL-24 was not statistically significantly increased in RA patients. IL-32 levels increased significantly in type 2 diabetes ($p=0.02$), while RA showed no significant difference.

**Conclusion.** Patients with RA have elevated levels of IL-32 expression and has a positive correlation with indicators of RA activity indicators such as ESR and RF. An increase in IL-24 and IL-32 in RA patients indicates a positive correlation between IL-24 and IL-32. Diabetic patients exhibit significantly elevated pro-inflammatory properties of IL-32.

**Keywords.** interleukin, rheumatoid arthritis, type 2 diabetes mellitus

## Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that primarily affects small joints. RA is characterized by symmetric peripheral polyarthritis. Morning stiffness that lasts more than an hour and discomfort in one or more joints that lasts weeks to months are common signs of RA.[1] The prevalence of RA around the world is estimated to be between 0.5% to 1%, with a prevalence rate that is four times higher in women than in males.[2] Patients with rheumatoid arthritis have peripheral insulin resistance that is associated with inflammatory indicators and returns to normal after treatment with glucocorticoids, which reduces the level of inflammation.[3] Systemic inflammation can increase the chance of getting diabetes in the future. C-reactive protein (CRP) and other indicators of active inflammation were linked to a higher risk of diabetes in patients with RA. Among RA-affected individuals, other convention-

**Corresponding author:** Ghadeer Osama Abd, e-mail: scipath21p1@sci.utq.edu.iq

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al risk factors for type 2 diabetes mellitus are also very common.[4]

Diabetes can be caused by an insufficient amount of insulin produced by the pancreas or an inappropriate response by the cells of the body to the insulin that is produced.[5] Insulin resistance is a pathological condition characterized by inadequate activation of insulin receptors (IR) by insulin. Insulin resistance is associated with diabetes. Under the influence of inflammatory cytokines, IRs are expressed ubiquitously on the cell surface of adipose tissue, muscle, and a great number of other cells, such as synovial cells and T-lymphocytes.[6] RA is frequently associated with organ dysfunction. In particular, persistent synovitis, which is the result of an abnormality in the immune system, causes abnormal bone and cartilage metabolism, in addition to a continuous increase in the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which results in irreversible destruction of joint tissue. Therefore, treatment must begin as soon as possible and be appropriate.[7] Patients with RA have a high prevalence of anticitrullinated peptide antibodies and rheumatoid factor (RF), which not only provide information on how the immune system is dysregulated, but also signals for clinical diagnosis and therapy of the disease.[8] Variants of RF include IgG, IgM, IgA, and IgE autoantibodies that are directed against antigenic determinants in the Fc fraction of IgG molecules. RFs are also known as autoantibodies (auto-Abs).[9]

The primary cause is unknown, and it is unclear how this inflammatory disease was triggered and began. In RA, genetic, cognitive, and psychosocial factors interact, according to the multidimensional hypothesis. The immune system mediates the spread of the disease.[10] Alterations in the number and composition of circulating blood cells, such as normochromic anemia, thrombocytosis, and lymphopenia, typically accompany systemic inflammation. This is in addition to an increase in neutrophil count. Therefore, measurement of inflammatory activity could be possible using components of circulating blood cells.[11] During the active phases of the disease, platelet counts in RA patients can increase. Platelet counts decrease as inflammation subsides, but their precise function remains unclear.[12] The erythrocyte sedimentation rate (ESR) is a diagnostic marker. Several factors, including the size, morphology, and quantity of red blood cells, as well as other plasma components such as immunoglobulins, affect RA levels.[13]

The cytokine known as IL-24 regulates cellular responses. IL-24 is associated with an increased risk of developing a number of autoimmune disorders, including psoriasis and rheumatoid arthritis (RA). IL-24 also exerts actions that are inflammatory in nature.[14] IL-24 is able to block the production of pro-inflammatory cytokines by increasing the expression of proteins that decrease the signaling of cytokines.[15] IL-24 and IL-20 are cytokines that are produced by leukocytes; nevertheless, these cytokines act most effectively in nonhematopoietic cells, particularly epithelial cells.[16] The antitumor, antibacterial, tissue remodeling and wound healing properties of IL-24 It can depend on the type of cell, the target and the stage of the immune response; the action of this cytokine may cause additional complications. Diabetes mellitus is one of the diseases influenced by IL-24 due to the effects of infections and inflammation.[17] Among the immune cells that generate IL-24 are T cells, B cells, natural killer (NK) cells, monocytes, and macrophages.[18]

IL-32 is a new pro-inflammatory cytokine that plays a crucial role in immune regulation.[19] IL-32 plays a crucial role in inflammatory disorders such as rheumatoid arthritis, ulcerative colitis, and Crohn's disease, according to previous research.[20] IL-32, also known as natural killer cell protein 4 (NK4),[21] has nine alternative transcript variants of the IL-32 gene. It was produced by epithelial cells and immune cells such as NK cells, T cells, and monocytes. Although the mechanism(s) by which IL-32 exerts its signaling capabilities are not fully understood,[22] IL-32 is capable of exerting its effects. IL-32 is an endogenous regulator that controls cytokine production, stimulating TNF-α production by macrophages. Repression of IL-32 decreased TNF production in human monocytes, demonstrating the essential pro-inflammatory properties of IL-32 and its close relationship with TNF-α.[23]

## Aim

The study sought to explain differences in CBC values among patients with RA and type 2 diabetes. It also looked at the levels of IL-24 and IL-32 in patients with both disorders, as well as the sensitivity to RF in those with RA.

## Material and methods

### *Study design*

The case-control study at the Al-Nasiriyah Teaching Hospital in Thi-Qar province, Iraq, was collecting one hundred blood samples from patients. The study period was September 2022 to February 2023. They were divided into four groups, with each group of 25 patients (n=25) as follows: the first group of patients with RA, the second group of patients with type 2 diabetes, the third group of RA patients in addition to type 2 diabetes patients, and a control group that included 25 healthy people. Ethical clearance was obtained from the Dhi-Qar Health Department in Dhi-Qar city, Iraq. The reference number for ethical approval is 1461/11/3.

The study considered patients suitable for participation if they disclosed a positive diagnosis of RA. They had an expert in rheumatology and joint problems per-

form the diagnostics. Patients were not allowed to participate in the study if they were pregnant, had a history of cancer, or had a disease caused by bacteria or a virus. Each patient had a biochemical examination of their blood as part of the diagnostic process. Demographic information of the patients, such as their gender, age, family history, smoking habit, chronic illness, and occupation.

Blood samples (5 mL) were collected and divided into two parts: the first part was 2 mL in EDTA tubes, which was used to determine hematological parameters such as red blood cell count (RBC), platelet (PLA) count, hemoglobin (Hb) and total white blood cells (WBC) using a complete automated hematology analyzer (Mindray BC-5000), and ESR was measured by using a Westergren method.[24] Blood is drawn and mixed with an anticoagulant so that it remains fluid, and the red cells will gradually settle to the bottom of the Westergren container. The second part was 3 mL of blood collected in gel tubes and centrifuge tubes at room temperature at 4000 rpm for 5 minutes. The serum was separated and analyzed to determine the concentration of immunological parameters. A sandwich enzyme-linked immunosorbent assay was used to measure IL-24 and IL-32. The plate has been precoated with the human immunological parameters antibody. The immunological parameters present in the sample are added and bind to antibodies coated in the wells. A biotinylated human immunological parameters is added and binds to the immunological parameters in the sample. Then streptavidin-HRP is added and binds to the antibody with biotinylated immunological parameters. After incubation, the unbound streptavidin-HRP was washed away during a washing step. The substrate solution is then added, and the color develops in proportion to the amount of human immunological parameters. The reaction is terminated by the addition of an acidic stop solution, and absorbance is measured at 450 nm by a semiautomated ELISA reader (ELx800), company and origin Bio Tek (USA). A fully automatic clinical chemistry analyzer (Dirui, CS-T180) was used to measure RF.

### *Statistical analysis*

All data in the current study were statistically analyzed with Microsoft Windows Excel (version 2019) and SPSS version 26 (IBM, Armonk, NY, USA) using one-way analysis of variance, least significant difference and independent sample t tests with p values of 0.05 and 0.01.

## Results

According to the current results, there was no identifiable difference in WBC count, neutrophils, Hb, or PLT between the groups compared to the control group; the percentage of monocytes decreased significantly in type 2 diabetic patients compared to the control group; LYM decreased significantly in rheumatoid patients compared to control groups; and RBC decreased significantly in rheumatoid patients when compared to the control groups. The level of ESR increased significantly in patients who had both conditions. This was followed by a significant increase in patients with rheumatoid disease, which was then followed by patients who were type 2 diabetic compared to the control group. at $p<0.05$, as shown in Table 1.

**Table 1.** Estimate of hematological parameters in studded groups[#]

| Groups Parameters | RA | T2DM | RA and T2DM | Control | p | LSD | F |
|---|---|---|---|---|---|---|---|
| | Mean±SD | | | | | | |
| WBC ($10^3$/µL) | 7.82±1.91 | 8.45±2 | 8.70 ±2.2 | 7.79±1.69 | 0.267 | Non-sig | 1.33 |
| Neutrophils (%) | 63.7±10.2 | 60.4 ±5.88 | 57.0 ±14.1 | 58.2±6.89 | 0.092 | Non-sig | 2.21 |
| Monocytes (%) | 6.95±1.87$^{ab}$ | 6.08 ±1.79$^{b}$ | 6.98 ±1.91$^{ab}$ | 7.61±1.98$^{a}$ | 0.045$^{*}$ | 1.06 | 2.78 |
| Lymphocytes (%) | 26.1±6.78$^{b}$ | 30.8 ±6.79$^{a}$ | 31.2 ±8.40$^{a}$ | 32.5±6.30$^{a}$ | 0.011$^{*}$ | 3.98 | 3.91 |
| RBC ($10^6$/µL) | 4.47±0.51$^{b}$ | 4.76 ±0.54$^{ab}$ | 4.60 ±0.74$^{b}$ | 4.95±0.54$^{a}$ | 0.036$^{*}$ | 0.33 | 2.97 |
| Hb (mg/dL) | 11.9±1.51 | 12.8 ±1.31 | 12.4 ±1.76 | 12.5±1.34 | 0.258 | Non-sig | 1.36 |
| PLT ($10^3$/µL) | 260.2±80.1 | 271.3 ±66.4 | 254.2 ±67.5 | 300.7±68.2 | 0.101 | Non-sig | 2.13 |
| ESR (mm/h) | 30.5±9.95$^{b}$ | 17.4 ±5.98$^{c}$ | 43.9 ±10.6$^{a}$ | 4.56±1.26$^{d}$ | <0.001$^{**}$ | 4.44 | 114.5 |

[#] F table for DF 96=2.305; each p-value has two stars indicating a high significant at p value 0.01, p. value has one star indicate significant at 0.05, while p-value without star indicates a nonsignificant difference, similar small letters above the means indicate the non-significant differences, while different letters indicate the significant differences, the LSD value is used to determine the significant differences between means in the ANOVA test, where we subtract any two means from the table and compare the result of the subtraction with the LSD value, if the value of the subtraction is equal to or higher than the LSD value, it indicates a significant difference, while if it is less, it indicates that there is non-significant difference, T2DM – type 2 diabetes mellitus

According to the most recent findings, there is a statistically significant increase in the level of RF in patients who have both diseases, followed by a statistically significant increase in patients with rheumatoid arthritis compared to the control group; however, there is no statistically significant difference between patients who only have type 2 diabetes compared to the control group. IL-24 did not statistically significant increase in rheumatoid patients compared to the control group. IL-24 also did not show a statistically significant decrease in diabetic patients compared to the control

group. IL-32 levels showed a significant increase in type 2 diabetes patients, but individuals with rheumatoid arthritis showed a nonsignificant difference compared to the control group at a $p<0.05$, as shown in Table 2.

**Table 2.** Assessment of immune markers, RF, IL-24, and IL-32 in studied groups[#]

| Immune parameters | RF (IU/mL) | IL-24 | IL-32 |
|---|---|---|---|
| Groups | | Mean±SD | |
| RA | 10.9±3.52$^{b}$ | 249.0±59.8$^{ab}$ | 37.3±7.82$^{ab}$ |
| T2DM | 5.88±0.21$^{c}$ | 226.4±52.5$^{b}$ | 40.6±12.2$^{a}$ |
| RA and T2DM | 15.8±4.85$^{a}$ | 228.9±52.4$^{b}$ | 35.2±9.07$^{b}$ |
| Control | 5.81±0.73$^{c}$ | 266.1±65.5$^{a}$ | 32.3±7.70$^{b}$ |
| p | <0.01$^{**}$ | 0.047$^{*}$ | 0.020$^{*}$ |
| LSD | 1.69 | 32.4 | 5.27 |
| F | 63.25 | 2.598 | 3.443 |

[#] F table for DF 96=2.305, T2DM – type 2 diabetes mellitus

## Discussion

According to Table 1, the results are consistent with Korkmaz et al. The study results did not show a clear difference between the mean counts of leukocytes, neutrophils, or lymphocytes among the hemogram parameters. The results of the study agree in WBC and neutrophils, but differ for lymphocytes.[25] As acute inflammation subsides or becomes chronic, peripheral leukocyte counts fall. During the active phase of the disease, the total leukocyte count (TLC) may grow or remain normal. Chronic inflammation can induce these effects. Hormonal changes, stress, food, lifestyle and sex might affect the total leukocyte count. Due to these factors, TLC values alone cannot predict chronic inflammatory activity.[26] The impacts of various pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, and IFN-, are involved in the pathways that can be responsible for the enhancement of abnormalities in the levels of certain hematological parameters when chronic inflammation is present.[27] The results disagree with the findings of Badawy et al.[28] According to the findings of another study by Larasati et al., about patients with type 2 diabetic patients, the result did not agree with this study.[29] According to Table 1, monocytes and macrophages were the primary or possibly the only, responder cells involved in the production of TNF-α in the systems. Most disease-modifying medicines used to treat RA are designed to reduce monocytes and cytokines that are produced from monocytes. Recent research has shown that monocytes and macrophages, as monocyte-generated well as dendritic cells, play an essential part in the generation of cytokines in RA joints after being stimulated by an immune complex.[30] The important part that TNF-α plays in causing leukocyte infiltration into an inflammatory joint: TNF inhibitors were responsible for a reduction in joint leukocyte counts, resulting in a decrease in clinical scores.[31]

The current result in Table 1 did not agree with the findings of another study with the PLT, but agreed in neutrophils and lymphocytes carried out by Fu et al., where the number of neutrophils and platelets detected in the blood of RA patients was found to be much higher, while the number of lymphocytes was significantly lower.[32] The inflammatory state of RA, which is characterized by elevated serum levels of IL-6 and TNF-α as well as other inflammatory substances, may be responsible for the increase in the number of neutrophils and platelets.[33] ; these inflammatory substances can encourage the development of neutrophils and platelets in the bone marrow as well as their release.[34] Crosstalk between coagulation markers and the inflammatory system suggests that platelets may play a significant role in inflammation and immunological regulation. This hypothesis is based on the observation that platelets are present when coagulation markers are present. Platelets, when activated, release pro-inflammatory platelet microparticles. These microparticles then interact with leucocytes, which ultimately results in joint and systemic inflammation in RA patients.[35]

The results in Table 1 agree with the finding of Chen et al. and are inconsistent with the finding of Mohammed et al.[36,37] Both patients who had an active form of the disease and patients who had a latent form of the condition had lower levels of hemoglobin. Anemia may be brought on by rheumatoid arthritis for a number of reasons, including a diminished response to erythropoietin, a pathologic iron homeostasis brought on by hepcidin, and a reduced lifespan for red blood cells. Cytokines are also known to have a direct harmful effect on erythropoietin.[38]

The results of the current study are shown in Table 1. These findings are consistent with the studies conducted by Khadim and Al-Fartusie.[39] Age is correlated with a higher ESR reading. ESR increases with patient age.[40] Al-Nimer and Ratha show in this study that ESR values are significantly higher in diabetic patients.[41] RBC can keep their distinctive shapes thanks to the assistance of a phospholipid bilayer and several transmembrane proteins. Proteins that act as receptors or transporters are responsible for the elasticity and structure of RBC, as well as their interactions with the biochemical environment. Proteins also play a role in how RBC respond to stimulation. Some of these proteins, such as calpastatin, have the potential to trigger inflammatory activities in other cells or become antigens in the autoimmune environment present in the physiology of an RA patient. Due to this, the structure and contacts of RBCs may be hampered by the transmembrane protein alterations, which is a potential explanation of the recognizable phenomena of increased sedimentation rates in plasma.[42] ESR is affected by blood fibrinogen levels; therefore, even non-inflammatory situations can elevate ESR: pregnancy, diabetes, end-stage renal illness, and heart disease. In multiple myeloma, monoclonal immuno-

globulin concentrations increase significantly, which also causes increased sedimentation.[43]

The results in Table 2 show consistency with the findings of Kragstrup et al., who discovered that the levels of IL-24 in RA patients' plasma were considerably higher than in normal people's plasma, are higher than those of the current study.[44] Another study by Kragstrup et al., which discovered that plasma concentrations of IL-20 and IL-24 were higher in early RA patients, was also higher than the current study.[45] This provides more evidence in favor of the concept that changes in IL-20 and IL-24 production occurring outside the synovial joints are associated with rheumatic disease. Peripherally activated mononuclear cells that have infiltrated the synovial joint might be a source of IL-20 and IL-24 that is complementary to other sources.[46] The current result agrees with the findings of Eswadi and AL-Hellawi's study, which demonstrated a fall in serum IL-24 level in diabetic patients as compared with the controls.[47] IL-24 deficiency in diabetic patients is dangerous. Despite neuropathies, nerve injuries, and wound nonhealing, this interleukin drop may lower patient immunity. IL-24 induces innate defense mechanisms in epithelial tissues during infection and inflammation to preserve tissue homeostasis.[48] IL-24 levels recorded in a prior investigation were not the same as the levels in the current study[49] The opposite findings were caused by a number of factors, including the smaller sample size for patients with RA in the current study and the fact that half of the RA patients chosen were inactive. In addition, variations in the types and dosages of biological treatments may result in changes in IL-24 levels.[50]

The current results in Table 2 are consistent with the finding of Mohammed et al. that RA patients can benefit from a decrease in IL-32 activity, and the pathophysiology of the disease depends heavily on the quantity of cytokines, and are inconsistent with Gui et al.[51,52] The fact that IL-32 expression is related to inflammatory indicators such as ESR provides further evidence that IL-32 has an important effect on the production of inflammation in joints that are afflicted by RA.[53] According to the study of conclusions of the Abid et al., IL-32 levels were considerably higher in diabetic patients than in the control group.[54] The findings are consistent with the study.

The findings in Table 2 are consistent with those of Al-Taee et al. and Hassoon et al., who reported that the RF titer in patients with RA was substantially greater than in the control group.[55,56] Because it is well known that rheumatoid factors contain two antigen-binding sites, when B cells are stimulated by an antigen, they begin to develop into antibody-producing plasma cells. These plasma cells can produce IgM, IgG, IgA, or IgE, depending on the stimulating antigen and the location of the infection.[57] Patients with RA who have a positive RF have immune complexes in their synovial fluids. This causes the immune complexes to attach to Fc receptors on the surfaces of macrophages or toll-like receptors, which in turn activates the production of TNF-α. This cytokine, in turn, supports the synthesis of a number of other molecules and cytokines that contribute to the development of inflammation. Ultimately, this cytokine is responsible for the development of inflammation.[30] The current study agrees with the finding of Ali et al. that individuals with RA with hyperinsulinemia had highly significant RF.[58]

### *Study limitations*

The study has several limitations. One of them is that the patients included in the study take medications that can influence the results. In addition, some patients have underlying viral or cancerous infections, which could affect outcomes. Most patients also lead an unhealthy lifestyle, which is another factor to consider. Furthermore, the presence of pregnant women among study participants introduces additional variability that can impact the findings.

## Conclusion

Patients with RA have higher expression of IL-32, which is positively with RA activity markers such as ESR and RF. A rise in patients with IL-24 and IL-32 in RA suggests a positive association between the two. Furthermore, diabetic patients have significantly higher pro-inflammatory levels of IL-32. Therefore, rheumatoid arthritis should be diagnosed using IL-24 and IL-32. Furthermore, IL-32 can help in the diagnosis of diabetes.

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## Declarations

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### *Author contributions*

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

### *Conflicts of interest*

The authors declare that they have no conflicts of interest.

***Data availability***

The data sets used and analyzed in this study have been made available from the corresponding author on a reasonable request.

***Ethics approval***

Ethical clearance was obtained from Dhi-Qar health department in Dhi-Qar city, Iraq. The reference number for ethical approval is 1461/11/3.

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**ORIGINAL PAPER**

# The effect of hypnotic suggestion on labor market attachment, functioning, and cognition after brain injury – a randomized controlled trial

Pernille Pedersen [1,2], Claus Løvshall [1], Christina Malmose Stapelfeldt [2,3], Claus Vinther Nielsen [1,2,4], Jonas Kristoffer Lindeløv [5]

[1] DEFACTUM, Central Denmark Region, Aarhus, Denmark
[2] Department of Public Health, Aarhus University, Aarhus, Denmark
[3] The Danish Clinical Quality Program – National Clinical Registries (RKKP), Aarhus, Denmark
[4] Department of Clinical Social Medicine and Rehabilitation, Gødstrup Hospital, Herning, Denmark
[5] Department of Communication and Psychology, Aalborg University, Aalborg, Denmark

**ABSTRACT**

**Introduction and aim.** Cognitive impairments after acquired brain injury (ABI) or concussion significantly affect work capacity. While hypnotic suggestion has shown promise in improving working memory and work ability, studies on its long-term effects on labor market attachment and cognition are lacking. The aim of this study was to estimate the long-term effect of hypnotic suggestion on labor market attachment, cognition, and functioning following acquired brain injury or concussion.

**Material and methods.** A randomized controlled trial (RCT) was conducted at a municipal vocational rehabilitation center in Denmark, among 87 patients aged 18 to 62 years who experienced ABI or concussion at least 6 months prior to the first therapy session. The study group were randomized according to the applied intervention: usual care (n=28), hypnosis (n=30) or mindfulness (n=29). Participants underwent baseline and 6-month follow-up assessments involving The Danish Register for Evaluation of Marginalization (DREAM), The Wechsler Adult Intelligence Scale – Fourth Edition (WAIS-IV), Impact on Participation and Autonomy questionnaire (IPA), The Working Memory Questionnaire and Trial Making Test related to working memory and functioning, assessing three dimensions: short-term storage, attention, and executive control.

**Results.** The primary outcome was the average number of weeks employed during the 12–24-month period post-inclusion. The mean number of weeks employed was 32.71 (SD: 22.31) in the usual care group, 35.97 (SD: 21.58) in the hypnosis group, and 32.90 (SD: 22.44) in the mindfulness group. All intervention groups had exhibited a working memory score of around 90, which improved to a range of 91 to 95 at the 6-month follow-up. No significant differences were found between the groups.

**Conclusion.** Brief hypnotic treatment at a municipal vocational rehabilitation center for people with ABI and concussion showed no significant advantage over mindfulness or usual care in labor market attachment, cognition, or family functioning. However, participants in the hypnosis group demonstrated improved social functioning at the 6-month follow-up compared to usual care.

**Keywords.** cognition, functioning, hypnosis, mindfulness, work

**Corresponding author:** Pernille Pedersen, e-mail: pelped@rm.dk

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## Introduction

People with vascular and traumatic brain injuries, such as acquired brain injury (ABI) or concussions, often face challenges in occupational competencies and cognitive abilities, including working memory.[1,2] These difficulties persist upon returning to work post-injury, contributing to significant vocational skills challenges and global implications for personal and economic well-being.[3] The incidence of chronic cognitive consequences after ABI in a Danish context ranges from 5,000 to 15,000 citizens annually, with at least one-quarter belonging to the working-age population.[4,5]

A common cognitive consequence following a stroke is diminished working memory, affecting people's ability to navigate complex situations and sustain concentration.[6] The compromised cognitive function poses a substantial obstacle to work, with return-to-work rates after a stroke ranging widely from 11 to 88%.[4,7,8] Among Danes aged 20–57 who had experienced a stroke, 62% were employed two years post-stroke.[9]

The effectiveness and cost-effectiveness of current cognitive rehabilitation approaches, specifically targeting work-related abilities for people with ABI and concussion, remain inconclusive.[10,11] Hypnosis has emerged as a promising intervention, leveraging its core mechanism of shaping expectancies in both pre- and post-injury stages.[12-15] Additionally, hypnotic sessions have been shown to enhance neural plasticity and improve pain regulation, offering potential for addressing cognitive and functional impairments following ABI.[16] Nevertheless, evidence supporting the efficacy of hypnotic treatments remains inconsistent, primarily due to small sample sizes and variability in clinical trial methodologies.[16]

A recent RCT demonstrated significant improvements in working memory following ABI through hypnotic suggestion.[17] This eight-session intervention successfully restored working memory capacity to levels observed in the healthy population, indicating substantial functional improvement. This was achieved through techniques such as age regression and visualizations of brain plasticity.[17] Other studies also showed clinically noteworthy effects in the cognitive domain with small-to-medium effect sizes following hypnotic suggestion.[18] Mindfulness-hypnosis exhibited impacts on fatigue, and in some certain studies, on depression and anxiety.[17,19,20] However, there is a research gap regarding the long-term effects (> 2 months) of hypnotic suggestion on labor market attachment and cognitive abilities.

This study aims to investigate whether the positive outcomes observed in Lindeløv et al.'s intervention can be sustained over an extended period.[17]

## Aim

Therefore, the aim of this study was to estimate the long-term effect of hypnotic suggestion on labor market attachment, cognition, and functioning.

We hypothesized that hypnotic suggestion would have positive long-term effects in each of these domains compared to both mindfulness (active control group) and usual care.

## Material and methods

### *Study design*

A three-arm RCT with 6- and 24-month follow-up was undertaken, comparing the effects of hypnotic suggestions, mindfulness, and usual care. The trial is registered at ClinicalTrials.gov under the identifier NCT05142007.

### *Study setting and population*

Recruitment, testing, and treatment took place from May 2017 to February 2021 at an outpatient municipal vocational rehabilitation center in Silkeborg, Denmark. People referred to the center were considered eligible for the study if they met the following inclusion criteria: 1) aged 18 to 62 years, 2) experienced ABI or concussion at least 6 months prior to the first therapy session as verified in the participant's medical journal, and 3) demonstrated a substantial risk of reduced labor market attachment, as assessed by the vocational rehabilitation center staff (social workers and physiotherapists) based on sequelae and previous employment status.

Participants were excluded based on criteria that could significantly diminish treatment effects for reasons unrelated to the effectiveness of the intervention. Exclusion criteria included: 1) progressive injuries, including dementias, 2) unemployed for more than 6 consecutive months immediately preceding the injury, 3) employment less than 50% of the time after finishing the latest education, 4) pensioned or recommended for pension due to old age, disability pension or both, and 5) ongoing mental disorders that require psychiatric treatment, with the exception of psychopharmaca for depression and anxiety (any subscale exceeding the per-instruction manual cutoffs on the Patient Health Questionnaire (PHQ).[21]

While hypnosis is generally considered a safe procedure across patient groups, one precaution was taken to screen out participants with a history of psychosis (PSQ), or substance abuse (PHQ) to avoid eliciting those behaviors.[21,22]

### *Sample size*

The effect sizes reported by Lindeløv et al. were so substantial that even the weakest model, with half the effect size of the weakest contrasts, achieved >90% power with only eight participants per group. However, such a small sample size is insufficient for a clinical study. Therefore,

we aimed for 30 participants per group (90 in total), as this was feasible within the available budget.

***Randomization and blinding***

A study coordinator utilized a computerized random sequence generator to allocated participants to the three intervention groups. We employed blocked randomization, with the constraint that multiples of five participants were to the hypnosis and mindfulness groups for logistical reasons. Stratification was implemented to ensure an equal number of participants in each arm and an equal ratio of participants with ABI to concussion in all three arms.

The coordinator communicated participants' allocations and scheduled appointments with a hypnotist. Participants were informed only about the two possible outcomes of the allocation: hypnosis or usual care, remaining unaware of the existence of two hypnosis groups (hypnosis and mindfulness). This strategy aimed to standardize treatment expectations across intervention groups, mitigating the potential confounding effect. The blinding was lifted after 6-month follow-up test for the last participant. The authors of this paper remained blinded until an agreed-upon analysis plan was established.

***Interventions***

*Usual care*

All participants were provided with usual care at the outpatient municipal vocational rehabilitation center in Silkeborg. This encompassed assistance to enhance labor market attachment for people with diminished work capacity due to ABI or concussion. Activities under usual care involved consultations with a social worker or neuropsychologist, participants in support patient groups, and engagement in mindfulness. The nature and frequency of these activities were tailored to individual needs, usually amounting to two activities per week.

*Hypnosis*

The hypnotic intervention comprised a series of four weekly one-hour treatment sessions aimed at enhancing working functions. These sessions involved the use of hypnotic suggestions to instantiate preinjury memory abilities in the present. Techniques included age regression, visualizations of neuroplasticity, and posthypnotic suggestions for continued improvement. The central theme of these suggestions was to make thinking effortless and reliable for the participant, ultimately leading to reduced fatigue, improved memory, and the avoidance of information overload.

Each session followed a manualized hypnosis script, dictated by three experienced hypnotists with several years of hypnotherapy practice, including working with brain injuries and concussions.[17] The only variation among hypnotists were in intonation and speed.

The treatment sessions adhered to a consistent strategy, encompassing four main steps: 1) re-instantiating pre-morbid brain functioning in the present, 2) reprogramming the unconscious mind for effectiveness, 3) regaining control and inducing relaxation, and 4) fostering a healthy self-image.[23]

Furthermore, hypnotic suggestions encouraged participants to adopt alternative emotional or cognitive appraisals of problems, disrupting habitual thought patterns.

*Mindfulness*

This group also involved four weekly one-hour treatments. Each treatment session utilized the same induction and termination procedures as the hypnosis group and was matched in duration. However, the suggestions within the sessions were derived from the Mindfulness-Based Stress Reduction method (MBSR), with no explicit mention of brain injury or working memory-related abilities. Therefore, the "mindfulness" intervention was formally a hypnosis with mindfulness suggestions, and this group served to isolate the specificity of the hypnotic suggestion, while also controlling for other influences such as placebo effects and retest effects.

Mindfulness sessions incorporated techniques such as body scanning to cultivate focused attention and open monitoring of thoughts in a non-judgmental manner. Specifically, these sessions aimed to train participants in letting worrying thoughts pass without fixation and promoting general relaxation and well-being, focusing solely on the hypnosis session. Importantly, there were no suggestions regarding the transfer of these abilities to other contexts.

***Data sources***

Participants underwent baseline and 6-month follow-up assessments involving questionnaires and tests related to working memory and functioning. Information on injury type, time since injury, gender and age was collected from questionnaires and cross-verified with patient records.

Employment status was obtained from The Danish Register for Evaluation of Marginalization (DREAM) (24). DREAM supplied weekly data on public transfer payments, including compensation benefits for sick leave, unemployment benefits, and disability pension. The specific type of social benefits in DREAM is recorded for each week if the person has received the benefit for at least 1 day. Termination of registration occurs following the first full week without receiving any social benefits and is interpreted as working. Previous validation studies demonstrated the reliability of DREAM data, comparing it against workplace-registered data on sick leave and self-reported information on income type.[24,25] Both studies concluded that DREAM provides accurate and valid data.

***Outcomes***

*Primary outcome*

The primary outcome measure was the number of weeks employed during the 12–24-month period following inclusion, as derived from DREAM data. Working was defined as not receiving any social benefits, excluding unemployment benefits (indicating fitness for duty without current employment), and flexible job arrangements (for people with a significantly and permanently reduced working capacity due to illness). Thus, the outcome was calculated as the total number of weeks during the 12–24-month period without receiving any benefits, even for a single day.

*Secondary outcomes*

The assessment focused on the number of weeks participants were employed during the initial 0–6 months after study inclusion. Participants' work status (yes/no) was determined 6 month (26 weeks) and 24 months (104 weeks) after inclusion. To ensure a sustained connection to the labor market, the criterion for working also required participants to have worked in the last 4 weeks leading up to each time points.

Cognitive tests and questionnaires were administered three times: at baseline (21–3 days before the first possible treatment date), at post-test (3–11 days after the last possible treatment date, and at the 6-month follow-up (6 months after the last possible treatment day ± 14 days).

The Wechsler Adult Intelligence Scale – Fourth Edition (WAIS-IV) (26) was utilized to measure Working Memory, employing subtest such as digit span, arithmetic and letter-number sequencing. Tests were scored, aggregated, and transformed into index scores ranging from 50 (worst) to 150 (best), as outlined in the WAIS-IV manual (26). The index exhibits favorable psychometric properties.[27]

The executive component of working memory was evaluated through the Trial Making Test (28), consisting of two parts (A and B) that assess visual attention and task switching. Total time in seconds for completing both parts A and B was recorded. Direct scores were obtained, and a log-ratio score (log(B/A)) was calculated to better capture the exponential increase in reaction times with decreasing ability, aiding in generalizing results across healthy and clinical groups.[28]

The Working Memory Questionnaire is a self-administered scale addressing three dimensions: short-term storage, attention, and executive control. Each dimension comprises 10 items, rated on a five-point Likert-type scale (0 to 4), with higher total scores (maximal score 120) indicating more difficulties or complaints. The questionnaire demonstrates good validity among people with brain injury.[29]

Functioning was evaluated using the Impact on Participation and Autonomy questionnaire, with two subscales: Family and Social, each containing 7 items.[30] Items were rated on a 5-point scale (0 to 4), and subscale scores were calculated using the median, following conventional scoring criteria (score range 0–4). Higher scores indicate more perceived restrictions on everyday functioning.

***Ethics***

Participants were required to disclose any adverse effects after each hypnosis session. Specifically, they were explicitly asked if they wished to withdraw from the study in the event of such effects. In cases of serious side effects, the blinding could be temporarily lifted for an individual participant. If a side effect was deemed severe enough to warrant halting the entire study, all blinding measures would be lifted to identify at-risk participants. Fortunately, no serious side effects were reported by the participants, and consequently, the procedure was never implemented. Approval was granted by the regional ethics committee (North Denmark Region) (May 15., 2017, no. N-20170022) and by the Danish Data Protection Agency (Sept. 18., 2015 and no. 2015-57-0001).

***Statistics***

Initially, baseline characteristics of participants across the three intervention groups were presented.

Differences in mean number of weeks employed during the 0–6- and 12–24-month periods after inclusion among intervention groups were analyzed using linear regression, with the group receiving usual care as reference for comparison to the two active groups. Analyses were conducted in both crude form and adjusted for months since injury (log-transformed), injury type, age, gender and baseline employment status. The log transformation of months since injury accounted for its asymptotic nature in spontaneous recovery, as expected a priori to be related to the treatment effect.[31]

Differences in work status between the intervention groups at 6- and 24-months' follow-up were analyzed through logistic regression models, again using the group receiving usual care as the reference. These analyses were conducted in both crude and adjusted models, accounting for baseline employment status and injury type.

For mean scores on questionnaires and tests at baseline and 6 months' follow-up, as well as the differences between these time points, linear regression was employed to analyze treatment effects at 6 months. The 6-month score served as the dependent, with the baseline score as a covariate and the group as a categorical predictor (usual care as the reference group). An adjusted model additionally included covariates for log-transformed months since injury, injury type, age, and gender.

Analyses regarding labor market attachment were conducted as intention-to-treat, while other outcomes were only collected and analyzed if participants adhered to the protocol. All point estimates are presented

with 95% confidence intervals (CI). Statistical significance was determined by a two-sided probability of $p<0.05$. Stata version 17 served as the statistical software (StataCorp LLC, Lakeway Dr, TX, USA).[32]

## Results

A total of 95 individuals were eligible for participation, and 87 agreed to participate and were randomized to usual care (n=28), hypnosis (n=30) or mindfulness (n=29) (Fig. 1).

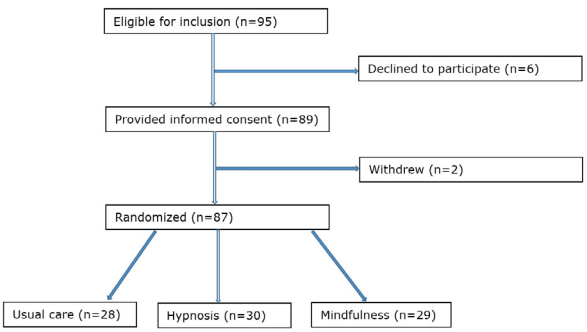


**Fig. 1.** Flow chart of the selection of participants in the study

Participants in the hypnosis group received an average 3.9 sessions, while those in the mindfulness group received 3.8 sessions.

The mean age of the participants ranged from 47.8 years in the usual care group to 50.7 years in the mindfulness group (Table 1). Two-thirds of the participants in the hypnosis and mindfulness groups were women, while women comprised three-quarters of the usual care group. Across all intervention groups, approximately 40% reported suffering from concussion, and the mean time since injury ranging from 22 to 25.6 months. At baseline, around 29% of participants were working at baseline in the usual care group, compared to 40% in the hypnosis groups.

**Table 1.** Baseline characteristics of the study population

| | Usual care n=28 (32.2%) | Hypnosis n=30 (34.5%) | Mindfulness n=29 (33.3%) |
|---|---|---|---|
| Age, mean (SD) | 47.8 (9.5) | 48.1 (8.3) | 50.7 (8.2) |
| Gender, n (%) | | | |
| Female | 22 (78.6) | 20 (66.7) | 19 (65.5) |
| Male | 6 (21.4) | 10 (33.3) | 10 (34.5) |
| Months since injury, mean (SD) | 22.0 (19.4) | 25.6 (26) | 23.8 (24.5) |
| Injury type, n (%) | | | |
| Concussion | 11 (40.7) | 12 (40.0) | 12 (41.4) |
| Injury | 16 (59.3) | 18 (60.0) | 17 (58.6) |
| Baseline employment status, n (%) | | | |
| Working | 8 (28.6) | 12 (40.0) | 10 (34.5) |

The mean number of weeks participants worked during the first 6 months after inclusion ranged from 10.36 in the usual care group to 12.77 in the hypnosis group (Table 2). Similarly, during the 12 to 24 months' follow-up period, the correspond varied from 32.71 in the usual care group to 35.97 in the hypnosis group. No statistically significant differences in treatment effects were observed in either the crude or adjusted analyses for either time intervals.

**Table 2.** Mean number of weeks working up to the 24-month follow-up in the three intervention groups*

| | Usual care (n=28) | Hypnosis (n=30) | Mindfulness (n=29) | p |
|---|---|---|---|---|
| Weeks working during | | | | |
| 0–6 months, mean (SD) | 10.36 (11.52) | 12.77 (12.03) | 10.45 (12.24) | |
| Diff from control, crude (CI) | 0 (ref) | 2.41 (-3.83;8.65) | 0.09 (-6.20;6.38) | 0.68 |
| Diff from control, adj.[a] (CI) | 0 (ref) | -0.32 (-4.28;3.65) | -1.67 (-5.67;2.33) | 0.67 |
| 12–24 months, mean (SD) | 32.71 (22.31) | 35.97 (21.58) | 32.90 (22.44) | |
| Diff from control, crude (CI) | 0 (ref) | 3.25 (-8.30;14.80) | 0.18 (-11.46;11.83) | 0.82 |
| Diff from control, adj.[a] (CI) | 0 (ref) | -0.73 (-12.32;10.86) | -2.34 (-14.03;9.34) | 0.92 |

*[a] – adjusted for months since injury (log), injury type, age and gender, baseline employment status

At 6-month of follow-up, 35% of participants in the usual care group were working, compared to approximately 40% in both the hypnosis and mindfulness groups (Table 3). By the 24-month follow-up, these figures had risen to around 55% in the usual care group and mindfulness groups, while 63% were working in the hypnosis group. No statistically significant differences in treatment effects were observed in the crude or the adjusted OR for working.

**Table 3.** Odds ratio (OR) for working at 6 and 24-month follow-up*

| | Usual care (n=28) | Hypnosis (n=30) | Mindfulness (n=29) | p |
|---|---|---|---|---|
| Working at | | | | |
| 6 months, n (%) | 10 (35.7) | 13 (43.3) | 12 (41.4) | |
| Compared to usual care, crude OR (CI) | 1 (ref) | 1.38 (0.48;3.96) | 1.27 (0.44;3.70) | 0.83 |
| Compared to usual care, adj. OR[a] (CI) | 1 (ref) | 0.84 (0.22;3.25) | 0.93 (0.24;3.57) | 0.97 |
| 24 months, n (%) | 16 (57.1) | 19 (63.3) | 16 (55.2) | |
| Compared to usual care, crude OR (CI) | 1 (ref) | 1.30 (0.45;3.72) | 0.92 (0.32;2.63) | 0.80 |
| Compared to usual care, adj. OR[a] (CI) | 1 (ref) | 1.21 (0.40;3.66) | 0.89 (0.30;2.69) | 0.86 |

*[a] – adjusted for baseline employment status and injury type

At baseline, all intervention groups had exhibited a working memory score of around 90, which improved to a range of 91 to 95 at the 6-month follow-up (Table 4). The log ratio for the executive working memory (where lower scores are better) was approximately 0.80 for participants in the hypnosis and mindfulness groups and 0.68 for those in the usual care group. At the 6-month follow-up, the scores were approximately 0.75 in both the usual care group and hypnosis groups, and 0.86 in the mindfulness group.

**Table 4.** Differences in working memory and functioning between intervention groups at baseline and 6-month follow-up*

| | Usual care n=28 | n | Hypnosis n=30 | n | Mindfulness n=29 | n | p |
|---|---|---|---|---|---|---|---|
| | Mean (SD) | | Mean (SD) | | Mean (SD) | | |
| **Working memory** | | | | | | | |
| Baseline | 90.88 (11.98) | 24 | 89.17 (12.90) | 29 | 88.11 (7.74) | 27 | |
| 6 months | 95.60 (11.20) | 15 | 92.62 (11.74) | 21 | 91.81 (8.58) | 21 | |
| Diff (baseline-6 months) | 0.87 (7.01) | 15 | 1.62 (6.12) | 21 | 3.38 (5.63) | 21 | |
| Diff from control at 6-month, crude (CI)[a] | 0 (ref) | | 0.36 (-3.99;4.07) | | 1.30 (-2.80;5.41) | | 0.74 |
| Diff from control at 6-month, adj. (CI)[b] | 0 (ref) | | -0.17 (-4.68;4.34) | | 0.66 (-4.06;5.38) | | 0.91 |
| **Executive working memory** | | | | | | | |
| Baseline | | | | | | | |
| Trail A | 41.79 (17.98) | 24 | 40.72 (14.58) | 29 | 40.37 (14.94) | 27 | |
| Trail B | 83.21 (38.91) | 24 | 93.34 (39.24) | 29 | 93.11 (33.63) | 27 | |
| Log(B/A) | 0.68 (0.24) | 24 | 0.80 (0.24) | 29 | 0.84 (0.33) | 27 | |
| 6 months | | | | | | | |
| Trail A | 36.47 (16.60) | 15 | 34.62 (13.06) | 21 | 31.05 (7.72) | 21 | |
| Trail B | 76.60 (38.03) | 15 | 75.67 (28.53) | 21 | 74.05 (22.95) | 21 | |
| Log(B/A) | 0.75 (0.20) | 15 | 0.76 (0.34) | 21 | 0.86 (0.32) | 21 | |
| Diff (baseline-6 months), Log(B/A) | 0.12 (0.18) | 15 | -0.01 (0.31) | 21 | 0.05 (0.28) | 21 | |
| Diff from control at 6-month, crude Log(B/A) (CI)[a] | 0 (ref) | | -0.07 (-0.25;0.10) | | -0.00 (-0.18;0.17) | | 0.59 |
| Diff from control at 6-month, adj. Log(B/A) (CI)[b] | 0 (ref) | | -0.08 (-0.26;0.11) | | -0.00 (-0.19;0.19) | | 0.58 |
| **Storage, attention, and executive control** | | | | | | | |
| Baseline | 48.08 (22.70) | 25 | 48.71 (22.34) | 24 | 55.41 (20.39) | 27 | |
| 6 months | 47.67 (20.99) | 18 | 34.36 (25.05) | 22 | 47.74 (22.89) | 19 | |
| Diff (baseline-6 months) | -6.88 (14.83) | 17 | -13.39 (18.05) | 18 | -13.26 (13.41) | 19 | |
| Diff from control at 6-month, crude (CI)[a] | 0 (ref) | | -8.53 (-19.19;2.14) | | -5.46 (-15.76;4.85) | | 0.27 |
| Diff from control at 6-month, adj. (CI)[b] | 0 (ref) | | -9.56 (-20.12;1.00) | | -7.17 (-17.65;3.32) | | 0.18 |
| **Functioning, Family** | | | | | | | |
| Baseline | 1.12 (1.01) | 25 | 1.13 (1.12) | 24 | 1.48 (1.12) | 27 | |
| 6 months | 1.39 (1.24) | 18 | 1.00 (1.11) | 22 | 1.47 (0.90) | 19 | |
| Diff (baseline-6 months) | 0.18 (0.81) | 17 | -0.06 (0.54) | 18 | -0.21 (0.71) | 19 | |
| Diff from control at 6-month, crude (CI)[a] | 0 (ref) | | -0.25 (-0.71;0.21) | | -0.30 (-0.76;0.16) | | 0.38 |
| Diff from control at 6-month, adj. (CI)[b] | 0 (ref) | | -0.42 (-0.89;0.04) | | -0.43 (-0.90;0.03) | | 0.11 |
| **Functioning, Social** | | | | | | | |
| Baseline | 0.76 (0.79) | 25 | 0.75 (0.69) | 24 | 1.09 (0.88) | 27 | |
| 6 months | 0.86 (0.80) | 18 | 0.41 (0.53) | 22 | 1.13 (0.60) | 19 | |
| Diff (baseline-6 months) | -0.06 (1.01) | 17 | -0.31 (0.64) | 18 | -0.11 (0.92) | 19 | |
| Diff from control at 6-month, crude (CI)[a] | 0 (ref) | | -0.44 (-0.86;-0.01) | | 0.20 (-0.22;0.63) | | 0.01 |
| Diff from control at 6-month, adj. (CI)[b] | 0 (ref) | | -0.49 (-0.92;-0.06) | | 0.26 (-0.18;0.70) | | 0.004 |

* [a] – controlled for baseline score, [b] – adjusted for baseline score, months since injury (log), injury type, age and gender

The baseline score on the Working Memory Questionnaire total score (covering storage, attention and executive control) was 48 for participants in the usual care and hypnosis groups, while it was 55 for participants in the mindfulness group. By 6-month follow-up, the scores had improved by 13 points in the two active intervention groups, whereas the score in the usual care group had deteriorated with 6.8 points.

Regarding the Impact on Participation and Autonomy questionnaire (IPA), the baseline score for family functioning was approximately 1.1 in both the usual care and hypnosis groups, slightly higher at 1.48 in the mindfulness group. At the 6-month follow-up, participants in the active intervention groups reported fewer problems, while the usual care group reported more problems. The IPA score for social context functioning was about 0.75 in both the usual care group and hypnosis group and 1.09 in the mindfulness group. While all groups experienced a decrease in social problems, the hypnosis group showed the most significant improvement at 6 months, with a statistically significant treatment effect in both crude and adjusted analyses. No significantly differences were found between groups for the other outcomes.

## Discussion

In this RCT, we compared the effects of hypnotic suggestion after 6- and 24- months' follow-up with mindfulness and usual care. There were no statistically significant differences in the mean number of weeks employed or work status at either 6 or 24 months of follow-up among the three intervention groups. However, participants assigned to hypnosis demonstrated a statistically significant improvement in social functioning at the 6-month follow-up compared to the usual care group. No differences were found between the groups regarding working memory or family functioning.

Interestingly, our study revealed that between 55% and 63% of participants were working at the 24-month follow-up. These percentages align closely with the 62% found in a Danish population of 20-57-year-old patients two years after stroke.[9] Contrary to our hypothesis, we did not observe a higher attachment to the labor market for participants in the hypnosis group compared to the other two groups. The sample size and the characteristics of participants included in the study may have influenced the results, as the average time since injury was approximately two years, suggesting that some participants might have been dealing with chronic conditions.

The relatively late onset of the intervention and its single-element nature might explain the lack of significant difference. Early rehabilitation has been recommended for improving functional outcomes and increasing the likelihood of returning to work.[1] The absence of workplace involvement in the intervention,

a factor identified in other studies, could also contribute to the lack of observed differences.[33]

Our study did not replicate the large effect sizes on working memory reported by Lindeløv et al. and contradicts earlier findings on the WAIS working memory index, the Trial Making Test or subjective assessments on the Working Memory Questionnaire.[17,34] Similarly, the mindfulness group did show improvement in fatigue, contrary to previous studies.[19,20] This raises questions about the comparability of the present study with earlier ones and whether they differ on critical variables.

The setting of our intervention at a municipal vocational rehabilitation center contrasts with previous studies on hypnosis that took place in research medical institutions.[17,18] The impact of different settings on perceived authority and treatment effectiveness should be considered, as expectancy plays a crucial role in the outcomes of hypnosis and placebos and for the success of brain injury rehabilitation in general.[14,15,23,35,36] Thus, the present null result may indicate that hypnotic suggestion for this patient group may require certain settings to be effective.

Moreover, participants in previous mindfulness studies were self-selected via newspapers, whereas our study included everyone meeting the inclusion criteria at the vocational rehabilitation center.[17,19,20] This may indicate that participants in the former RCTs were more motivated to improve their functioning, suggesting a potential limitation in the effectiveness of hypnotic suggestion for a group facing vocational challenges.

The lack of efficacy in the present study may, in part, also be attributable to the short treatment duration of only 4 sessions (4 hours total) compared to 8 sessions in Lindeløv et al. (8 hours total) and 9 sessions in Johansson et al. (27 hours total plus home training).

While our RCT found no differences between the groups, the results underscored a higher attachment to the labor market at 24 months' follow-up compared to 6 months', indicating the duration of symptoms after ABI and the gradual but continual progress experienced by the persons. The reversible nature of the disease is suggested by most secondary outcomes, even though no significant difference was found between the groups.

### *Study limitations and strengths*

The study had several strengths, including the blinding of both participants and researchers. The utilization of the DREAM register helped mitigate the risk of bias by ensuring full follow-up and minimizing misclassification. Additionally, employing a broader and validated set of questionnaires and cognitive tests expanded the scope of outcomes assessed compared to previous studies.

However, the study had its limitations, notably the small sample size, resulting in low statistical power to detect differences between groups. The impact of COVID-19 prevented the inclusion of more participants or complete follow-up on all enrolled participants. Furthermore, a lower-than-expected signup ratio necessitated an extension of the inclusion period. At 6-month follow-up, the dropout rate for secondary outcomes ranged between 37% and 46 % across the three groups, leading to reduced power for detecting difference between intervention groups.

In terms of the intervention, mindfulness was integrated into the usual care program, with approximately a quarter of participants engaging in at least one session. Unfortunately, records of specific participants were unavailable, precluding per-protocol analyses and potentially obscuring differences between the groups.

The cause, location, and severity of the participants' injuries were characterized by scanning their medical records. However, the data quality and completeness were insufficient for detailed analysis, and as a result, some injury characteristics remain unknown. This limits the ability to explore how specific injury profiles may have influenced treatment outcomes and may affect the generalizability of the findings to other populations with varying injury characteristics. Additionally, the timing of cognitive tests was not standardized across groups, which may have introduced bias in cognitive assessments. The study also lacked control for the amount of time participants spent in standard care, which could have confounded the comparison between groups. Furthermore, we did not measure hypnotic susceptibility, which may have influenced the outcomes. Lastly, the specific reasons for participant exclusion were not fully documented, limiting transparency regarding the selection process.

## Conclusion

Brief hypnotic treatment at a municipal vocational rehabilitation center for people with ABI and concussion showed no significant advantage over mindfulness or usual care in labor market attachment, cognition, or family functioning. However, participants in the hypnosis group demonstrated significantly improved social functioning at the 6-month follow-up compared to usual care.

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## Declarations

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***Author contributions***

Conceptualization, P.P., C.L., C.M.S., C.V.N. and J.K.L.; Methodology, P.P., C.L., C.M.S., C.V.N. and J.K.L.; Formal Analysis, P.P.; Investigation, J.K.L.; Resources, J.K.L. and C.V.N.; Data Curation, J.K.L.; Writing – Original Draft Preparation, P.P., C.L. and J.K.L; Writing – Review & Editing, P.P., C.L., C.M.S., C.V.N. and J.K.L.; Visualization, P.P. and J.K.L.; Supervision, C.V.N.; Project Administration, P.P., C.L. and C.V.N.; Funding Acquisition, J.K.L. and C.V.N.

***Conflicts of interest***

The authors declare that they have no conflict of interest.

***Data availability***

Data supporting the findings of this study are available from the last author upon reasonable request, subject to institutional and ethical approval.

***Ethics approval***

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the regional ethics committee (North Denmark Region) (May 15., 2017, no. N-20170022) and by the Danish Data Protection Agency (Sept. 18., 2015 and no. 2015-57-0001).

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**ORIGINAL PAPER**

# Silica nanoparticles from melon seed husk improves atherogenic, hematologic and oxidative stress indices in male Sprague Dawley rats exposed to Ni, Al and Ni/Al mixtures

Chidinma P. Anyachor [1], Awolayeofori Dokubo [2], Doris N. Ajibo [3], Baridoo Donatus Dooka [1], Theresa C. Ugwu [4], Danladi C. Husaini [5], Orish E. Orisakwe [1,6]

[1] African Centre of Excellence for Public Health and Toxicological Research (ACE-PUTOR), University of Port Harcourt, Port Harcourt, Nigeria
[2] Department of Biochemistry Faculty of Science, Rivers State University, Port Harcourt, Nigeria
[3] Department of Experimental Pharmacology and Toxicology, Faculty of Pharmacy, University of Port-Harcourt, Port-Harcourt, Nigeria
[4] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Madonna University Elele, Rivers State, Nigeria
[5] Department of Pharmacology and Toxicology, Faculty of Health Sciences, University of Belize, Belmopan, Belize
[6] Advanced Research Centre, European University of Lefke, Lefke, Northern Cyprus, Mersin, Turkey

**ABSTRACT**

**Introduction and aim.** With the increased awareness from circular bioeconomy that focuses on 'no waste' generation mantra, various technologies have been developed to valorize these wastes into useful products, including melon seed husk. The aim of the study was to evaluate the effects of silica nanoparticles from melon seed husk (SiNPs MSH) against Ni, Al, and Ni/Al mixture-induced hematotoxicity and lipotoxicity in Sprague Dawley rats.

**Material and methods.** Fifty-six male Sprague Dawley, 6 to 8 weeks and weighing 220 to 250 g, were randomly allocated to eight groups (n=7). Group 1 received deionized water only (control), groups 2, 3 and 4 (exposed groups) received the Ni/Al mixture, 0.2 mg/kg Ni and 1.0 mg/kg Al, while groups 5 to 8 received the Ni/Al mixture, Ni, and Al plus 100, 200 and 400 mg/kg of SiNPs respectively for 90 days. Blood samples were collected for biochemical investigation.

**Results.** Ni, Al and Ni/Al groups showed significant ($p<0.05$) alteration ($p<0.05$) in the classic lipid profile, hematological and oxidative stress markers compared to the control. Co-administration with SiNPs did not show significant ($p>0.05$) difference in these parameters compared to the control.

**Conclusion.** MSH SiNPs reversed Ni, Al, and Ni/Al mixture mediated hemotoxicity and elevated superoxide dismutase, catalase, and glutathione probably via metal chelation.

**Keywords.** hematotoxicity, lipid profile, nickel and aluminum, oxidative stress, silica nanoparticles

**Corresponding author:** Orish E. Orisakwe, e-mail: orishebere@gmail.com

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## Introduction

The processing of plant food products by the agri-food industry is now deemed useful given their enriched levels of bioactive components such as phenols, peptides, carotenoids, anthocyanins, fatty acids, fibers, and enzymes.[1] These bioactive compounds are often used in the production of functional foods, adjuvants, and drugs against acute and chronic diseases.[2] The presence of complex molecules such as cellulose, hemicelluloses, lignin and antinutritional compounds such as cyanogenic glycosides, oxalates, phytates, and trypsin inhibitors have hindered the utilization of these agro wastes, leading to large scale disposal in the environment and associated pollution.[3] With the increased awareness from circular bioeconomy that focuses on 'no waste' generation mantra, various technologies have been developed to valorize these wastes into useful products, including melon seed husk. Due to growing food waste (FW) issues and related environmental concerns, research into sustainable biotransformation of various types of FW and valorization of various agricultural waste has led to the research of a variety of high value commodities of interest over the past decades.[4] The utilization of melon seeds husk into various commodities of interest is limitless with a rich source of essential amino acids, vitamins and minerals, and fatty acids.[5] The abundance of noxious metals and metalloids in the earth crust, and their non-biodegradability confer on them the status of long environmental persistence from where they reach other environmental matrices naturally or through various anthropogenic activities.[6-8] Environmental pollution by heavy metals is now recognized as a huge toxicological menace.[9,6]

## Aim

In view of occupational and daily human exposure to various mixtures of these metals and metalloids, and not just individual elements, the aim of the present study was to evaluate the ameliorative effects of silica nanoparticles from melon seed husk against Ni, Al, and Ni/Al mixture-induced lipotoxicity, atherogenic, hematological, and oxidative stress indices in male Sprague Dawley rats.

## Material and methods

### *Collection of plant materials and chemicals*

The melon seed husk was procured from the local market in Abuja, Nigeria and taken to the Department of Plant Science and Biotechnology, University of Port Harcourt for identification. All chemicals and reagents used for this study were analytical grade obtained from reputable companies.

### *Preparation of melon seed husk ash*

The melon husk was washed carefully and several times in running tap water and dried in direct sunlight for six days before further processing. The clean and dried husks were measured before loading. The accurate amount of melon seed husk (2 kg) was subjected to heat treatment by carefully packing them in batches in heat resistant petri dishes in muffle furnace and calcinated at a temperature of 750°C for six hours to produce an off-white ash which was cooled to room temperature to obtain the melon seed husk ash.

### *Synthesis of silica nano particles from melon seed husk ash*

The sol-gel precipitation method was adapted to facilitate the precipitation from silica nanoparticles of the melon seed husk ash with slight modification.[10] The chemical reaction that provided the silica nanoparticles formation was expressed as shown in equations (1) and (2).

$SiO_2 + 2NaOH \quad Na_2SiO_3 + H_2O$ - (1)

$Na_2SiO_3 + H_2SO_4 \quad SiO_2 + NaSO_4 + H_2O$ - (2)

An equivalent sample weight of 5.00 g ash was carefully 0.5 N sodium hydroxide solution in 250 mL volumetric flask. The solution was heated for 30 minutes in a water bath and allowed to cool at room temperature prior to filtration. Hydrochloric acid was added dropwise manner until neutralized. The precipitated silica nanoparticles were carefully washed with double distilled and centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and pellets were dried in an oven at a temperature of 120° C for 24 hours and re-calcinated at 450° C for 1 hour to yield pure nanosilica. The characterization, particle size analysis of the silica nanoparticle from melon seed husk extract, X-ray (XRD) analytical pattern of the fabricated sample of silica nanoparticle from melon seed, the nano-silica size distribution range (DLS) and transmission electron microscopy of the silica nanoparticle from melon seed husk extract are shown in supplementary files 1, 2 and 3 respectively.

### *Experimental design*

Fifty-six male Sprague Dawley rats free of disease and deformity, aged 6–8 weeks old; weighing 220 to 250 g was used in this study. The animals were randomly assigned to eight groups, each with seven animals, and were acclimatized for two weeks prior to the start of the study. The animals were kept in standard laboratory conditions (a daily light period of 12 h and temperature of 21±2°C) and were provided with *ad libitum* food and water. The experiment was carried out according to the guidelines of the Research Ethics Committee of the University of Port Harcourt. The accepted study protocol was assigned UPH/CEREMAD/REC/MM86/037 as the reference number.

### *Exposure and establishment of Ni, Al and Ni/Al-induced toxicity*

To induce experimental poisoning, aluminum chloride and nickel chloride were administered orally at a dose of

1 mg/kg.[11] and 0.2 mg/kg body weight daily, respectively, for 90 days, respectively, three times a week.[12]

***Treatment intervention protocol***

The SiNP treatment groups were administered at concentrations of 100, 200 and 400 mg/kg.[13] Labeled low-dose, medium-dose, and high dose for 90 days by oral cannula and treated for 90 days as shown in Table 1.

**Table 1.** Treatment protocol for experimental animals

| S/No. | Groups | Treatment Procedure | Identification |
|---|---|---|---|
| 1 | Normal control | Deionized water | Control |
| 2 | Ni/Al mixtures exposed | 0.2 mg/kg Ni+1 mg/kg Al | Ni/Al mixtures |
| 3 | Ni only exposed | 0.2 mg/kg Ni | Ni |
| 4 | Al only exposed | 1 mg/kg Al | Al |
| 5 | Test 1 | 0.2mg/kg Ni+1 mg/kg Al +100mg/kg SiNPs | Ni/Al+100 mg/kg SiNPs+ |
| 6 | Test 2 | 0.2 mg/kg Ni+1.0 mg/kg Al+200 mg/kg SiNPs | Ni/Al+200 mg/kg SiNPs+ |
| 7 | Test 3 | 0.2 mg/kg Ni+1mg/kg Al + 400 mg/kg SiNPs | Ni/Al + 400 mg/kg SiNPs+ |
| 8 | Test 4 (positive control) | 100 mg/kg SiNPs only | 100 mg/kg SiNPs |

***Collection of blood samples for serum biochemistry***

After 90 days of treatment, rats in each group were sacrificed under pentobarbital (50 mg/kg IP) anesthesia, blood samples were collected from the abdominal artery in heparinized tubes and centrifuged to obtain plasma from which high-density lipoprotein (HDL-c) and low-density lipoprotein (LDL-c) + very low-density lipoprotein (VLDL-c) fractions were later obtained.[14] Simultaneously, additional blood samples were collected for hemology.[15] These samples were placed in tubes for serum separation, incubated at room temperature for 60–90 min and then centrifuged for 10 minutes.

***Lipid profile markers determination***

Lipid profile markers (total cholesterol, triglycerides, HDL-c, LDL-c, and VLDL-c) were measured enzymatically in a series of coupled reactions using a biochemical assay kit (Randox, Crumlin, United Kingdom). The enzymatic hydrolysis of cholesterol from its ester form by cholesterol esterase and further oxidation of cholesterol to produce hydrogen peroxide as a byproduct. Triglycerides are hydrolyzed to produce glycerol and oxidized using glycerol oxidase. Hydrogen peroxide ($H_2O_2$), the reaction by product was quantitatively measured at 500 nm.

***Determination of atherosclerotic cardiovascular disease risk***

Plasma atherogenic index, atherogenic coefficient, cardio risk ratio (Castelli's Index I and II), cardio protective index and small dense low density lipoprotein particles were used to estimate the estimation of atherosclerotic cardiovascular disease. AIP was calculated using equation (1), AC (2), CRI-I and II (3) and (4), cardio protective (5) and sdLDL-c (6) and sdLDL-c (5).

$$\text{Atherogenic index of plasma (AIP)} = Log\ \frac{TG}{HDL-c} \quad \text{- (1)}$$

$$\text{Atherogenic Coefficient (AC)} = \frac{(TC-HDL-c)}{HDL-c} \quad \text{- (2)}$$

$$\text{Castelli's risk Index (CRI-I)} = \frac{TC}{HDL-c} \quad \text{- (3)}$$

$$\text{Castelli's risk index (CRI- II)} = \frac{LDL-C}{HDL-c} \quad \text{- (4)}$$

$$\text{Cardio protective Index (CPI)} = \frac{sdLDL-C}{lbLDL-c} \quad \text{- (5)}$$

$$\text{sdLDL-c mg/dL} = 0.580\ (\text{non–HDL-C}) + 0.407\ (\text{dLDL-C}) - 0.719(\text{CLDL-c}) - 12.05 \quad \text{- (6)}$$

[non HDL-c=TC-HDL-c, large buoyant LDL-c (lbLDL-c)=LDL-c-sdLDL-c, and calculated LDL-c (CLDL-c)=TC-HDL-c- TG/5]

***Oxidative stress markers***

The lipid peroxidation which is marked by malondialdehyde (MDA), is evaluated by thiobarbituric acid reactive substances at 532 nm wavelength.[16] Nitric oxide (NO) was estimated at 540 nm wavelength.[17] Superoxide dismutase (SOD) activity was estimated by the rate of inhibition of pyrogallol auto-oxidation obtained by reporting the changes in absorbance, for 3 min (at 30 s intervals), at 420 nm.[18] Glutathione (GSH) levels and glutathione peroxidase (GPx) activity were determined at 450 nm and 340 nm, respectively.[19,20] A slight modification of technique was used for the catalase (CAT) activity assay, given that tissue catalase cleaves hydrogen peroxide which can be determined at 240 nm using a spectrophotometer.[21]

***Statistical analysis***

Data were expressed as mean±standard deviation (SD). Microsoft Xlstat 2014 (Microsoft, USA) was used in performing variance analysis and Kruskal-Wallis tests to check whether the concentration of the biomarkers was significantly different between groups. Data analysis involved performing descriptive statistics on metal and biomarkers concentration before ANOVA was used to establish whether there was significant difference in the concentration of heavy metals and biomarkers between groups.). Multivariate analysis was used to determine the level of association between dependent variables and independent variables in this study. All significant differences were at a $p<0.05$.

## Results

### *The effect of SiNPs from melon seed husk MSH on the serum lipid profile*

The effect of SiNPs on the serum lipid profile of rats exposed to Ni, Al, and Ni/Al mixtures is presented in Figure 1. Classic lipid parameters in groups exposed to Ni, Al, and Ni/Al mixtures showed significant ($p<0.05$) increase in the concentrations of TC, TG, LDL-c and VLDL-c, while significant ($p<0.05$) decrease in HDL-c concentration was observed compared to the control. The group co-administered with SiNP and Ni/Al mixtures (Ni/Al mixtures+100mg/kg SiNPs, Ni/Al mixtures+200 mg/kg SiNPs and Ni/Al mixtures +400 mg/kg SiNPs), showed no significant ($p>0.05$) difference in TC compared to the control, while significant ($p<0.05$) difference ($p <0.05$) was observed compared to the Ni, Al and Ni/Al exposed groups. There was a significant ($p<0.05$) decrease ($p <0.05$) in TG in the SiNP 100 mg/kg group compared to the control group. HDL-c values showed a significant ($p<0.05$) increase in test groups treated with Ni/Al mixture + 200 mg/kg SiNP and Ni/Al mixture + 400 mg/kg SiNP and were comparable to control. LDL-c showed a significant ($p<0.05$) decrease ($p <0.05$) in the Ni/Al mixture + 200 mg/kg SiNP, the Ni/Al mixture + 400 200 mg/kg SiNP and the Ni/Al mixture + 100 mg/kg SiNP compared to the exposed groups Ni, Al and Ni/Al. However, the group treated with LDL-cin 100 mg/kg of SiNP was significantly ($p<0.05$) different from the control. VLDL-c in all test groups treated with SiNP showed significant ($p<0.05$) decrease ($p <0.05$) compared to the Ni, Al and Ni+Al exposed groups.

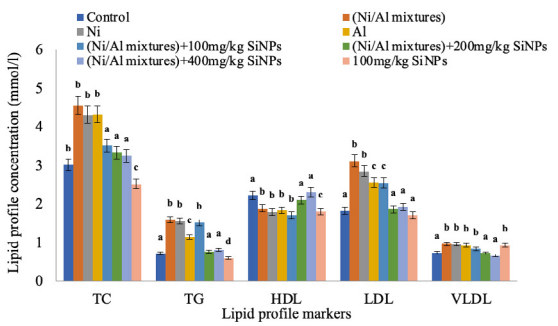


**Fig. 1.** Effect of SiNP from melon seed husk on classic lipid profile of rats exposed to Ni, Al and Ni/Al mixture (TC total cholesterol, LDL-c – low-density lipoprotein cholesterol HDL-c high-density lipoprotein cholesterol, VLDL-c very low-density lipoprotein cholesterol, TG triglyceride, values are expressed as mean±SD, superscripts with different letters are significantly different

The effect of SiNP on cardiovascular risk indices of rats exposed to Ni, Al and Ni/Al mixtures showed that AIP values ranged from -11.64 to -0.05 with an average value of -0.28 while sdLDL-c values range from -11.64 to -10.36 with an average value of -11.01 (mmol/L); however, risk values for atherosclerosis indicated low for exposed and treated groups, Table 2.

**Table 2.** The effect of SiNPs on the cardiovascular atherogenic risk indices of rats exposed to Ni, Al, and Ni/Al mixture *

| Groups | AIP | AC | CRI-I | CRI-II | lbLDL-c | sdLDL-c | CPI |
|---|---|---|---|---|---|---|---|
| Control | -0.50 | 0.37 | 1.37 | 0.83 | 13.14 | -11.32 | -0.86 |
| Ni/Al mixture | -0.08 | 1.42 | 2.42 | 1.65 | 14.04 | -10.93 | -0.78 |
| Ni | -0.06 | 1.41 | 2.41 | 1.60 | 13.86 | -11.02 | -0.78 |
| Al | -0.21 | 1.36 | 2.36 | 1.40 | 13.74 | -11.19 | -0.82 |
| Ni/Al mixture+ 100mg/kg SiNPs | -0.05 | 1.06 | 2.06 | 1.48 | 13.57 | -11.06 | -0.82 |
| Ni/Al mixture+ 200 mg/kg SiNPs | -0.44 | 0.58 | 1.58 | 0.88 | 13.21 | -11.35 | -0.86 |
| Ni/Al mixture+ 400 mg/kg | -0.44 | 0.48 | 1.48 | 0.87 | 13.22 | -11.30 | -0.85 |
| 100 mg/kg SiNPs | -0.50 | 0.38 | 1.38 | 0.94 | 13.08 | -11.37 | -0.87 |

* AIP atherogenic index of plasma, AC atherogenic coefficient, CRI castelli risk index, lbLDL-c – large buoyant lipoprotein cholesterol, sdLDL-c, small dense lipoprotein cholesterol, CPI, cardioprotective index

**Table 3.** Multivariate regression analysis showing the association sdLDL-c with the classic lipid parameters model 1*

| Parameter | β | *p* |
|---|---|---|
| Intercept | -12.05 | 3.72E-47 |
| TC | -0.139 | 5.18E-43 |
| TG | 0.1438 | 3.26E-42 |
| HDL-c | 0.139 | 1.9E-42 |
| LDL-c | 0.407 | 2.82E-43 |

* values are significant, $p<0.05$, β – regression coefficient

**Table 4.** Multivariate regression analysis showing the association of sdLDL-c with cardiovascular atherogenic risk indices model 2*

| Parameters | β | *p* |
|---|---|---|
| Intercept | -21.13 | 0.000114 |
| AIP | 0.58 | 0.003024 |
| AC | -0.28 | 0.063182 |
| Non-HDL-c | -0.13 | 0.119931 |
| lbLDL | 0.78 | 0.000972 |

* values are significant, $p<0.05$, β – regression coefficient

Multiple regression analysis performed with sdLDL-c concentration as a dependent variable and TC, TG, HDL-c and LDL-c as independent variables, Table 3. This stepwise regression analysis was identified as (model 1) and significant variables ($p<0.05$; $R2=1$) and the standard error of the estimate as ($4.61x10^{-16}$ mmol/L). The best fit of the linear regression equation was as follows:

$$y=-12.05 -0.14TC+0.14TG+0.14HDL-c+0.407 LDL-c \quad - (1)$$

Multiple regression analysis with the concentration of sdLDL-c as the dependent variable AIP, AC, Non-HDL, and lbLDL-c as independent variables was performed Table 4. This stepwise regression analysis was identified as (model 2) and significant variables ($p$<0.001; $R^2$=0.99) and the standard error of the estimate was 0.0083 mmol/L. The best fit of the linear regression equation was as follows:

y=-21.13 +0.58AI-0.28AC-0.133NonHDL-c+0.78lb

**Table 5.** Effect of SiNPs from melon seed husk on the hematological profile of rats exposed to the Ni, Al and Ni/Al mixture*

| Groups | PCV | WBC | RBC | Hb | Platelet |
|---|---|---|---|---|---|
| Control | 48.00±5.66$^{a}$ | 16.95±3.46$^{a}$ | 8.35±0.21$^{a}$ | 15.65±1.48$^{a}$ | 726.50±45.96$^{a}$ |
| Ni/Al mixture | 25.50±2.12$^{b}$ | 22.60±3.39$^{b}$ | 4.25±0.07$^{b}$ | 11.25±0.21$^{b}$ | 513.00±59.40$^{b}$ |
| Ni | 25.50±4.95$^{b}$ | 22.55±0.78$^{b}$ | 3.65±0.49$^{b}$ | 10.85±0.21$^{b}$ | 531.50±28.99$^{b}$ |
| Al | 28.00±5.78$^{b}$ | 20.60±2.26$^{b}$ | 3.40±0.42$^{b}$ | 10.75±0.35$^{b}$ | 517.00±63.64$^{b}$ |
| Ni/Al mixture + 100 mg/kg SiNPs | 48.50±2.12$^{a}$ | 13.70±3.96$^{ac}$ | 8.15±0.21$^{a}$ | 16.00±0.57$^{a}$ | 640.00±32.53$^{ab}$ |
| Ni/Al mixture + 200 mg/kg SiNPs | 48.50±2.12$^{a}$ | 16.00±4.53$^{a}$ | 8.00±0.85$^{a}$ | 15.25±0.92$^{a}$ | 682.00±77.78$^{ab}$ |
| Ni/Al mixture + 400 mg/kg SiNPs | 50.50±2.12$^{a}$ | 15.s75±4.67$^{a}$ | 8.65±0.07$^{a}$ | 16.55±1.34$^{a}$ | 705.00±84.85$^{a}$ |
| 100 mg/kg SiNPs | 48.50±4.95$^{a}$ | 11.35±1.34$^{c}$ | 8.30±0.00$^{a}$ | 14.85±0.78$^{a}$ | 627.00±56.57$^{ab}$ |

***** White blood cell, PCV – packed cell volume PCV, hemoglobin Hb, red blood cell, P – platelet, values expressed as mean±SD, superscripts with different letters are significantly different

**Table 6.** Effect of SiNPs from melon seed husk on the hematological profile of rats exposed to the Ni, Al and Ni/Al mixture*

| Groups | N | L | M | E | MCV | MCH | MCHC |
|---|---|---|---|---|---|---|---|
| Control | 13.00 ±2.83$^{a}$ | 82.00 ±0.71$^{a}$ | 2.00 ±1.41$^{a}$ | 1.00 ±0.00$^{a}$ | 57.10 ±5.23$^{a}$ | 18.65 ±1.20$^{a}$ | 32.75 ±0.92$^{a}$ |
| Ni/Al mixture | 23.50 ±2.83$^{b}$ | 71.00 ±4.24$^{b}$ | 3.50 ±0.71$^{b}$ | 2.00 ±1.41$^{b}$ | 52.30 ±3.54$^{b}$ | 17.90 ±0.99$^{b}$ | 30.55 ±0.35$^{b}$ |
| Ni | 22.50 ±3.54$^{b}$ | 72.00 ±5.65$^{b}$ | 3.00 ±1.41$^{b}$ | 2.50 ±0.71$^{b}$ | 51.15 ±1.77$^{b}$ | 17.40 ±0.14$^{b}$ | 30.25 ±1.20$^{b}$ |
| Al | 24.50 ±2.12$^{b}$ | 70.50 ±3.54$^{b}$ | 3.50 ±0.71$^{b}$ | 1.50 ±0.71$^{a}$ | 53.10 ±2.97$^{b}$ | 16.10 ±0.85$^{b}$ | 29.95 ±0.21$^{b}$ |
| Ni/Al mixture + 100 mg/kg SiNPs | 15.50 ±0.71$^{a}$ | 80.50 ±4.24$^{a}$ | 2.50 ±2.12$^{a}$ | 1.50 ±0.71$^{a}$ | 61.30 ±4.10$^{a}$ | 19.00 ±1.56$^{a}$ | 32.70 ±0.28$^{a}$ |
| Ni/Al mixture + 200 mg/kg SiNPs | 14.00 ±4.24$^{a}$ | 81.50 ±4.95$^{a}$ | 2.00 ±0.00$^{a}$ | 1.50 ±0.71$^{a}$ | 58.00 ±5.66$^{a}$ | 18.40 ±1.13$^{a}$ | 31.70 ±1.27$^{a}$ |
| Ni/Al mixture + 400 mg/kg SiNPs | 16.50 ±3.54$^{a}$ | 80.50 ±4.24$^{a}$ | 1.00 ±0.00$^{a}$ | 1.00 ±0.00$^{a}$ | 57.90 ±5.09$^{a}$ | 18.20 ±0.28$^{a}$ | 33.50 ±2.26$^{a}$ |
| 100 mg/kg SiNPs | 13.00 ±1.41$^{a}$ | 84.00 ±2.83$^{a}$ | 2.00 ±1.41$^{a}$ | 1.00 ±0.00$^{a}$ | 57.00 ±0.42$^{a}$ | 18.30 ±0.28$^{a}$ | 33.80 ±1.41$^{a}$ |

* N neutrophils, L – lymphocytes, M monocytes, E – eosinophils, MCV – mean corpuscular volume, mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration of MCHC, values are expressed as mean±SD, superscripts with different letters are significantly different

***The effect of SiNPs on the hematological profile of rats exposed to Ni, Al, and Ni/Al mixtures***

The effect of SiNPs on the hematologic profile of rats exposure to Ni, Al, and Ni/Al mixtures is presented in Tables 5 and 6.

**Table 7.** Percentage change in the hematological profile of rats exposed to Ni/Al mixtures, Ni and Al with respect to the control, test groups co-administrated with SiNP*

| Groups | PCV | WBC | RBC | Hb | Platelets | N | L | M | E | MCV | MCH | MCHC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 46.44 | -44.66 | 54.44 | 31.28 | 22.96 | -53.26 | 11.96 | -81.82 | -50 | 11.65 | 7.55 | 7.30 |
| CombSiNPs | 45.14 | -29.31 | 54.89 | 30.03 | 28.36 | -80.77 | 13.21 | -66.67 | -100 | 8.61 | 8.13 | 7.63 |
| SiNPs only | 45.70 | -93.11 | 54.62 | 26.26 | 16.99 | -80.77 | 15.28 | -66.67 | -100 | 8.45 | 6.37 | 10.53 |

* test groups co-administered with SiNP (CombSiNP) (HMM + 100 mg/kg SiNP, HMM + 200 mg/kg SiNP, HMM + 400 mg/kg SiNP) and SiNPs only (100 mg/kg SiNP), PCV packed cell volume, WBC – white blood cell, RBC red blood cell, N – neutrophils, L lymphocytes, M – monocytes, E eosinophils, MCV – mean corpuscular volume, MCH mean corpuscular hemoglobin, MCHC mean corpuscular haemoglobin concentration

There were significant (p<0.05) changes (p <0.05) in all measured hematological parameters in the groups exposed to Ni, Al and Ni/Al mixtures compared to the control and test groups coadministered with SiNPs. The WBC values in the Ni/Al mixture + 100 mg/kg SiNP and 100 mg/kg SiNP, platelets (Ni/Al mixture + 100 mg/kg SiNP, Ni/Al mixture + 200 mg/kg SiNP and 100 mg/kg SiNP only) groups decreased significantly (p<0.05) in comparison to the control. In Ni/Al mixtures, Ni and Al exposed groups Lymphocytes (L), MCV, MCH and MCHC had a significant (p<0.05) decrease (p <0.05) while neutrophils (N), monocytes (M) and eosinophils (E) significant (p<0.05) increased significantly (p <0.05) compared to the control. The average % reduction was observed to be in the following order: 54.9% (RBC)<-45% (PCV)<-30.1% (Hb)< 28% (platelets)<13.21% (lymphocytes)<8.6% (MCV) < 8.13% (MCH) < 7.6% (MCHC). While the average percentage elevation was observed to be in the following order and +16.04% (E) <29.10% (WBC) <66.67 %(M) < 80.76% (N) with respect to the control. The test groups had an average increase in WBC to be 60.0% (Ni/Al mixture+100 mg/kg SiNP) and 93.1% (100 mg/kg SiNPs) while platelets had an average of 59.6 % for the test groups (Ni/Al mixture + 100 mg/kg SiNP, Ni/Al mixture + 200 mg/kg SiNP and 100 mg/kg SiNP) respectively with respect to the metals and Ni/Al, Ni and Al only exposed groups. Coadministration of SiNPs caused significant (p<0.05) increase in PCV, RBC, Hb and platelet counts, while significant (p<0.05) reduction comparable to the control was observed for WBC, N, M and E. The percentage reduction for WBC was observed for -29.31 which was the three times the value obtained for the SiNPs only treated

group (-93.11) and 1.5 times the control group (-44.66), respectively. The E% reduction was twice the value. The value of N was over 60 % reduction. The % increase for PCV and RBC were greater than 40%, Hb and platelets greater than 20%, L, MCV, MCH and MHCH were greater than 5% as shown in Table 7.

***The effect of SiNPs on the oxidative stress markers in the blood of rats exposed to Ni, Al, and Ni/Al mixtures***

The effect of SiNPs on oxidative stress markers in the blood of rats exposed to Ni, Al, and Ni/Al mixtures is presented in Figure 2.

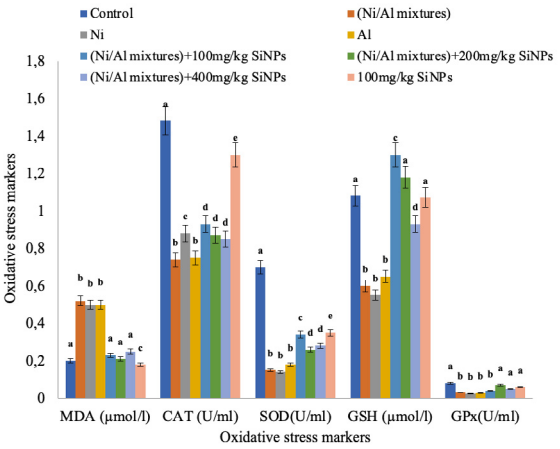


**Fig. 2.** SiNP treatment on oxidative stress markers in rats exposed to Ni, Al and Ni/Al mixture, values are expressed as mean±SD, superscripts with different letters are significantly different

In the Ni, Al and Ni/Al mixture exposed groups, there was a significant ($p<0.05$) increase ($p<0.05$) in MDA levels compared to the control and the groups co-administration with SiNP. CAT, SOD, GPx, and GSH concentrations decreased significantly ($p<0.05$) in groups exposed to Ni, Al and Ni/Al mixture only compared to control and test groups co administered with SiNP. Coadministration of Ni, Al, Ni/Al mixture groups with SiNPs showed remarkable reduction in MDA levels and increase in SOD, CAT, and GPx.

## Discussion

Nickel (Ni) is a known environmental toxicant that can be found in various sources such as air pollution, industrial emissions, and tobacco smoke.[22-25] On the other hand, Al is a widely used element and can be found in various food additives, medications, and cosmetic products.[26-28] There has been a surge in public health concerns regarding its potential impact on human health, including disruption of normal functioning of lipid metabolism, the driving force behind progression of cardiovascular disease (CVD) hematotoxicity, and induction of oxidative stress markers.[29]

Previous studies have evaluated the effect of Ni and Al on their potential effects on lipid metabolism, including their impact on LDL, VLDL, TC, and TG levels in rats.[30,31] In this study, rats exposed to Ni, Al, and Ni/Al mixtures had significant increase in TC, TG, LDL-c, and VLDL-c while the HDL-c showed a significant decrease. These observations may be linked to interference in the synthesis and metabolism of lipoproteins that are responsible for transporting cholesterol and other lipids throughout the body.[32,33] Ni-induced lipotoxicity may involve interference with enzymes that play a critical role in lipid synthesis and metabolism.[34] Ni exposure has been linked to inhibition of major enzymes such as acetyl-CoA carboxylases and fatty acid synthases, essentially required for the synthesis of triglyceride and cholesterol.[35] The mechanisms behind Al effects on the lipid profile may also be attributed to interference with key enzymes involved in lipid metabolism and transport, as well as expression of genes responsible for lipid metabolism.[36,37] In the test group co-administration with SiNPs, the lipid profile markers were similar to the control. HDL-c is regarded as a good cholesterol crucial for the transportation of cholesterol from peripheral tissues to the liver for metabolism. The specific effects of Ni and Al on HDL-c may have occurred through reverse cholesterol transport.

Lipid oxidation emanates from an abnormal lipid profile, since increased LDL-c levels are deemed the main contributor to atherosclerosis.[38] Elevated plasma levels of LDL and VLDL-c seen in noncommunicable diseases NCDS like hypertension and obesity, are now known as the primary health issues globally.[39] The different ratios or combinations of these lipid profiles are vital tools used in the prediction and detection of high-risk individuals than just the lipid parameters alone.[38] There are three ratios of lipid profile indices namely atherogenic coefficient (AC), atherogenic index of plasma (AIP), and Castelli's risk indices that employed as markers of lipid atherogenic risk (CRIs). Calculated lipid fraction ratios have tended to replace the more conventional lipid profile parameters in clinical settings in the assessment of cardiovascular risk since the atherogenic index specifies the degree of the probable rate of atherosclerosis as a marker of CVD.[40,41]

Changes in lipid metabolism can significantly affect cardiovascular health and increase the risk of developing heart disease.[42,43] In this study, an attempt to better characterize the atherogenic potential of the measured lipid profile, the following parameters as indices for cardiovascular atherogenic risk markers were integrated. AIP, AC, cardiorisk ratio index (CRR-I), cardio-protective index (CPI), high buoyant lipoprotein cholesterol (lbLDL-c), and small dense lipoprotein cholesterol (sdLDL-c). The indices suggested a low risk of atherosclerosis with classification values that fall within the range of -0.3 to 0.1 (low risk), 0.1 to 0.24 (moderate) and >0.24 (high) for AIP.[44,45]

Generally, studies have shown that heavy metals are associated with increased atherogenic indices.[46-48] However, some can also lead to a lower AIP. An AC value greater than 3 is considered an abnormal value of cardiovascular risk.[44] The values computed for the exposed and treated groups were lower than the low 3 and reflect the atherogenic potential of the entire lipoprotein fraction to be low. Castelli risk indices CRI-I and CRI-II, CPI are both atherogenic and protective lipid fractions to evaluate cardio atherogenic disease (CAD).[49] However, the lipid indices obtained for these markers were below the reference limit of (>4 and >3, respectively).[50] sdLD-cL has been shown to be more atherogenic than lbLDL-c to promote the formation of atherosclerotic plaques, sdLDL-c provides longer time and more chances of its penetration into the subendothelial space.[50] sdLDL-c is more sensitive to oxidation; stronger binding ability to proteoglycans and glucosamines located in the endothelium lining.[51-53] Decrease in the sdLDL-c may be attributed to rapid inhibition of oxidative modification of sdLDL-c in the treated group.[51] This can prevent accumulation of oxidized LDL-c that are associated with plaque instability in coronary and carotid artery disease and other metabolic syndrome.[52] Furthermore, elevated sdLDL-c is generally accompanied with reduced HDL-c and elevated levels of TG.[54] There was a significant correlation and association of classic lipid markers and atherogenicity in Ni/Al exposed groups and reduced levels of atherogenic lipids and a lower AIP in SiNPs.

Ni and Al can exert a negative impact on the hematological profile of humans. Several studies have reported the influence of these elements on the hematopoetic system through different routes of exposure.[55-58] In this study, rats exposed to Ni, Al, Ni/Al mixture had significant alterations in the hematologic profile. Elevated exposure to Ni is associated with potential hemolysis.[59-61] Al exposure impacts red blood cell production and disrupts iron homeostasis, absorption, transportation, and iron utilization, ultimately affecting packed cell volume levels in rats.[62-64] Rats exposed to Ni, Al, and Ni/Al and Ni/Al mixtures had significant increase in neutrophils, monocytes, and eosinophils compared to control, as well as decreased platelet counts that can further lead to platelet dysfunction and impairment.[65-67] Co administration of SiNPs tended to attenuate hemolysis. Nutraceuticals are known to ameliorate Ni and Al mediate hematotoxicity in rats.[68-70] SiNPs may contain antioxidants and metal chelating bioactive compounds.[71-73] SiNPs can chelate Ni and Al ions to accelerate fecal elimination and offer at least the benefit of SiNPs in the present study.[74] Ni and Al exposure can trigger inflammation and hematotoxicity that can be ameliorated by SiNPs treatment. [52,71]

MDA is considered a marker of lipid peroxidation. Other studies have shown that Ni and Al exposure is accompanied by elevated MDA levels.[75-77] In this study there was a significant decrease in CAT, SOD, GPx and GSH) and increased MDA after exposure to Ni/Al, Ni, Al. Ni exposure decreases the activity of antioxidants in tissues just like Al exposure.[74-82]

## Conclusion

The toxicity of the Ni, Al and Ni/Al binary mixtures can cause a decrease in the HDL-c and increase in TC, TG, LDL-c, and VLDL-c cholesterol in rats, which may be attributed to interference with key enzymes involved in the metabolism and transport as the well as expression of genes responsible for lipid metabolism. Ni, Al Ni/Al mixtures may interfere with hematopoiesis via oxidative stress mechanism.

SiNPs may ameliorate Ni, Al, and Ni/Al-mediated lipotoxicity via antioxidant activity, and metal chelating activity suggesting a beneficial role of SiNPs in attenuating cardiovascular risk. Together, SiNPs from melon seed husk can improve atherogenic, hematological and oxidative stress indices in male Sprague Dawley rats exposed to Ni, Al, and Ni/Al mixture.

## Declarations

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***Authors' contributions***

Conceptualization, O.E.O.; Methodology, C.P.A., A.W., D.N.A., B.D.D. and T.C.U.; Validation, O.E.O.; Formal Analysis, C.P.A., A.W., D.N.A., B.D.D. and T.C.U.; Investigation, C.P.A. and B.D.D.; Data Curation, C.P.A., A.W., D.N.A., B.D.D. and T.C.U.; Writing – Original Draft Preparation, C.P.A., A.W., D.N.A., B.D.D. and T.C.U.; Writing – Review & Editing, A.W., D.N.A., T.C.U. and O.E.O.; Supervision, O.E.O.; Project Administration, O.E.O.

***Conflicts of interest***

The authors confirm that there were no conflicts of interest.

***Data availability***

All data have been provided.

***Ethics approval***

The experiment was carried out according to the guidelines of the Research Ethics Committee of the University of Port Harcourt. The accepted study protocol was assigned UPH/CEREMAD/REC/MM86/037 as the reference number.

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**ORIGINAL PAPER**

# Correlation between serum gamma glutamyl transferase with atherogenic index of plasma with angiographic severity in patients with coronary artery disease

Vignessh Raveekumaran [1], Arun Prasath [2], Sivasankar Masilamani [3], Jenifer Florence Mary [4]

[1] Department of General Medicine, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry, India
[2] Department of Cardiology, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry, India
[3] Department of Nephrology, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry, India
[4] Department of Community Medicine, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Pondicherry, India

**ABSTRACT**

**Introduction and aim.** Coronary artery disease (CAD) is a leading cause of morbidity and mortality worldwide. Gamma-glutamyl transferase (GGT) has been found to be involved in the pathogenesis of CAD. The aim of the study was to study the correlation between serum GGT and the atherogenic index of plasma (AIP) with angiographic severity in patients with CAD.

**Material and methods.** This was an analytical cross-sectional study performed in 150 CAD patients in a tertiary-care teaching hospital in Puducherry, India. The patients were categorized as ST-elevated myocardial infarction (STEMI), non-ST-elevated MI (NSTEMI) and unstable angina. Routine biomarkers including troponin-I, AIP, GGT, and angiographic severity were calculated by applying a Gensini score (GS).

**Results.** The mean age of the study participants was 55.7±10.2 years, predominantly males. The GGT and GS was higher in STEMI group followed by NSTEMI and unstable angina groups ($p<0.001$ and 0.016, respectively). This indicates that GGT could be a potential biomarker for CAD, specifically in STEMI. AIP was shown to be statistically significant in unstable angina patients ($p=0.029$). GGT and GS showed a positive correlation with each other, and were statistically significant ($r=0.1685$, $p=0.0387$).

**Conclusion.** Elevated serum GGT levels were positively correlated with angiographic severity of CAD with stronger associations in patients who had STEMI.

**Keywords.** angiographic severity, atherogenic index of plasma, coronary artery disease, gensini score, serum gamma-glutamyl transferase

## Introduction

Cardiovascular disease (CVD), such as ischemic heart disease and stroke are the major cause of death, accounting for 17.7 million fatalities.[1] According to the World Health Organization (WHO), India is responsible for one-fifth of these deaths globally, particularly

**Corresponding author:** Sivasankar Masilamani, e-mail: drsivasankar1984@gmail.com

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among the younger population.[1] CVDs affected Indians a decade earlier than the rest of the world,[2] with highest rate of coronary artery disease (CAD) and considered as a primary cause of death and morbidity in India.[3] According to the Global Burden of Disease (GBD) report, India has a mortality from CVD of 272 per 100,000, higher than the global average of 235 per 100,00.[2] India has the greatest prevalence of acute coronary syndrome (ACS) and ST-elevation myocardial infarction at the moment (STEMI).[1,4]

Patients with CAD are at an increased risk of developing subsequent cardiac events and mortality. Traditional risk variables and prognostic risk models, on the other hand, are insufficient to account for the development of CAD. Thus, identifying novel prognostic indicators is critical for more aggressive secondary prevention in individuals with CAD.[5]

Inflammation is a major contributor to the development of atherosclerosis. Changes in the blood levels of specific inflammatory indicators can influence the development and progression of atherosclerosis, as well as on the risk of thrombotic consequences. Gamma-glutamyl transferase (GGT) has been linked to the development of CAD and its mortality.[6,7] It is the membrane-bound enzyme involved in glutathione catabolism (G-SH) and promotes the oxidation of low-density lipoprotein (LDL) and the generation of reactive oxygen species (ROS), that contribute to the atherosclerotic process.[8] Various literature implies that elevated serum GGT can be used as a predictive biomarker for variety of illnesses including conditions such as liver function, excessive alcohol intake, and oxidative stress.[6,9–11] Similarly, the atherogenic index of plasma (AIP) is a novel index composed of triglycerides and high-density lipoprotein (HDL) cholesterol that is used to quantify blood lipid levels and commonly used as optimal indicator of dyslipidemia and associated diseases especially CVD.[5]

Currently, serum GGT level is considered as the risk factor for CVD.[12] Yet, the prognostic usefulness of GGT levels in predicting CV and all-cause mortality in individuals with CAD is controversial.[9,11,13–15] Pooled studies, established an association between an elevated GGT level and an increased risk of cardiovascular and all-cause mortality in the general population.[16–18] Also, it can be considered as an early marker for atherosclerosis, and also as an independent biomarker for coronary artery calcification.[19-23] Even with the Framingham offspring study, was one of the first epidemiological studies to examine the relationship between GGT levels and CVD risk, resulted that GGT may be used to forecast metabolic and CV risks associated with the beginning of metabolic syndrome and acute CVD, as well as to determine mortality.[24]

## Aim

With this background, in this study we want to determine the correlation between serum GGT and AIP with angiographic severity in patients with CAD.

## Material and methods

### *Study setting and design*

The study was conducted in the General Medicine and Cardiology outpatient departments (OPD) in a tertiary care teaching hospital in Puducherry, India. State-of-the-art equipment at this tertiary care facility empowers specialists to perform a wide range of cardiac management and treatments, including surgery. The present study was a hospital-based analytical cross-sectional study. Data collection was done for the period of January 2020 to April 2021.

### *Study population*

The inclusion criteria for the study considered were adult patients over 18 years of age, admitted to the Department of Medicine and Cardiology, who had ACS, including STEMI, NSTEMI, and unstable angina diagnosed on a clinical basis involving relevant history, biochemical test, and electrocardiogram (ECG) recording and patients with chronic stable angina. Patients with a history of myocardial infarction (MI), coronary intervention, congestive heart failure (CHF), history of alcoholic liver disease, chronic obstructive pulmonary disease (COPD), recent alcohol intake (<3 weeks), respiratory failure, renal failure, on medications such as oral contraceptives, statin therapy, and antiepileptic drugs (phenytoin, phenobarbital, carbamazepine) were excluded from this study.

### *Sample size and sampling technique*

Considering the prevalence of CAD to be 9.7%,[25] as the leading cause of disability in 2016, with 5% absolute precision and 95% as the confidence interval, the calculated sample size was 135. With an attrition rate, the final sample size was 148.5, rounded to the highest figure of 150 patients. A consecutive sampling technique was used to include all patients eligible for the study according to inclusion criteria until the desired sample size was achieved.

### *Data collection procedure*

After obtaining informed consent, data was collected using a patient proforma. It includes demographic details, risk factors, comorbidities, anthropometry, and previous medical and clinical history. A trained postgraduate paid visit to the and collected data by a face-to-face interview. The confidentiality, anonymity, and privacy of the participants were guaranteed throughout the study.

***Operational definitions***

STEMI is defined as ST-elevation of ≥0.1m V in >1 limb leads or ≥0.2 mV in contiguous chest leads or left bundle branch block (LBBB) on presentation to the hospital. Those without ST elevations were diagnosed with UA or NSTEMI differentiated by the presence of cardiac enzymes.

NSTEMI is defined as those who have persistent or transient ST segment depression or T wave inversion, flat T waves, pseudo-normalization of T waves or no ECG changes at presentation with elevated cardiac enzymes however those without elevated cardiac enzymes will be defined as unstable angina.

***Laboratory procedure***

Peripheral blood was drawn in an EDTA containing tube and stored for biochemical experiments within 24 hours after admission. The glucose oxidase method was used to detect fasting blood glucose and post-prandial glucose. Blood lipid indexes, including triglycerides, total cholesterol, HDL, LDL, and serum GGT levels, were measured by the 902 automatic nano auto analyzer (Hitachi, Tokyo, Japan). AIP was calculated by using the formula,

$$AIP = log\ log\ (triglycerides\ /\ HDL)$$

Where AIP less than 0.11 is associated with a low risk of CVD; the values between 0.11 and 0.21 and higher than 0.21 are associated with intermediate and increased risks, respectively.[26]

Angiographic severity is calculated by applying the Gensini severity score (GS).[27] It is a measure of the severity of coronary stenosis (luminal narrowing) and its location. A severity coefficient was given for each segment as follows: 1-point for <25% obstruction, 2-points for 26–50% obstruction, 4-points for 51–75% obstruction, 8-points for 76–90% obstruction, 16-points for 91–99% obstruction, 32-points for complete occlusion (100%). The score is multiplied by the factor which depends on the functional significance of the area supplied by that segment (5 for the left main coronary artery (LMCA), 2.5 for the proximal segment of the left anterior descending artery (LAD) or circumflex artery, 1.5 for the middle segment of the LAD artery, 1 for the apical segment of the LAD artery or the middle or distal segment of the circumflex artery or the entire segment of the right coronary artery, 0.5 for other small branches of the coronary artery. Consequently, total digital GS were obtained that indicated the severity of CAD.

***Ethical approval***

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committees and with the Helsinki Declaration (as revised in 2013). Written informed consent was obtained from the patient for the publication of this case report and accompanying images. This study was approved by the Institutional Ethics Committee (MGMCRI/Res/01/2019/33/IHEC/104).

***Data analysis***

Data were entered in MS EXCEL (Ver_2007) software and analysis was performed using Statistical Package for the Social Sciences (SPSS) software (version 24.0, IBM, Armonk, New York, USA). Categorical variables were measured in terms of frequencies and percentages. Continuous variables were expressed as mean and standard deviation (SD) or median with interquartile range (IQR). Data were analyzed according to the type of variables and the normal distribution between two groups. ANOVA was performed between three groups. Correlation was made between the GGT, and GS score. Statistical significance was considered as a $p<0.05$ for the analyzed data.

## Results

Among 150 study participants, the mean age was 55.7±10.2 years and ranged from 29–76 years. The demographic and baseline laboratory parameters are presented in Table 1. Cardiac enzymes were taken and presented in Table 2. The mean AIP was 0.41±0.19 (95% confidence interval (CI): 0.380, 0.442) and the GS score were 47.75±44.68 (95% CI: 37.008, 52.365).

**Table 1.** Demographic and baseline laboratory parameters among study participants (n=150)*

| Variables | n (%) or mean±SD | 95% CI |
|---|---|---|
| Age (in years) | 55.69±10.13 | 54.060–57.317 |
| **Gender** | | |
| Male | 102 (68) | |
| Female | 48 (32) | |
| **Anthropometric indices** | | |
| WC (cm) | 38.05±3.53 | 37.485–38.621 |
| BMI (kg/m$^2$) | 31.97±3.96 | 31.328–32.604 |
| **Blood glucose and lipid profile** | | |
| FBG (mg/dL) | 109.95±7.50 | 108.747–111.16 |
| PPBG (mg/dL) | 165.56±21.55 | 162.097–169.029 |
| Total cholesterol (mg/dL) | 147.23±34.98 | 141.613–152.864 |
| Triglycerides (mg/dL) | 176.44±27.39 | 172.039–180.847 |
| HDL-c (mg/dL) | 28.70±7.21 | 27.542–29.861 |
| LDL-c (mg/dL) | 165.89±17.96 | 162.993–168.768 |
| VLDL-c (mg/dL) | 31.61±23.63 | 27.793–35.42 |

* WC – waist circumference, BMI – body mass index, FBG – fasting blood glucose, PPBG – post-prandial blood glucose, HDL-c – high density lipoprotein cholesterol, LDL-c – low-density lipoprotein cholesterol, VLDL-c – very low-density lipoprotein cholesterol, CI – Confidence interval, SD – standard deviation

**Table 2.** Cardiac biomarkers among the study participants (n=150)*

| Variables | mean±SD | 95% CI |
|---|---|---|
| CPK NAC (U/l) | 430.32±257.6 | 188.402–326.790 |
| CK MB (IU/l) | 33±9.96 | 31.398–34.602 |
| Troponin I (ng/mL) | 258.58±243.48 | 201.909–285.066 |
| GGT (U/L) | 80.73±39.96 | 74.302–87.155 |
| AIP | 0.41±0.19 | 0.380–0.442 |
| GS score | 47.75±44.68 | 37.008–52.365 |

* AIP – atherogenic index of plasma, CPK NAC – creatine phosphokinase-N acetyl cysteine, CK MB – creatine kinase myocardial band, GGT – gamma glutamyl transferase, GS – Gensini severity score, CI – confidence interval, SD – standard deviation

Among study participants, 82 (54.7%) patients had STEMI, 36 patients (24%) had NSTEMI, and 32 (21.3%) had unstable angina. The gender-wise distribution of CAD was assessed and found a male preponderance, where 36.7% of the male patients had STEMI, 18% and 13.3% of the patients had NSTEMI and unstable angina, respectively. CADs were compared with the baseline laboratory parameters and are presented in Table 3. BMI, triglycerides, HDL-c, and LDL-c were statistically significant for the development of CAD. The association between CAD types with cardiac biomarkers was done and found that CK-NAC, CK-MB, troponin I and GGT were statistically significant for the development of CAD. Furthermore, the AIP and GS score were also found to be statistically significant for the development of CAD (Table 4).

**Table 3.** Association of CAD types with anthropometric and laboratory parameters among study participants (n=150)*

| Variables | STEMI Mean±SD | NSTEMI Mean±SD | Unstable angina Mean±SD | F ratio, p |
|---|---|---|---|---|
| Age | 54.83±10.73 | 54.97±8.33 | 58.72±10.12 | 1.841, 0.162 |
| WC (cm) | 38.18±3.67 | 37.89±3.14 | 37.90±3.70 | 0.120, 0.887 |
| BMI (kg/m$^2$) | 32±3.93 | 30.72±4.13 | 33.21±3.47 | 3.858, 0.023 |
| **Laboratory parameters** | | | | |
| FBG (mg/dL) | 110.24±7.99 | 109.73±7.67 | 109.47±6.06 | 0.143, 0.867 |
| PPBG (mg/dL) | 166.04±22.44 | 166.24±22.59 | 163.53±18.25 | 0.179, 0.836 |
| CHO (mg/dL) | 148.72±38 | 150.18±29.68 | 140.03±32.47 | 0.883, 0.416 |
| TGL (mg/dL) | 176.89±27.58 | 168.38±21.55 | 184.62±30.9 | **3.129, 0.047** |
| HDL-c (mg/dL) | 27.31±6.83 | 34.32±6.81 | 25.75±4.87 | **19.203, <0.001** |
| LDL-c (mg/dL) | 167.89±16.22 | 159.45±22.14 | 168.15±15.43 | **3.229, 0.042** |
| VLDL-c (mg/dL) | 30.93±25.17 | 35.76±21.38 | 30.81±22.61 | 0.201, 0.818 |

* STEMI – ST elevated myocardial infarction, NSTEMI – non-ST elevated myocardial infarction, WC – waist circumference, BMI – body mass index, FBG – fasting blood glucose, PPBG – post-prandial blood glucose, CHO – total cholesterol, TGL – triglycerides, HDL-c – high density lipoprotein cholesterol, LDL-c – low-density lipoprotein cholesterol, VLDL-c – very low-density lipoprotein cholesterol, SD – standard deviation, ANOVA was performed

**Table 4.** Association of types of CAD with cardiac biomarkers among the study participants (n=150)*

| Variables | STEMI Mean±SD | NSTEMI Mean±SD | Unstable angina Mean±SD | F ratio, p |
|---|---|---|---|---|
| CPK NAC (U/l) | 402.70±325.13 | 607.65±253.51 | 89.25±45.94 | 3.57, 0.030 |
| CK MB (IU/l) | 53.69±48.04 | 82.08±76.31 | 92.19±61.07 | 4.41, 0.014 |
| Troponin I (ng/mL) | 181.68±79.17 | 63.48±36.90 | 8.49±4.87 | 109.52, <0.001 |
| GGT (U/L) | 139.98±26.85 | 64.40±12.31 | 37.09±13.36 | 290.45, <0.001 |
| AIP | 0.38±0.18 | 0.411±0.18 | 0.49±0.28 | 3.610, 0.029 |
| GS score | 51.92±45.98 | 51.31±46.75 | 43.07±23.75 | 4.225, 0.016 |

* AIP – atherogenic index of plasma, GS – Gensini severity score, STEMI – ST elevated myocardial infarction, NSTEMI – non-ST elevated myocardial infarction, CPK NAC – creatine phosphokinase-N acetyl cysteine, CK MB – creatine kinase myocardial band, GGT – gamma glutamyl transferase, SD – standard deviation, ANOVA was performed

The mean GS score was performed for patients with CAD types and resulted that in patients with STEMI. The mean score was 51.92±45.98, for patients with NSTEMI and unstable angina was 51.31±46.75 and 43.07±23.75, respectively. They were statistically significant (F=4.225, p= 0.016) among the three groups, implying that the GS score was varied between the groups. In STEMI group, it was high and followed by in NSTEMI and unstable angina groups (Fig. 1).

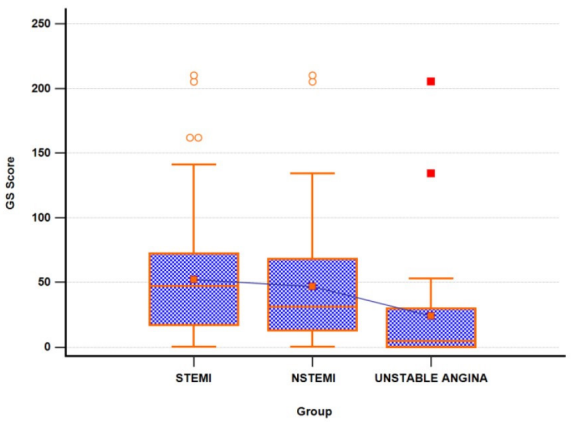


**Fig. 1.** Comparison of angiographic severity using GS score between three groups (in STEMI group, the GS score was high, followed by NSTEMI and unstable angina groups)

The GGT was compared with the GS Score to determine the correlation between the GGT and the severity of CAD with GS score and scatterplot was made (Fig. 2). The present study observed that GGT and GS score had positive correlation with each other. Therefore, when the GGT increases, the GS Score also increased. This correlation was statistically significant (r=0.168, p=0.0387).

## Discussion

In this present study, the correlation between serum GGT and angiographic severity in patients with CAD

was performed in 150 patients and found that GGT was positively correlated with the GS score, implying that increasing GGT will subsequently increase the angiographic severity among the patients with CAD.

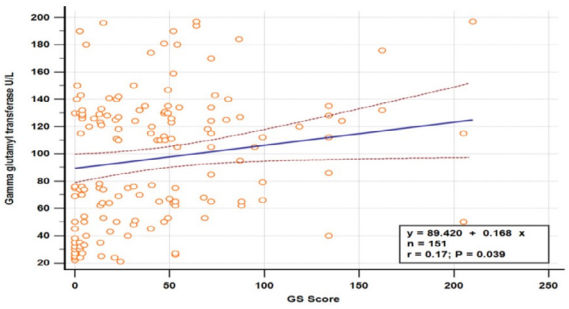


**Fig. 2.** Scatter plot for comparison of the GGT and GS score (GGT and GS score were positively correlated with each other)

The mean age of patients with STEMI at the time of presentation was 54.83±10.73 years in this present study, which is consistent with the findings of the CREATE registry, and another study from the southern Indian state of Tamil Nadu.[28,29] Male preponderance was detected in all age groups of patients with STEMI, and the sex ratios observed in both the younger and older age groups in our study were comparable to the study conducted by Holay et al.[30]

GGT is a serum and cell surface enzyme that contributes to G-SH equilibrium, a vital component of the body's defense against free radicals. Once GGT hydrolyzes G-SH extracellularly, glycine and cysteine are generated.[31,32] The synthesis of G-SH, the main antioxidant involved in the defense against oxidative stressors, is facilitated by the amino acids that are subsequently transported into the cell.[21] On the other hand, the extracellular cysteine-glycine combination stops $Fe^{3+}$ from being reduced to redox active $Fe^{2+}$.[31–33] This process results in the formation of peroxide, oxidized LDL, and free oxygen radicals. Thus, more oxidized LDL receptors are present on the cell surface as a result of increased oxidative stress, which facilitates the entry of LDL/GGT complexes into the plaque.[24,31,33] The development and progression of atheromatous plaque in the arteries are caused by these processes.[11,21,24,31,32] The idea that GGT directly contributes to the development of atherosclerosis is supported by research results that show GGT activity within the atherosclerotic plaque.[31]

According to the findings of Kittleson et al., increased GGT activity, even when it is within the normal range, is related to increased oxidative stress.[34] In our study, we found that patients with CAD presented with higher GGT. Many studies established that GGT is associated with cardiovascular disease in the form of atherosclerosis development and its degree of CAD and consistent with our study findings.[35,36] Among them, STEMI patients had higher levels of GGT, compared to NSTEMI and unstable angina, which was similar to the study by Breitling et al.,[14] Kunutsor et al.[37] Various studies demonstrated that patients with STEMI have elevated GGT and considered as an independent predictor of premature mortality.[13,17,35,36,38] Thus, from all these studies, it appears that GGT levels are associated with not only the development of atherosclerosis, but also with the development of CAD where the results of the current investigation were also consistent with the findings of the previous study.

In our study, we found that serum GGT, HDL-c, LDL-c and triglycerides were statistically significant and associated with CAD. Our findings were consistent with the study by Aksakal et al., where the serum GGT, diabetes, HDL-c, eGFR, and ejection fraction were all independent predictors of a high SYNTAX score.[15] Also, study done by Mao et al., among the Chinese population, showed that serum GGT and CAD had a favorable relationship indicating that GGT is a novel biomarker for CAD.[39]

From our study, we found a statistically significant correlation between the serum GGT and atherogenic severity in CAD patients. When comparing patients with <50% blockage in their coronary arteries with healthy controls and patients with <50% obstruction, the level of GGT in patients with obstruction was higher.35 Similarly, study done by Sheikh et al. showed that association was present between serum GGT and CAD.[12] Also, the study showed that every 10 unit rise in serum GGT was found to be robust predictor of existence of the early CAD.[12]

In a prospective cohort study, Hartopo et al., evaluated the link between AIP value and significant adverse cardiovascular events in patients with acute MI who were admitted to critical care throughout their stay, which was in line with the present study.[40] In the study it has found that a low AIP value, as opposed to a high AIP value, was an independent predictor of all-cause death in patients with acute MI who were receiving intensive hospitalization.[40]

The major strength of this study is that we assessed serum GGT as the novel biomarker along with the evidence of AIP and GS score. Additionally, the detailed categorization of cardiovascular conditions including STEMI, NSTEMI, and unstable angina enables a nuanced understanding of each subgroup. Furthermore, the study uses a broad range of clinical and biochemical parameters, including CPKMB, CPKNAC, troponin I, and GGT, facilitating a detailed investigation of cardiac health.

Every study always presents with the limitations. The main limitation of the study includes the smaller sample size. Being a cross-sectional study, the temporality of the association could not be assessed. Also, this was a single-centric hospital study, in which the results cannot be extrapolated to the general population. Lastly, potential confounding factors, such as medication use

and lifestyle factors, were not extensively controlled or discussed, which could affect the study's results.

## Conclusion

Therefore, in our study, an elevated serum GGT is correlated with the angiographic severity of CAD assessed by the GS score. Stronger associations were observed in patients with STEMI. Similarly, we found a positive correlation between the serum GGT and GS score and significant statistically.

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## Declarations

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***Author contributions***

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

***Conflicts of interest***

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

***Data availability***

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

***Ethics approval***

This study was approved by the Institutional Ethical Committee (MGMCRI/Res/01/2019/33/IHEC/104).

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**ORIGINAL PAPER**

# Assessing the impact of sleeve gastrectomy on micronutrient levels and inflammatory markers – a case-control study

Abbas Mosad Ajeed [1], Nabaa Hassan Naif [2], Aliaa Hashim Farag [3], Osama A. Mohsein [4,5]

[1] Al-Kunooze University College, Faculty of Pharmacy, Basra, Iraq
[2] Department of Biochemistry, Faculty of Medicine, University of Kerbala, Kerbala, Iraq
[3] Basic Sciences, Dentistry College, Uruk Private University, Baghdad, Iraq
[4] Department of Medical Laboratory Techniques, Mazaya University College, Thi-Qar, Iraq
[5] Thi-Qar Health Directorate, Al Habbobi Teaching Hospital, Thi-Qar, Iraq

**ABSTRACT**

**Introduction and aim.** Sleeve gastrectomy has become one of the most common surgical procedures in the world recently, due to its role in promoting weight loss and reducing the risk of obesity-related diseases. This study aims to determine the levels of inflammatory and nutritional factors in patients undergoing gastric sleeve surgery.

**Material and methods.** A case-control study was conducted including 150 patients who underwent gastric sleeve surgery and 50 healthy participants as a control group. Inclusion criteria included patients aged 45–65 years with a BMI ≥35 kg/m$^2$. Levels of interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), adiponectin, interferon-gamma, IL-10, IL-1β, monocyte chemoattractant protein-1, fasting blood glucose (FBG), serum amyloid A, iron, ferritin, calcium, and vitamin D3 were measured using enzyme-linked immunosorbent assay.

**Results.** The study revealed that the control group (22.8±3.6 kg/m$^2$) had a significantly reduced BMI compared to the patients who underwent sleeve gastrectomy (35.5±7.1 kg/m$^2$, ($p<0.001$). Elevated levels of C-reactive protein, IL-6, TNF-α, and FBG were seen in the postoperative group, although adiponectin levels were dramatically reduced ($p<0.001$). Furthermore, the postoperative patients manifested significantly reduced levels of iron, calcium, and vitamin D3, suggesting a profound insufficiency in these vital nutrients and their possible consequences on their long-term well-being.

**Conclusion.** The study results indicate that patients who underwent gastric sleeve surgery had significantly lower levels of iron, ferritin, calcium, and vitamin D3, compared to the control group. This is due to the effect of surgery on the absorption of nutrients, which causes a deficiency in vitamins and minerals necessary for bone and body health.

**Keywords.** nutrient absorption, sleeve gastrectomy, vitamin D3 deficiency

## Introduction

The condition of obesity is defined by an abnormal buildup of adipose tissue. The major constituents of many disorders linked to obesity are metabolic abnormalities and chronic inflammation caused by the buildup of surplus fat.[1] Obesity has reached epidemic proportions worldwide, accounting for more than 1.9 billion overweight and approximately 650 million obese adults. There is greater morbidity and mortality in patients with severe obesity, especially classes II body mass index (BMI) 35 to 39.9

**Corresponding author:** Osama A. Mohsein, e-mail: osamaakram889@gmail.com

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kg/m$^2$) and III (BMI greater than 40 kg/m$^2$). In addition, obesity is a recognized risk factor for the development of comorbid conditions such as cardiovascular disease, type 2 diabetes mellitus (T2DM), malignancy, asthma, osteoarthritis, chronic back pain, obstructive sleep apnea, non-alcoholic fatty liver disease, and gallbladder diseases.[2] Bariatric surgery (BS), sometimes referred to as metabolic surgery, is advised for the treatment of obesity in persons who meet the specified criteria: individuals diagnosed with T2DM and having a BMI equal to or above 30 kg/m$^2$; those with a BMI ranging from 30 to 34.9 kg/m$^2$ who have not seen substantial weight loss or improvements in other health conditions after thoroughly exploring all non-surgical alternatives; and individuals with a BMI above 35 kg/m$^2$, irrespective of the severity of their other health conditions. Through the adoption of a basic dietary strategy (BS), overweight or obese persons can reduce the negative consequences of T2DM, high blood pressure, dyslipidemia, and non-alcoholic fatty liver disease. Indeed, there are benefits to BS that extend beyond mere reduction of body weight.[3,4] Bariatric surgery has been associated with a temporary increase in inflammatory markers as part of the immune system's response to weight loss and tissue remodeling. Empirical evidence highlights this phenomenon, with elevations observed in matrix metallopeptidase-9 (MMP-9), C-reactive protein (CRP), and proteins associated with monocyte activation. Additionally, the expression of Toll-like receptors TLR-2 and TLR-4, which recognize Gram-positive bacterial residues and endotoxins, is also affected. Nuclear factor kappa-β (NF-κB) activation is stimulated, leading to its translocation to the nucleus. Notably, cluster of differentiation (CD14) expression shows an increase following bariatric surgery, reflecting the body's adaptive inflammatory response during the post-surgical phase.[5,6] Prior studies have demonstrated that the effects of BS often lead to significant improvements within the first year, particularly in enhancing insulin sensitivity. However, inflammatory markers may initially increase following surgery due to the body's immune response to tissue injury and the metabolic stress associated with rapid weight loss. This transient rise reflects the activation of pathways involved in wound healing and tissue remodeling. In individuals with T2DM, a notable decrease in inflammatory markers and an increase in insulin sensitivity are observed over time after sleeve gastrectomy, as the inflammatory response stabilizes and metabolic improvements take effect.[7,8] Furthermore, individuals may experience a reduction in their food intake following surgery due to the following factors. The pre-meal cognitive and sensory signals that induce hunger, weight gain concerns, abdominal pain, nausea, vomiting, difficulty swallowing due to texture, and satiety have all been associated with reduced eating in individuals with Binge Eating Syndrome. Flavor exerts a direct influence on dietary patterns by altering individuals' food preferences and portion sizes. Modifications in dietary patterns following surgery might be attributed to alterations in gut-derived compounds such as hormones, nutrients, bile acids, bacteria, and neuronal signals that affect the brain's balance and pleasure-related regions.[9,10] Alterations in hormones can also influence individuals' dietary choices. This may result in feelings of anxiousness and fluctuations in mood. There exists a correlation between anxiety and excessive indulgence due to insufficient self-restraint levels. The examination of the Dutch Eating Behavior Questionnaire (DEBQ) reveals that individuals who engage in more uncontrolled or emotional eating experience greater difficulty in achieving weight loss following surgery. To the best of our understanding, no research has examined the impact of BS on individuals' moods and dietary patterns in Saudi Arabia.[11,12] prior research has demonstrated that laparoscopic sleeve gastrectomy (LSG) is a highly efficacious approach for managing morbid obesity. Although it is crucial to achieve weight loss and reduce the number of illnesses simultaneously after BS, these therapies also include difficulties and nutritional hazards. Both Roux-en-Y gastric bypass and LSG models have a restricted and inefficient capacity to absorb nutrients, potentially resulting in famine.[13,14]

## Aim

The study aims to determine the levels of inflammatory and nutritional factors in patients undergoing gastric sleeve surgery.

## Material and methods

### *Study design and setting*

Case-control study conducted at Nasiriyah General Hospital and Al-Habbobi Teaching Hospital during the period from 1/1/2023 to 1/8/2024.

### *Participants*

Between 2023 and 2024, 150 patients had Sleeve Gastrectomy procedures. The control group comprised 50 healthy individuals with a normal BMI of 18.5 to 24.9 kg/m$^2$. The inclusion criteria were male and female patients aged 45 to 65 years with a BMI of 35 kg/m$^2$ or more who had a sleeve gastrectomy procedure between 2023 and 2024 and were monitored for one year. Excluded from consideration were pregnant women, patients who had already undergone bowel surgery, and those who had received a diagnosis of psychological illnesses. An 80% response rate was achieved in the trial. Within the original sample of 300 patients who had sleeve gastrectomy between 2023 and 2024, 50 records were removed because of duplications, while an additional 50 patients were excluded because of insufficient data or complications necessitating surgical intervention.

***Blood sampling and measurement***

10 mL of blood was drawn from each participant and placed in a gel tube and left at room temperature for 15 minutes until clotting. Serum was separated using a centrifuge at 3500 rpm for 15 minutes and the serum was isolated under sterile conditions and stored at -20°C until use. Levels of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), adiponectin (Adp), interferon-gamma (IFN-γ), IL-10, IL-1β and monocyte chemoattractant protein-1 (MCP-1) were measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations (Bio-Techne, USA, catalog numbers: D6050, DTA00C, DY1065, DIF50, D1000B, DY201, and DCP00 respectively. Levels of fasting blood glucose (FBG), serum amyloid A (SAA), iron, ferritin, calcium and vitamin D3 were measured using a Cobas E4 11 device according to the manufacturer's recommendations (Roche, German).

***Statistical analysis***

Statistical analysis is often used to analyze quantitative data and provides methods for data description and simple inference for continuous and categorical data. The technique involves collecting information to assess the link between two statistical data sets. This study gives all data as the mean accompanied with standard deviation. Statistical analyses for variables with a normal distribution were performed using SPSS (version 26, IBM, Armonk, NY, USA), employing both the dependent t-test (two-tailed) and the independent t-test (two-tailed). The Mann-Whitney U test and the Wilcoxon test are utilized for variables exhibiting non-normal distribution. Statistical significance was defined as $p<0.05$.

***Ethical approval***

All patients participating in this study were fully informed about its purpose and procedures, and their verbal consent was obtained prior to sample collection. The study was conducted in accordance with ethical guidelines and was approved by the Committee on Publication Ethics at the Thi-Qar Health Directorate, Al-Habboubi Teaching Hospital, No 3324 in January 1, 2023.. However, we acknowledge the potential limitations of relying solely on verbal consent and recognize the importance of ensuring comprehensive ethical compliance in future studies.

## Results

***Comparison of sociodemographic characteristics between gastric sleeve patients and healthy controls***

The study results showed that the mean age among gastric bypass patients was 45.2±10.4 years, compared to 44.6±9.8 years in the control group, and there was no statistically significant difference between the two groups (p=0.75). The gender distribution was equal between males and females in both the patient group and the control group (p>0.999). However, the BMI was significantly higher in gastric bypass patients (35.5±7.1) compared to the control group (22.8±3.6) (p<0.001). The percentage of smokers among gastric bypass patients was 40% compared to 30% in the control group, but this difference was not statistically significant (p=0.15). In addition, the prevalence of diabetes was significantly higher among gastric bypass patients (60%) compared to 10% in the control group, with a statistically significant difference (p<0.001) as shown in Table 1.

**Table 1.** Sociodemographic characteristics of study participants

| Variable | Patients (n=150) | Controls (n=50) | p |
|---|---|---|---|
| Age, years (mean±SD) | 45.2±10.4 | 44.6±9.8 | 0.75 |
| Gender (male/female) | 75/75 | 25/25 | >0.999 |
| BMI, kg/m$^2$ (mean±SD) | 35.5±7.1 | 22.8±3.6 | <0.001 |
| Smoking (%) | 40% | 30% | 0.15 |
| Diabetes mellitus (%) | 60% | 10% | <0.001 |

***Inflammatory biomarker levels in gastric sleeve patients compared to healthy controls***

The results of the study showed that CRP levels were significantly higher in gastric bypass patients after surgery (8.5±2.4 mg/L) compared to the control group (2.1±1.2 mg/L), with a statistically significant difference (p<0.001). IL-6 levels were significantly higher in gastric bypass patients (15.3±5.8 pg/mL) compared to the control group (5.2±2.0 pg/mL) with a statistically significant difference (p<0.001). In addition, TNF-α levels were significantly higher in gastric bypass patients (18.7±6.1 pg/mL) compared to the control group (7.3±3.4 pg/mL) with a statistically significant difference (p<0.001) as shown in Table 2.

**Table 2.** Analysis of CRP, IL-6, and TNF-α levels post-surgery

| Group | CRP, mg/L (mean±SD) | p |
|---|---|---|
| Post-surgery patients | 8.5±2.4 | <0.001 |
| Controls | 2.1±1.2 | |
| **Group** | **IL-6, pg/mL (mean±SD)** | **p** |
| Post-surgery patients | 15.3±5.8 | <0.001 |
| Controls | 5.2±2.0 | |
| **Group** | **TNF-α, pg/mL (mean±SD)** | **p** |
| Post-surgery patients | 18.7±6.1 | <0.001 |
| Controls | 7.3±3.4 | |

***Metabolic and inflammatory marker analysis in gastric sleeve patients versus healthy controls***

Following surgery, individuals who underwent a gastric bypass presented with markedly elevated FBG levels (112.5±28.3 mg/dL) compared to those in the control group (89.4±14.6 mg/dL). The observed disparity was statistically significant (p<0.001). A substantial statistical difference (p<0.001) was seen in the levels of adi-

ponectin between gastric bypass patients (5.9±2.7 μg/mL) and the control group (12.3±4.1 μg/mL). Furthermore, those who underwent gastric bypass surgery had notably elevated SAA levels (36.7±12.1 mg/L) in comparison to the control group (18.2±7.9 mg/L), and this difference was statistically significant (p<0.001) as shown in Table 3.

**Table 3.** Evaluation of fasting blood glucose, adiponectin, and serum amyloid A levels gastric sleeve patients versus healthy controls

| Group | FBG, mg/dL (mean±SD) | p |
|---|---|---|
| Post-surgery patients | 112.5±28.3 | <0.001 |
| Controls | 89.4±14.6 | |
| **Group** | **Adiponectin, μg/mL (mean±SD)** | **p** |
| Post-surgery patients | 5.9±2.7 | <0.001 |
| Controls | 12.3±4.1 | |
| **Group** | **SAA, mg/L (mean±SD)** | **p** |
| Post-surgery patients | 36.7±12.1 | <0.001 |
| Controls | 18.2±7.9 | |

### *Cytokine and chemokine profiles in gastric sleeve patients compared to healthy controls*

A statistically significant difference was seen in IFN-γ levels between gastric bypass patients after surgery (22.5±8.9 pg/mL) and the control group (10.8±4.3 pg/mL) with a significance level of p<0.001. A statistically significant difference (p<0.001) was seen in the levels of IL-10 between the gastric bypass patients (7.2±2.9 pg/mL) and the control group (3.5±1.8 pg/mL). Significantly higher IL-1β levels were seen in gastric bypass patients (14.9±5.7 pg/mL) compared to the control group (6.4±3.1 pg/mL), with a statistically significant difference (p<0.001). The levels of MCP-1 were markedly elevated in patients who underwent gastric bypass (265.4±92.6 pg/mL) compared to the control group (145.3±58.2 pg/mL), creating a statistically significant disparity (p<0.001) as shown in Table 4.

**Table 4.** Assessment of IFN-γ, IL-10, IL-1β, and MCP-1 levels profiles in gastric sleeve patients compared to healthy controls

| Group | IFN-γ, pg/mL (mean±SD) | p |
|---|---|---|
| Post-surgery patients | 22.5±8.9 | <0.001 |
| Controls | 10.8±4.3 | |
| **Group** | **IL-10, pg/mL (mean±SD)** | **p** |
| Post-surgery patients | 7.2±2.9 | <0.001 |
| Controls | 3.5±1.8 | |
| **Group** | **IL-1β, pg/mL (mean±SD)** | **p** |
| Post-surgery patients | 14.9±5.7 | <0.001 |
| Controls | 6.4±3.1 | |
| **Group** | **MCP-1, pg/mL (mean±SD)** | **p** |
| Post-surgery patients | 265.4±92.6 | <0.001 |
| Controls | 145.3±58.2 | |

### *Iron and ferritin levels in gastric sleeve patients versus healthy controls*

The study showed a significant decrease in iron levels in gastric bypass patients after surgery (55.2±15.3 μg/dL) compared to the control group (85.7±20.4 μg/dL), with a statistically significant difference (p<0.001). Ferritin levels, an indicator of body iron stores, were also significantly lower in gastric bypass patients (22.5±8.6 ng/mL) compared to the control group (55.3±12.7 ng/mL) with a significant difference (p<0.001). These results indicate a significant decrease in iron stores in patients after gastric bypass surgery as shown in Table 5.

**Table 5.** Serum iron and serum ferritin levels in gastric sleeve patients versus healthy controls

| Group | Iron, μg/dL (mean±SD) | p |
|---|---|---|
| Post-surgery patients | 55.2±15.3 | <0.001 |
| Controls | 85.7±20.4 | |
| **Group** | **Ferritin, ng/mL (mean±SD)** | **p** |
| Post-surgery patients | 22.5±8.6 | <0.001 |
| Controls | 55.3±12.7 | |

### *Calcium and vitamin D3 levels in gastric sleeve patients compared to healthy controls*

The study results showed a significant decrease in calcium levels in gastric bypass patients after surgery (8.4±0.9 mg/dL) compared to the control group (9.5±0.7 mg/dL), with a statistically significant difference (p<0.001). Vitamin D3 levels were also significantly lower in patients after surgery (18.6±7.3 ng/mL) compared to the control group (30.2±10.5 ng/mL), with a significant difference (p<0.001). These results indicate a significant deficiency in both calcium and vitamin D3 in patients after gastric bypass surgery, which may affect their bone and psychological health in the long term as shown in Table 6.

**Table 6.** Post-surgery nutritional deficiencies in calcium and vitamin D3

| Group | Calcium, mg/dL (Mean±SD) | p |
|---|---|---|
| Post-surgery patients | 8.4±0.9 | <0.001 |
| Controls | 9.5±0.7 | |
| **Group** | **Vitamin D3, ng/mL (Mean±SD)** | **p** |
| Post-surgery patients | 18.6±7.3 | <0.001 |
| Controls | 30.2±10.5 | |

## Discussion

BS is widely considered the most efficacious method for weight loss. The weight loss during BS can be ascribed to several variables, including as hormonal fluctuations, reduced food intake, reduced absorptive capacity and gastrointestinal secretions, and adverse effects associated with operations, including food aversion and frequent vomiting. The above provided data demonstrates a significant disparity in BMI, smoking habits, and diabetes

mellitus between patients with gastric sleeve and healthy individuals. These findings align with other studies.[15-17] In order to achieve their long-term weight and health objectives, individuals undergoing gastrectomy must meticulously adhere to their dietary and lifestyle recommendations. Varying degrees of compliance with the dietary recommendations provided by the nutritionists to the participants of the study were observed. Furthermore, the participants shown a strong will to avoid calorie-dense items by strictly adhering to the instructions. Adopting such practice will significantly impact their overall health and body weight.[18] An underlying factor contributing to the unexpectedly high degree of treatment adherence is the relatively brief duration following the surgery, which lasted only 12 months. Given the current knowledge, patients are highly inclined to adhere to the guidelines about their diet and lifestyle both prior to and immediately following surgery. Nevertheless, the level of devotion diminishes with time.[19] Furthermore, studies have demonstrated that about one to two years following surgery, individuals typically exhibit reduced eating habits, lower hunger levels, and reduced mental health issues. Additionally, adherence to a diet, use of supplements, and overall improvement in overall well-being tend to improve.[20] The data presented in Table 2 highlights a significant elevation in CRP levels among post-surgery patients (8.5±2.4 mg/L) compared to the control group (2.1±1.2 mg/L), with a $p<0.001$, indicating statistical significance. These findings align with studies documenting an initial surge in inflammatory markers following bariatric surgery. For example, Radi S et al. A similar pattern was observed by Hentilä et al. who attributed the increased CRP levels to an acute-phase inflammatory response of the body as a consequence of transition to tissue injury and metabolic stress in the postoperative recovery phase. In contrast, long-term studies, exemplified by the work of Randell et al., noted a progressive decline in CRP levels at six months to one year after surgery, which was associated with reduced adiposity and systemic inflammation. This seems to indicate that though there is an acute inflammatory response from bariatric surgery, the long-term weight loss and metabolic benefits outweigh any adverse effects. Other researchers noted that the difference in short- and long-term studies is possibly due to the timing in tissue repair and the effect of other external factors like diet and stress. For the impact of inflammatory markers after bariatric surgery, this highlights the need to examine both short and longer-term outcomes.[21,22] The analysis shows significantly higher IL-6 levels in post-surgery patients (15.3±5.8 pg/mL) compared to controls (5.2±2.0 pg/mL, $p<0.001$), consistent with studies like Smidowicz et al., that attribute this rise to the acute-phase response and tissue repair following surgery. However, long-term studies, such as Ko et al., report decreased IL-6 levels six to twelve months post-surgery, correlating with reduced visceral fat and systemic inflammation. This suggests that while IL-6 elevation is a natural post-operative response, it is transient and decreases as metabolic health improves over time.[23,24] Alterations in diet can also modify the composition of gut microbiota and the equilibrium between pro-inflammatory and anti-inflammatory gut microbiota groups, therefore exacerbating inflammation. The potential impact of BS on the gastrointestinal axis may result in reduced consumption of fruits and vegetables among our participants, therefore influencing their overall nutritional intake.[25,26] Patients who underwent gastrointestinal surgery exhibited elevated levels of TNF-α and SAA, as well as increased levels of IFN-γ, IL-10, IL-1, and MCP-1, in comparison to healthy controls.[27] Post-surgery patients had significant nutritional deficiencies of calcium and vitamin D3 levels ($p<0.05$) comparing to controls, as demonstrated in Table 6. Calcium levels post-surgery were significantly lower than in controls (8.4±0.9 mg/dL vs. 9.5±0.7 mg/dL, $p<0.001$). Likewise, vitamin D3 status was markedly poorer in patients post-surgery (18.6±7.3 ng/mL) than in controls (30.2±10.5 ng/mL, $p<0.001$). This is in accordance with studies like Steenackers et al. which stated that bariatric surgery may result in malabsorption or altered nutrient metabolism due to decreased intestinal surface area and dietary restrictions. Prolonged deficiency in these areas can lead to effects on the bones as well as immune function, making it very important to evaluate and assess with regularity whether supplementation and/or post-surgical nutrition is appropriate.[28] That study also noted a marked increase in calcium at the 1- and 12-month follow-ups for the LSG group, which mirrors the findings of our study. The effect may be due to the activation of bone remodeling during BS, which may alter calcium homeostasis. The increase in participants can be attributed to the fact most people now adhere upper to guideline vitamin and mineral supplementation, along with supplementation with daily 600 mg calcium carbonate tablets.[29] Moreover, our results provided evidence that the concentration of plasma levels of vitamin D were significantly higher, in comparison to pre-operative levels, at the 3- and 12-months postoperative time-points. Preoperative vitamin D deficit and insufficiency were highly prevalent during the trials with levels from 33.1 (23.9) nmol/L at recruitment to 57.1 (23.1) nmol/L 12 months after the operation when they were regularly supplemented. It is advised to take a daily supplement of vitamin D3 to prevent vitamin D deficiency post- SG, which can be around 2000 to 4000 IU. During a period of just eight weeks, every single one of the patients in our investigation with either a deficiency or insufficiency of vitamin D was given 50,000 IU of vitamin D3 dietary supplements.[30,31] Data indicates dramatic drops in iron and ferritin levels for

patients after surgery, relative to controls. Iron levels were significantly decreased after surgery (55.2±15.3 μg/dL) compared to controls (85.7±20.4 μg/dL, p<0.001). Ferritin levels were also found to be significantly lower in post-surgery patients (22.5±8.6 ng/mL) when compared to controls (55.3±12.7 ng/mL, p<0.001). Similar studies including Ahmed et al. and Gudzune et al., who linked impaired iron absorption after bariatric surgery to low gastric acid production and altered gastrointestinal anatomy. This suggests that post-surgery patients need to receive complementary nutritional assessment and supplementation on a regular basis to prevent anemia, and its related health problems.[32,33] Visceral fat presents a susceptibility to insulin resistance, and gastric bypass results in early and also significant decrease in body weight. In addition, it shrinks this tissue. The short-term increase in serum adiponectin levels can be attributed to the reduction in visceral fat mass occurring post-surgery, an organ that produces low levels of adiponectin. But then, as the loss of weight progresses and the body adjusts to the fresh state of affairs, blood adiponectin levels may start to diminish smoothly as part of a new homeostasis of fat metabolism. This reduction could also be attributed to variations in insulin sensitivity and the improved inflammatory status of the organism in the post-surgery period.[34] The data show major changes in adiponectin and serum amyloid A (SAA) levels after surgery as compared to controls. Post-surgery patients had significantly decreased adiponectin levels (5.9±2.7 μg/mL) compared to controls (12.3±4.1 μg/mL, p<0.001), whilst SAA levels were significantly higher in post-surgery patients (36.7±12.1 mg/L) compared to controls (18.2±7.9 mg/L, p<0.001). These results align with studies like Barron et al. and Stoica et al. to indicate that the decrease of adiponectin can be explained by the acute metabolic alterations and transient inflammation that occur after surgery. In contrast, higher levels of SAA demonstrate an inflammatory acute-phase response, found most frequently in post-surgical settings. This reflects the complex relationship between inflammation and metabolic adaptations that occurs in the post-operative recovery phase.[34,35] After gastric surgery, the vitamin deficiencies are well documented. Generally, BS-induced signs of hunger are represented by protein-energy imbalances and deficiencies of micronutrients including cobalamin, folate, calcium, iron, transferrin, and fat-soluble vitamins. Because bipolar disorder decreases gastric acid, it also reduces the bioavailability of iron. This situation is further complicated by the patient's insufficient vitamin intake.[36] Thus, compliance with the dietary recommendations given after surgery to avoid deficits and maximize the chance for the success immediately is paramount. Below are thorough guidelines for determining a diet, food selection, supplementation, nutrition therapy, and treatment of common GI disorders.[37] Iron deficiency can occur due to decreased absorption hence the need for supplementation to prevent nutritional deficiencies. To reduce the risk of iron deficiency post-operatively, all of our patients were given oral ferrous sulphate in a 190 mg dose every 12 hours. According to Lefebvre et al., the transferrin IBC ratio was 62.7 at baseline and decreased to 61.8 after 12 months. Moreover, soybean meal seems to be the initial feed component with the highest concentration of factors that were considered detrimental to Fe absorption, with a previous study showing an initial prevalence of iron insufficiency in serum at 29.8% decreasing to 15.6% after a year 10.[38,39] The data in this table reflects major changes in some of the main inflammatory responses after bariatric surgery. In particular, higher expression levels of IFN-γ, IL-10, IL-1β and MCP-1 were found in post-surgery patients compared to the control group, maintaining an inflammatory response after surgery, which might be related to the physiological changes that occur after surgery. Post-surgery patients had significantly higher levels of IFN-γ (22.5±8.9 pg/mL) than control patients (10.8±4.3 pg/mL, p < 0.001). IFN-γ: a cytokine central to the Th1 immune response, elevated in inflammatory conditions and during infection. This rise in IFN-γ may also be a reflection of the acute-phase inflammatory response induced by surgery.[40] Anti-inflammatory cytokine IL-10 was elevated in the post-surgery patients (7.2±2.9 pg/mL) compared with the controls (3.5±1.8 pg/mL, p<0.001). Although IL-10 is mainly involved in limiting inflammatory responses to prevent immune-mediated damage to tissues, the increase in IL-10 that we observed post-surgery could potentially function as a mechanism to compensate for the increased inflammatory state triggered by surgery. Li et al. also showed a comparable response, suggesting that the acute inflammation immediately following surgery is paired with an increase in IL-10, which might represent a regulatory response aimed at dampening inflammation and preventing chronic damage.[41] Moreover, significantly elevated concentrations of IL-1β (14.9±5.7 pg/mL in postsurgery patients, vs 6.4±3.1 pg/mL in controls, p<0.001) is consistent with this premise. IL-1β is proinflammatory cytokine and its increase after surgery correlates with results of Trahtemberg et al., this coincides with increased levels of IL-1β early post-operatively as a result of tissue damage and follow-up inflammation. The increased IL-1β is linked with many metabolic and immune changes that may includ insulin resistance which can have implications for post-injuncture metabolic health.[42] MCP-1 (265.4±92.6 pg/mL in post-surgery patients vs. 145.3±58.2 pg/mL in controls, p<0.001) was significantly upregulated, as has also been reported by Salman et al. who reported that MCP-1 levels are increased by bariat-

ric surgery. MCP-1 is important in recruiting monocytes to sites of inflammation and its elevation indicates ongoing processes of inflammation. This increase may participate in adipose tissue remodeling and may be involved in the reprogramming of the immune system during rapid weight loss.[43] Additionally, some visceral fat may remain after surgery, which is an important source of these inflammatory proteins, contributing to their continued elevation. MCP-1 also plays a role in attracting immune cells to promote wound healing after surgery, while SAA is elevated as part of the inflammatory response and altered metabolism in the body during the recovery period. These changes are usually transient and levels gradually return to normal with full recovery.[44,45] A small number of people in the study showed a low level of emotional eating, but about 64.4% of them showed an average level of this behavior. Most of the people who took part (58.1%) displayed a low level of external eating, while 41.9% displayed a moderate level of this habit. Many research studies have shown that stress, grief, and disappointment have a big effect on both emotional and outward eating habits.[46] Questioned about whether they felt depressed or hopeless along with the urge to eat, 45% of the people who took part said "occasionally." Along with this, there is a strong link between higher DEBQ scores linked to emotional and external eating and weight loss plans not working as well.[47] A person's psychological eating habits may affect their ability to adapt to changes in their food after surgery, which can either help or hurt their weight loss efforts. Several factors affect how people eat after surgery, which is a complicated process.[48] Dietary changes after bariatric surgery depend on factors like gender, type 2 diabetes, genetics, and the specific type of surgery. It is also thought that the signaling routes between the gut and the brain affect how people eat differently after having gut surgery.[49]

## Conclusion

The study results indicate that gastric sleeve surgery results in a significant decrease in iron, ferritin, calcium, and vitamin D3 levels in patients compared to the control group. This may be explained by the effect of surgery on reducing the absorption of these important nutrients, which increases the risk of mineral and vitamin deficiencies essential for bone health and general body functions. Therefore, patients after surgery need close nutritional monitoring and nutritional supplements to compensate for this deficiency and avoid future health problems.

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## Declarations

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***Author contributions***

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

***Conflicts of interest***

There is no conflicts of interest.

***Data availability***

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

***Ethics approval***

The study was conducted in accordance with ethical guidelines and was approved by the Committee on Publication Ethics at the Thi-Qar Health Directorate, Al-Habboubi Teaching Hospital, No 3324 in January 1, 2023.. However, we acknowledge the potential limitations of relying solely on verbal consent and recognize the importance of ensuring comprehensive ethical compliance in future studies.

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**ORIGINAL PAPER**

# Evaluation of lipid profile, malondialdehyde, hemoglobin and ferritin in Iraqi women with polycystic ovarian syndrome

Ashwaq A. Shenta [1], Abbas D.M. Al-Maliki [1], Mufid K. Abou-Turab [2]

[1] Department of Chemistry, College of Education for Pure Sciences, University of Basrah, Basrah, Iraq
[2] Department of Biology, College of Education for Pure Sciences, University of Basrah, Basrah, Iraq

**ABSTRACT**

**Introduction and aim.** The concept of polycystic ovarian syndrome (PCOS) is defined as a biochemical complex statement that affects many young and adult females (single and married). This case presents a variety of medical and biological concerns related to the reproductive system. The aim of the study was to investigate and estimate the levels of the lipid profile, malondialdehyde, hemoglobin and ferritin in women with PCOS.

**Material and methods.** Blood samples were collected from 180 women who were divided into 100 PCOS patients and 80 healthy women according to the variables of age and body mass index. Triglycerides (TG) levels were measured in blood sera by spectrophotometric method, total cholesterol (TC) levels were estimated using enzymatic methods and the high-density lipoprotein (HDL) was determined by the HDL-phosphotungstic acid precipitation method.

**Results.** Total cholesterol, triglyceride, low-density lipoproteins, and very low-density lipoproteins recorded a highly significant increase ($p<0.001$) whereas high-density lipoproteins decreased significantly ($p<0.001$) in women with PCOS women when compared to the control group depending on age and body mass index variables. The results showed that hemoglobin, ferritin and malondialdehyde levels increased significantly ($p<0.001$) in female PCOS compared to the healthy group according to age and body mass index variables.

**Conclusion.** The importance of thorough medical management of PCOS includes minimizing oxidative stress, metabolic function, and lipid profiles for avoidance of chronic health conditions.

**Keywords.** age variable, body mass index, ferritin, lipid profile, polycystic ovarian syndrome

## Introduction

Polycystic ovarian syndrome (PCOS) is defined as a pathologically and biologically complex condition that takes place in the reproductive system belonging to women during their reproductive age. This syndrome leads to various biochemical disorders and clinical changes caused by many factors, such as physiological conditions and changes in the chemical systems of enzymes, hormones and vitamins.[1,2]

Many studies indicated that PCOS may increase the chance of developing certain diseases such as hypertension, diabetes, uterine cancer, and infertility. The severity of PCOS could also lead to alterations in the concentrations of enzymes, hormones, vitamins, uric acid, creatine, creatinine, malondialdehyde, lipids and trace elements.[3,4] Also, blood proteins such as hemoglobin, ferritin and albumin may differ in their levels in females affected by polycystic ovarian syndrome.[5]

**Corresponding author:** Ashwaq A. Shenta, e-mail: ashwaqshenta568@gmail.com

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Therefore, oxidative stress, which is influenced by the balance between oxidants and antioxidants in the body, can fluctuate in response to changes in female sex hormones. This imbalance contributes to various disorders in both the clinical and biochemical systems of a woman's reproductive health. On the other hand, genetic inheritance, pathogenesis, and insulin resistance are considered distinct biochemical mechanisms that help explain the clinical abnormalities associated with PCOS. For example, insulin resistance is often a contributing factor to obesity in many women with PCOS.[6,7]

Various factors have biochemical roles in altering the clinical, physiological, and biochemical aspects of this syndrome, including age, body mass index (BMI), marital status, smoking, blood group, family history, and infection of other diseases.[8] Numerous studies have been conducted on PCOS and its etiology. While these studies have shown the pathophysiological processes associated with PCOS, the underlying causes remain unclear and require further explanation. Continuous research and investigation of the oxidant-antioxidant balance associated with PCOS shows DNA damage and genomic disorders that occur in the living cell, especially in the mitochondria. Therefore, this biochemical and clinical evidence suggests a link between oxidative stress and reduced fertility.[9,10]

Recent advances in PCOS management of PCOS include an improved understanding of its pathophysiology, the use of letrozole for infertility, and the emphasis on lifestyle changes such as diet, exercise, and weight loss that can enhance metabolic health and reproductive results.[11] The most important achievements in PCOS include the formation of the 2018 International Evidence-Based PCOS Guideline and the establishment of an early career researcher network that will improve evidence synthesis and future research which both have made breakthroughs in the current understanding of the etiology and genetics of PCOS.[12]

There have been many controversies related to PCOS, such as its distinct nature, unclear etiology, differing diagnostic standards (eg, Rotterdam criteria vs. Androgen Excess Society), and the focus on treating symptoms rather than causes, all of which result in conflicting guidelines and individual experiences influencing medical behavior.[13]

Many different biochemical parameters are necessary in polycystic ovarian syndrome such as lipid profile, various hormones and enzymatic antioxidants, vitamins, urea, trace elements, malondialdehyde, C-reactive protein, and liver enzymes according to the severity of PCOS and their correlations with some variables such as age, blood group, marital status, and BMI.[14,15]

## Aim

Therefore, current research was performed to evaluate and investigate the levels of lipid profile, malondialdehyde (MDA), ferritin and hemoglobin in Iraqi female PCOS patients according to age and BMI.

## Material and methods

180 women were divided into two groups. The first contains 100 women affected by PCOS, and the second is composed of 80 healthy females. These females were also divided into three categories according to their age represented by the first (14 to 24 years), the second (25 to 35 years) and the third (36 to 45 years). These women were also split into three groups according to their BMI (normal, overweight, and obese) and they were checked to make sure they had no other diseases.

### *Ethical approval*

All ethical approval was obtained according to the official order with the number (592) on (26/4/2022) which was decided by the Basra Health Directorate Training and Human Unit - Knowledge Management Center/Research department.

### *Location of blood sampling*

Blood samples were obtained from different women with PCOS and from healthy women at 9:00 AM at the Basra Teaching Hospital of children and women in the Basra governorate in the Republic of Iraq. The blood samples were obtained from all women by a trained nurse. Five milliliters of venous blood were withdrawn from PCOS females and control group, then the samples were placed in vacationer tubes and centrifuged at a velocity equal to 5000 RPM for six minutes. Subsequently, sera was gathered and maintained at 20°C until the day of estimation of clinical and biochemical markers. The remaining blood was kept in special tubes to separate the blood plasma. Subsequently, the red blood cells were gently rinsed with sodium chloride (9% w/v). Subsequently, the mixture underwent a lysation process using deionized water with a ratio of (1:1 w/v).[16,17]

### *Assessment of clinical biochemical markers*

The concentrations of the lipid profile were estimated as follows:

Triglycerides (TG) levels were measured in blood sera by spectrophotometric method, where triglycerides were hydrolyzed with lipase enzyme to form glycerol and fatty acids. Then glycerol reacts with adenosine triphosphate (ATP) catalyzed by glycerol kinase to produce glycerol-3-phospate and adenosine diphosphate. Glycerol-3-phospate afterward is oxidized by oxygen catalysed by glycerol phosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide. Finally, 4-chlorophenol and 4-aminoanti-quinonemine react with hydrogen peroxide in the presence of peroxidase enzyme to produce quinonimine complex and water. the complex has a pink color, and its absorbance is proportional to the

amount of triglycerides. This test occurs at a wavelength of 500 nm.[18]

Total cholesterol (TC) levels were estimated in the serum of the blood using enzymatic methods. In the beginning, cholesterol ester is hydrolyzed by cholesterol esterase to form cholesterol and fatty acids, free cholesterol was oxidized in the presence of cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide. After that hydrogen peroxide reacts with phenol and 4-amino-antipyrine catalyzed by peroxidase enzyme to produce a pink quinonimine pigment. Finally, the complex absorbance was measured at 500 nm.[19]

The high-density lipoprotein (HDL) was determined by the HDL-phosphotungstic acid precipitation method. The principle includes the precipitation of LDL, VLDL, and chylomicrons by phosphotungstic acid and $MgCL_2$. Then HDL was estimated by centrifugation depending on the total cholesterol amount.[20]

Very low-density lipoproteins (VLDL) were calculated from the following equation:[21]

$$VLDL = Triglycerides / 5$$

Low-density lipoprotein (LDL) levels were measured using the following equation:[22]

$$LDL = Total\ cholesterol - [HDL + VLDL]$$

Hemoglobin concentrations were estimated from the conversion of hemoglobin in the presence of potassium ferric cyanide and potassium cyanide. The absorbance of the colored complex was then calculated at 540 nm.[23]

MDA levels were measured spectrophotometrically by the reaction between barbituric acid and malondialdehyde, which forms a pink complex. Then its absorbance was determined at a wavelength of 535 nm.[24]

Ferritin levels were measured by the Cobas method to form a sandwich complex, then the absorbance is measured depending on the calibration curve.[25]

### *Statistical analysis*

The concentration values in the current study were expressed by mean±standard deviation (SD) for both groups (PCOS patients and healthy group). According to the variables of age and BMI. The social science statistical program (SPSS, version 25, IBM, Armonk, NY, USA) was carried out for all data on levels belonging to lipid profile, malondialdehyde, ferritin and hemoglobin for both patients and control group using various univariate programs. Their regression coefficient values were used to differ among the means belonging to female PCOS and the control group. The *p*-value was calculated from the column 'SIG' (2-tailed) in the independent samples. This test was used to assess the equality of means between groups. Two conditions were considered: one assumed equal variances. And the other does not assume equal variances.

## Results

The biochemical importance of clinical investigation into chemical markers shows the advantages of following any change that can occur in the stages of the disorder. The lipid profile is one of the clinical parameters that must be estimated.[26] Table 1 shows the concentrations of the lipid profile that is presented by TG, TC, HDL, LDL and VLDL in female patients according to the age variable.

**Table 1.** Activity levels of TC, TG, HDL, LDL and VLDL in PCOS patients and control group according to age variable[a]

| Age category (year) | Women Groups | TC (mg/dL) | TG (mg/dL) | HDL (mg/dL) | LDL (mg/dL) | VLDL (mg/dL) |
|---|---|---|---|---|---|---|
| First (14–24) | Control (n=28) | 121.286 ±9.55 | 86.369 ±24.762 | 38.514 ±7.707 | 70.638 ±17.708 | 17.272 ±4.953 |
| | PCOS (n=34) | 201.058 ±26.757*** | 221.271 ±102.606** | 26.665 ±6.41*** | 97.819 ±26.314*** | 44.448 ±20.401** |
| Second (25–35) | Control (n=27) | 118.087 ±12.052 | 84.436 ±37.313 | 41.593 ±8.126 | 68.772 ±17.798 | 16.912 ±7.433 |
| | PCOS (n=36) | 195.747 ±18.723*** | 211.417 ±115.327** | 26.055 ±7.799*** | 108.977 ±18.74*** | 42.28 ±23.066** |
| Third (36–45) | Control (n=25) | 114.64 ±11.871 | 68.336 ±20.107 | 39.218 ±6.048 | 64.214 ±15.522 | 13.667 ±4.021 |
| | PCOS (n=30) | 207.783 ±26.876*** | 240.488 ±87.792** | 26.455 ±5.309*** | 97.036 ±21.873*** | 48.094 ±17.558** |

[a] The levels were expressed as mean±SD, *** – p<0.001, ** – p<0.01, * – p<0.05

The values were obtained for the activity levels of TG, TC, HDL, LDL, and VLDL. TC recorded different concentrations equal to 201.058±26.757, 195.747±18.723 and 207.783±26.876 mg/dL corresponding to the first, second and third age categories in female PCOS patients, respectively, while TG showed assorted values of concentrations represented by 221.271±102.606, 211.417±115.327 and 240.488±87.792 mg/dL for the same age categories in women with PCOS. HDL concentrations were estimated to be equal to 26.665±6.41, 26.055±7.799 and 26.455±5.309 mg/dL for the first, second and third age groups, respectively, in women with PCOS, while LDL recorded different concentrations in patients with PCOS and these levels were assessed to be equal to 97.819±26.314, 108.977±18.74 and 97.036±21.873 mg/dL at the same age categories, respectively. Regarding VLDL, the concentrations were calculated to be equal to 44.448±20.401, 42.28±23.066 and 48.094±17.558 (mg/dL) corresponding to the first, second and third age categories.

According to the results obtained, it was found that the bad cholesterols (TC, TG, LDL, VLDL) were significantly increased in the PCOS patients when compared to the control group, while HDL (good cholesterol) was noticed to be lower when comparing its levels in the same groups.

BMI is a very important variable to monitor the severity of polycystic ovarian syndrome and its correlation with biochemical markers.[27] Therefore, the lipid profile was estimated for all classes of lipids according to the body mass index, as shown in Table 2.

**Table 2.** TC, TG, HDL, LDL, VLDL and non-HDL activity levels in PCOS patients and control group according to the variable body mass index

| BMI | Women Groups | TC (mg/dL) | TG (mg/dL) | HDL (mg/dL) | LDL (mg/dL) | VLDL (mg/dL) |
|---|---|---|---|---|---|---|
| Normal | Control (n=28) | 116.945 ±10.118 | 72.809 ±29.253 | 40.707 ±8.087 | 66.41 ±19.458 | 16.292 ±5.593 |
| | PCOS (n=31) | 200.262 ±25.775*** | 200.573 79.663** | 27.168 ±5.558*** | 98.392 ±22.318*** | 43.684 ±21.23** |
| Overweight | Control (n=27) | 117.062 ±10.175 | 81.403 ±28.055 | 39.008 ±6.968 | 68.978 ±14.13 | 14.575 ±5.837 |
| | PCOS (n=34) | 198.54 ±23.225*** | 218.44 ±106.152** | 26.03 ±6.564*** | 104.897 ±25.679*** | 40.58 ±16.22** |
| Obese | Control (n=25) | 120.216 ±13.427 | 85.444 ±30.12 | 39.554 ±7.275 | 68.478 ±17.931 | 17.087 ±6.025 |
| | PCOS (n=35) | 205.058 ±24.557*** | 251.681 ±116.84** | 26.029 ±7.538*** | 101.609 ±20.543*** | 50.047 ±23.218** |

[a] The levels were expressed as mean±SD, *** – p<0.001, ** – p<0.01, * – p<0.05

**Table 3.** Activity levels of MDA, hemoglobin, and ferritin in PCOS patients and control group according to age variable[a]

| Age category (year) | Women Groups | MDA (µmol/L) | Haemoglobin (g/dL) | Ferritin (ng/mL) |
|---|---|---|---|---|
| First (14–24) | Control (n=28) | 2.313 ±0.047 | 11.096 ±0.754 | 15.845 ±5.639 |
| | PCOS (n=34) | 3.977 ±0.603*** | 13.278 ±0.742** | 65.891 ±13.336*** |
| Second (25–35) | Control (n=27) | 2.308 ±0.019 | 10.751 ±0.849 | 16.381 ±3.76 |
| | PCOS (n=36) | 3.672 ±0.479*** | 13.288 ±0.522** | 62.275 ±11.875*** |
| Third (36–45) | Control (n=25) | 2.308 ±0.051 | 10.527 ±0.832 | 13.613 ±4.719 |
| | PCOS (n=30) | 3.832 ±0.588*** | 13.218 ±0.899** | 60.754 ±11.757*** |

[a] The levels were expressed as mean±SD, *** – p<0.001, ** – p<0.01, * – p<0.05

TC recorded levels, according to body mass index (normal, overweight and obese) represented by 200.262±25.775, 198.54±23.225 and 205.058±24.557 mg/dL respectively, while TG showed various concentration values equal to 200.573±79.663, 218.44±106.152 and 251.681±116.84 mg/dL in PCOS patients in normal, overweight and obese categories, respectively, according to the BMI variable. HDL concentrations were recorded as equal to 27.168±5.558, 26.03±6.564 and 26.029±7.538 mg/dL for the same BMI statements, respectively, while LDL concentrations were equal to 98.392±22.318, 104.897±25.679 and 101.609±20.543 mg/dL in women with PCOS according to the same BMI categories. Concerning VLDL, the concentration values were found to be equal to 43.684±21.23, 40.58±16.22 and 50.047±23.218 mg/dL in female PCOS female patients for the same BMI statements above.

The activity levels of MDA, ferritin, and hemoglobin showed a significant correlation with different age groups. This suggests that age may influence these biochemical markers, highlighting the importance of considering age-related factors in health assessments.

MDA, hemoglobin and ferritin showed various concentration values that were equal to 3.977±0.603 µmol/L, 13.278±0.742 g/dL and 65.891±13.336 ng/mL in PCOS patients according to age factor in the first category, while the same biochemical parameters recorded different concentrations equal to 3.672±0.479 µmol/L, 13.288±0.522 g/dL and 62.275±11.875 ng/mL in the second age category, while in the third age category, the activity levels of MDA, hemoglobin and ferritin were measured equivalent to 3.832±0.588 µmol/L, 13.218±0.899 g/dL and 60.754±11.757 ng/mL respectively.

The results show a significant increase in MDA, hemoglobin, and ferritin, with ferritin showing the most significant difference between the healthy group and the control group.

There is a clinical and biochemical association between the BMI variable and the levels of MDA, ferritin, and hemoglobin. Therefore, these biochemical markers recorded various concentration values depending on the type of BMI, as indicated in Table 4.

**Table 4.** Activity levels of MDA, hemoglobin and ferritin in PCOS patients and control group according to body mass index variable[a]

| BMI | Women Groups | MDA (µmol/L) | Hemoglobin (g/dL) | Ferritin (ng/mL) |
|---|---|---|---|---|
| Normal | Control (n=28) | 2.304 ±0.017 | 10.714 ±0.78913 | 15.313 ±4.212 |
| | PCOS (n=31) | 3.902 ±0.616*** | 13.116 ±0.531** | 65.049 ±13.232*** |
| Overweight | Control (n=27) | 2.317 ±0.014 | 10.811 ±0.818 | 15.524 ±5.085 |
| | PCOS (n=34) | 3.754 ±0.492*** | 13.28 ±0.711** | 61.895 ±12.403*** |
| Obese | Control (n=25) | 2.294 ±0.069 | 10.868 ±0.912 | 15.265 ±5.259 |
| | PCOS (n=35) | 3.822 ±0.594*** | 13.409 ±0.91** | 62.107 ±11.659*** |

[a] The levels were expressed as mean±SD, *** – p<0.001, ** – p<0.01, * – p<0.05

In Table 4 it was found that the levels of MDA, hemoglobin and ferritin were reported to be 3.902±0.616 µmol/L, 13.116±0.531 g/dL and 65.049±13.232 ng/mL in PCOS patients, according to the normal statement of body mass index. However, the same biochemical parameters showed various concentrations represented by 3.754±0.492 µmol/L, 13.28±0.711 g/dL and 61.895±12.403 ng/mL in female PCOS in the overweight category of BMI. In the obese category, the concentration values of MDA, hemoglobin, and ferritin were equal to 3.822±0.594 µmol/L, 13.409±0.91 g/dL and 62.107±11.659 ng/mL, respectively.

## Discussion

PCOS is a complex clinical, biochemical and physiological disorder which affects many women, both single and married causing different alterations and health complications in the reproductive system of women leading to the occurrence of many biological problems in the woman's body.[28,29] In the current research, PCOS was followed and investigated clinically by the estimation of the biochemical variables represented by the lipid profile (TC, TG, HDL, LDL and VLDL), malondialdehyde, ferritin and hemoglobin in accordance with age and body mass index factors. It is known that PCOS, as a complex statement, affects most women for various reasons, therefore the severity of the biological disorder can be followed by the evaluation of many clinical markers.

From the results of the current research, it was found that the lipid profile is a very necessary marker to investigate the significant changes in the levels of TC, TG, HDL, LDL and VLDL concentrations in PCOS patients compared to healthy women which may cause some irregularities in the body of women. TC, TG, LDL and VLDL levels showed a highly significant increase ($p<0.001$), whereas HDL decreased significantly decreased ($p<0.001$) in female PCOS compared with the control group. Ibrahim et al. and Swetha et al. both reported findings consistent with these results.[30,31] An atherogenic lipid profile, an important warning sign of cardiovascular disease, could originate from PCOS, due to this analysis. Because insulin resistance, which frequently occurs in women with PCOS, increases VLDL formation and diminishes HDL levels – both of which are associated with a higher chance of atherosclerosis – it is likely that insulin resistance results in dyslipidemia.[32,33]

It was noticed that the highest concentrations of TC, TG, LDL, and VLDL according to the age variable were seen mainly in the third age categories and the lowest levels of the same markers were observed in the first and second age categories, respectively. However, HDL showed the exact opposite trend, with HDL levels decreasing as age increases. The observed trend might be caused by an increase in insulin resistance and metabolic dysfunction that often become worse with age in PCOS, which additionally results in lipid abnormalities.[34] Variations in lipid concentrations associated with the biochemical mechanisms of PCOS are linked to an atherogenic lipid profile. Therefore, diet and physical activity could be used to manage these lipid imbalances. Studies have shown that weight loss and insulin sensitivity treatments (eg, diet and exercise) can reduce the risk of cardiovascular disease in PCOS patients. It is important to treat these lipid abnormalities early in PCOS patients to prevent the development of cardiovascular disease.[35,36]

Furthermore, the correlation between lipid profile concentrations of PCOS patients and body mass index was considered. Various concentration values for TC, TG, HDL, LDL and VLDL in female PCOS females according to the state of body mass index (normal, overweight, and obese). In PCOS patients, it was observed that women with higher BMI levels had higher levels of TC, TG, LDL, and VLDL, whereas those with lower weights had the lowest amounts. However, HDL levels showed a negative correlation with body weight, decreasing as weight increased.

This pattern reveals how obesity promotes an amplification of lipid issues in PCOS. Dyslipidemia is mainly caused by insulin resistance and inflammation, both of which become worse by obesity. The correlation between worsening lipid profiles and a higher body mass index indicates the need of weight management in PCOS.[37] The clinical chemical mechanism between lipid profile and BMI was suggested by the increased prevalence of hypertension in women with PCOS which is linked to many variables such as obesity, hyperandrogenism, insulin resistance, and autonomic dysfunction. Obesity in women with PCOS increases the risk of cardiovascular disease with dysfunctional blood pressure. Additionally, androgen levels and the ongoing use of oral birth control in women with PCOS can lead to variations in lipid profile levels, possibly leading to an increase or decrease.[38,39] More specifically, excessive androgen levels found in women with PCOS could increase cardiovascular risk through changing lipid transport and liver metabolism, which can contribute to lipid abnormalities.[40]

The biochemical indicators represented by malondialdehyde, ferritin, and hemoglobin are very important variables and have medicinal correlation and biological significance with PCOS.

In PCOS, hemoglobin levels might be higher owing to some disorders such as obesity and insulin resistance, but there is still no consistent relationship between Hb levels and PCOS, and individual results can differ depending on other health factors. De Medeiros et al. reported a moderate significant increase ($p<0.001$) in hemoglobin levels in PCOS patients which was consistent with the results collected in this investigation.41 On the other hand, Alvarez-Blasco et al. obtained different results, seeing a significant decrease in patients with Hb levels in PCOS compared to the healthy group.[42] The heightened levels of hemoglobin might be related to altered metabolic pathways and chronic low-grade inflammation. Higher hemoglobin levels might result from erythropoiesis due to inflammation and insulin resistance. However, additional research is required to further clarify the relationship between hemoglobin and PCOS.[43]

The results obtained show that hemoglobin levels increase in PCOS patients as weight increases (highest levels shown in the obese category) while decreasing as patients become older (lowest levels seen in the third age category). This confirms the theory that elevated blood hemoglobin concentrations are an outcome

of metabolic dysfunction in PCOS, especially insulin resistance and obesity. Women's hemoglobin levels could decrease as a result of disturbances in their metabolic processes as they age.[41]

Furthermore, malondialdehyde levels significantly increased ($p<0.001$) in PCOS patients compared to the healthy group. Deba et al. and Sabuncu et al. both found similar results.[44,45] MDA is a marker of inflammation and oxidative stress, which has been shown to be higher among women with PCOS. Since oxidative stress damages tissues and causes metabolic dysfunctions, including insulin resistance, it plays a role in the development of PCOS.[35]

Although the results in the normal BMI group show the highest concentrations, the main trend shows a moderately significant increase in MDA levels of MDA as BMI increases. This unexpected result in the lowest BMI group could be influenced by many factors, such as underlying metabolic or hormonal conditions that can increase oxidative stress. On the contrary, the same trend is seen when looking at the results of MDA according to age, as its levels show a steady increase as age increases. Also, the first (youngest) age group displays the highest levels. This could indicate that although oxidative stress and inflammation increase with age and BMI.[46] This relationship between MDA with age and BMI should be further investigated to confirm or disprove these results.

Increased malondialdehyde in oxidative stress, especially during pregnancy, can be necessary to achieve the diagnosis of PCOS, which means that MDA is a significant indicator of following PCOS progression.[47] This reinforces how oxidative stress plays an active role in the pathophysiology of PCOS, demonstrating that high levels of MDA can lead to the development of PCOS in conjunction with its adverse effects.[48]

Ferritin, an indicator of iron storage, is usually elevated in obese and insulin resistant women and is correlated to the severity of PCOS. High levels of ferritin signify elevated inflammation and oxidative damage, which contribute to the metabolic dysfunctions observed in PCOS, similar to MDA, which is another sign of oxidative stress.[49] Since ferritin is similar in its function as a marker of oxidative stress to MDA, its levels were also significantly ($p<0.001$) in PCOS patients compared to the healthy group. Sharifi et al. and Al-Hakeim et al. both concluded that ferritin levels increased in female PCOS due to the increase of oxidative stress.[50,51]

## Conclusion

In summary, the outcomes of this research highlight all the complicated metabolic and biochemical changes attributed to PCOS. Substantial changes in the lipid profile, including higher levels of TC, TG, LDL, and VLDL and decreased HDL, point to an atherogenic risk profile in women affected by PCOS. Obesity and insulin resistance worsen these lipid abnormalities, demonstrating the important role of weight management and early treatment to reduce cardiovascular risks. Furthermore, it became apparent that PCOS patients showed substantially higher levels of oxidative stress signals such as ferritin and MDA, demonstrating that inflammation and oxidative damage play an active role in the pathophysiology of the condition. Furthermore, there were significant connections involving hemoglobin levels and both BMI and metabolic dysfunction, revealing that raised hemoglobin might indicate a sign of insulin resistance and abnormal metabolic processes. With every aspect considered, these findings draw attention to the importance of thorough medical management of PCOS, with a special focus on minimizing oxidative stress, metabolic function, and lipid profiles to avoid chronic health conditions, notably cardiovascular disease.

## Declarations

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***Authors' contributions***

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

***Conflicts of interest***

The authors have disclosed no conflicts of interest.

***Data availability***

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

***Ethics approval***

In this study, the ethical approval with the number (592) on (26/4/2022) was acquired from the Basrah Health Department – Training and Human Development – Center of Knowledge Management/Research Division.

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**ORIGINAL PAPER**

# Translation and psychometric evaluation of the Diabetes Education Questionnaire (DATE-Q) from English to Marathi – assessing reliability, validity, and cross-cultural equivalence

Sharvari J. Kulkarni [1], Gabriela Lima de Melo Ghisi [2], Varoon C. Jaiswal [1], Pournima Pawar [1], Deepali R. Kharatmal [1], Mithali M. Kangude [1]

[1] Department of Cardiovascular and Respiratory Physiotherapy, MAEER's Physiotherapy College Talegaon Dabhade Pune Maharashtra, India
[2] KITE Research Institute, University Health Network, Toronto, Canada

**ABSTRACT**

**Introduction and aim.** The DiAbeTes Education Questionnaire (DATE-Q) is a self-administered tool created to evaluate disease-related knowledge and knowledge of five core components of rehabilitation: exercise, diet, psychological well-being, self-management and complications. The aim was to translate and adapt the DATE-Q into Marathi language and to evaluate the validity and reliability among diabetes and prediabetic patients.

**Material and methods.** The study was carried out following standard stepwise Beaton and COSMIN guidelines to complete the translation and psychometric validation of the questionnaire. The pre-final version was evaluated in 30 individuals with diabetes or prediabetes. Test-retest reliability and internal consistency were assessed among 200 individuals with type 2 diabetes using Cronbach's alpha and intraclass correlation coefficients respectively.

**Results.** The original and translated versions did not conceptually differ from each other. DATE-Q has ten elements that were culturally adjusted. Based on suggestions from the expert group and the results of the pilot tests, cross-cultural modifications were made. The value of 0.935 for Cronbach's alpha shows a very high level of internal consistency. For single and average measures, the intraclass correlation coefficient is 0.985 and 0.993 resp. which indicates an excellent level of reliability.

**Conclusion.** The DiAbeTes Education Questionnaire is a reliable and valid tool for evaluating knowledge among Marathi-speaking patients.

**Keywords.** cross cultural evaluation, diabetes mellitus, health education, psychometric evaluation, reliability, validity

## Introduction

Type 2 diabetes (DM2) is a chronic condition requiring ongoing self-management to prevent serious complications such as cardiovascular disease (CVD), nephropathy, and retinopathy.[1–4] In India, DM2 is a growing public health problem, with cases projected to rise from 74.2 million in 2021 to 124.9 million by 2045.[5] Cardiovascular complications remain a leading cause of mortality in DM2 patients, who are 2–4 times more likely to experience major events than those without diabetes.[6]

**Corresponding author:** Varoon C. Jaiswal, e-mail: drvaroon@gmail.com

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Effective self-management, supported by diabetes education, is essential for maintaining blood glucose levels, preventing complications, and promoting behavioral changes.[7] However, education is limited, particularly in socioeconomically disadvantaged populations.[8] Standardized tools are essential to evaluate diabetes knowledge and guiding education programs.[9] Although existing instruments like the Diabetes Knowledge Questionnaire (DKQ-24) and the Diabetes Knowledge Assessment Scale (DKN-A) assess aspects of diabetes management, they may not address all necessary domains comprehensively.[10,11]

The Diabetes Education Questionnaire (DATE-Q), validated in low-resource settings like Brazil, provides a holistic approach by assessing five domains: self-management, long-term complications, physical activity, healthy eating and psychosocial well-being.[9,12] Translating and validating this tool into Marathi, the official language of Maharashtra, will allow its use among a population of more than 120 million, ensuring culturally and linguistically appropriate evaluation of diabetes knowledge and education outcomes. This research aims to improve diabetes education and self-management for Marathi speaking people, ultimately improving health interventions and outcomes.

## Aim

The aim was to translate and culturally adapt the DATE-Q into Marathi and evaluate its reliability, validity, and cross-cultural equivalence for use among Marathi speaking populations.

## Material and methods

### *Design and procedures*

The cross-sectional and experimental study was conducted by following standard stepwise Beaton and COSMIN guidelines for the translation and psychometric validation of the questionnaire.[13,14] Permission was obtained from the original author and the Institutional Ethics Committee (EC/NEW/INST/2019/377/183).

Step 1: Forward translation. The translation process began with forward translation. Two bilingual translators, Translator 1 (T1) and Translator 2 (T2), both fluent in English and Marathi, were selected. Marathi was their native language. T1, an assistant professor in the physiotherapy department, had knowledge of DM2 and the concept measured by the questionnaire. T2, without medical background, was unfamiliar with the construct of the questionnaire. Both translators independently submitted their translated versions to the study coordinator, along with written explanations for their translation choices.

Step 2: Synthesis of forward translations. The next step involved synthesizing the translations from T1 and T2 under the guidance of the study coordinator. The two versions were combined to create a single translated version.

Step 3: Back translation followed, with two bilingual translators (BT1 and BT2) translated the combined T1 and T2 version back into English. Both back translators were native English speakers, without prior medical training, and were blinded to the original DATE-Q questionnaire and its purpose. The two translated versions were compared with the original questionnaire to assess the precision and comprehension of the terms.

Step 4: Expert committee review. An expert committee consisting of a diabetologist, a methodologist, health professionals, language experts, and translators reviewed the translated questionnaire. They provided feedback on the necessary changes to ensure cultural and linguistic appropriateness.

Step 5: Pilot test. A pilot study was conducted with 30 patients attending the cardiovascular and respiratory physiotherapy department. The patients completed the pre-final version of the questionnaire and their feedback was collected. This step was designed to assess the relevance, clarity, and completeness of the questionnaire's statements, response options, and instructions.

To identify potential problems, the interviewer recorded the full responses to each statement and calculated the number of items marked as unclear or misunderstood by theparticipants. The clarity of each statement was assessed using a Likert scale from 1 ("I do not understand anything") to 4 ("I understand completely"). The questionnaire, consisting of 20 items, was rated on this scale. The pre-final version was revised based on participants' feedback to address any issues identified.

Following this step, the tool was administered to a sample of 200 individuals to assess the reliability of the questionnaire. The scores for each domain were calculated based on the number of items and the minimum and maximum possible scores for each domain.

To assess test-retest reliability, the translated questionnaire was administered to patients with DM2. Participants completed the questionnaire, and their total scores were recorded. After 7 to 21 days, the questionnaire was re-administered to the same participants, and their scores were recalculated.[9] Differences between the initial and subsequent scores were analyzed to determine the consistency and reliability of the results.

### *Participants and setting*

For the psychometric evaluation, following the recommendation of Hair and Anderson ,which suggests that each item of the questionnaire should have at least 10 participants, a sample size of 200 was determined. Participants who were native Marathi speakers and diagnosed with DM2 (HbA1C level of 6.5% and above) or prediabetes (HbA1C level between 5.7% and 6.5%)[16] were included in the study.[15,16]

The study was carried out in a tertiary care teaching hospital, using purpose-sampling to select participants. Informed consent was obtained from all individuals.

### *DATE-Q questionnaire*

The DATE-Q was originally developed in English by Ghisi et al. to serve as a concise and accessible tool for use in clinical and research settings.[12] The DATE-Q consists of 20 true/false/I don't know items written in plain language, making it suitable for self-administration. The elements are divided into five domains: self-management, long-term complications, activeness, healthy eating, and psychosocial well-being, which guided the selection of the statements included in the tool. Each correct answer earns 1 point, with a maximum score of 20 points, evenly distributed across the five domains, allowing for a maximum of 4 points per domain.

- Domain 1: Self-management: items 2, 12, 16, 18
- Domain 2: Long-term complications: items 1, 3, 6, 11
- Domain 3: Being active: items 4, 8, 13, 17
- Domain 4: Healthy eating: items 5, 9, 14, 20
- Domain 5: Psychological Well-being: Items 7, 10, 15, 19

Correct answers for interpretation:

- True: Statements 1, 4, 6, 7, 8, 10, 11, 12, 14, 18, 19, 20
- False: Statements 2, 3, 5, 9, 13, 15, 16, 17

### *Data analysis*

Data were entered into Microsoft Excel and analyzed using the Social Sciences Statistical Package (SPSS) version 28 (SPSS Inc., Chicago, IL, USA). Internal consistency was assessed by calculating Cronbach's alpha values greater than 0.70 were considered acceptable, indicating a strong correlation between items and with the total score. 17 The reliability was evaluated using the intra-class correlation coefficient (ICC) by administering the same test to participants at two different times to assess the consistency of the scores.

For Cronbach's Alpha, 0.70-0.79 is acceptable, 0.80–0.89 is good and ≥0.90 is excellent. ICC values greater than 0.75 indicate good reliability and values above 0.90 indicate excellent reliability.

## Results

### *Translation and cultural adaptation*

Table 1 presents the original words or phrases that were translated and culturally adapted by an expert committee, based on feedback from the prefinal version tested with individuals diagnosed with DM2. Adjustments made were intended to improve the clarity and understanding of the questionnaire items.

The prefinal version was tested for cultural relevance with 30 individuals diagnosed with DM2. Most of the participants rated the items as 2 or 3, indicating some confusion. As a result, certain words and phrases were retranslated and culturally adapted in consultation with the expert committee. Based on these findings (Table 1), five elements were modified to improve clarity and cultural relevance. For example, in item nine, "canned soup" was replaced with "processed food," as canned soup is not typically consumed in Maharashtrian cuisine. Furthermore, sentences in items 2, 5, 8, 10, 15, and 18 were rephrased to improve coherence and clarity. For items 5, 7, 9, 13, alternative words for "discomfort" were used to ensure greater understanding among patients.

**Table 1.** Cultural adaptation made after the pre-final version of the questionnaire

| Q.NO | Words or phrases from the original version | Words according to translators and expert committee and their rating on Likert scale | Final changes according to the survey made during the pre-final version and their rating on Likert scale |
|---|---|---|---|
| 02 | "after eating a meal" | जेवल्यानंतर | जेवणानंतर |
| 04 | "bands" | बँड | पट्टा |
| 05 | "large"<br>"Prevent"<br>"And" | मोठ्या<br>मदत<br>आणि | जास्त<br>प्रतबिंध<br>व |
| 07 | "And"<br>"Prevent you from becoming overwhelmed" | आणि<br>दबून जाण्यापासून रोखू शकते. | व<br>असण्यामुळे भारावून टाकण्यापासून प्रतबिंध करू शकते |
| 08 | "manage" | व्यवस्थापन करण्यात | व्यवस्थापनेची |
| 09 | "canned soup"<br>"choices" | कॅन केलेला<br>नविडी असतात. | तयार कविा प्रक्रयिा केलेले अन्न<br>नविड असते |
| 10 | "to help" | मदत करण्याचा | मदतीचा |
| 13 | "Sores"<br>"ulcers"<br>"should check" | फोड<br>तपासा | जखम<br>तपासले पाहजिे |
| 15 | "does not affect" | परणिाम होत नाही. | परणिाम करत नाही |
| 16 | "carbohydrate"<br>"acting" | कार्बोहायड्रेट<br>अभनिय | कर्बोदके<br>परणिामकारक |
| 17 | "your"<br>"zone" | तुमचे<br>क्षेत्रामध्ये | तुमची<br>पातळीवर |
| 18 | "Oral"<br>"If you take" | तोडी<br>घेतल्यास | तोडाद्वारे<br>घेत असाल |
| 20 | "foods from plants" | वनस्पतीपासून<br>अन्न | वनस्पतीजन्य<br>पदार्थ |

### *Psychometric evaluation*

The validity of the content, an essential aspect of the adaptation and validation process, was carefully considered, especially when adapting the instruments for use in a foreign country and language. For this study, the validity of the content was established through validation by a panel of experts, including a diabetologist, a methodologist, forward and backward translators, health professionals and language experts.

Of the 200 respondents, 69.5% were male (139) and 30.5% were female (61), with an average age of 61.68 years (SD=14.23). The mean level of HbA1c among the participants was 7.11 (SD=0.91). Table 2 presents a summary of the responses to all 20 items, showing the mean

scores (mean), variability (standard deviation), and the number of respondents (n). Each question (Q1 to Q20 post-test values) was analyzed, with the mean providing the central tendency of the responses, and the standard deviation indicating the degree of variation around the mean. In particular, complete agreement (mean=1, SD=0) was observed for the items Q8post, Q9post, Q10post and Q20post, indicating a high level of consensus among participants for these items.

**Table 2.** Mean and standard deviation for each question (n=200)

| Q | | Mean | Std. deviation |
|---|---|---|---|
| 1 | मधुमेहासह जगताना, गुंतागुंत टाळण्यासाठी तुमचे रक्तदाब आणि कोलेस्टेरॉल व्यवस्थापित करणे महत्वाचे आहे. | 0.85 | 0.36 |
| 2 | जेवणानंतर दोन तासांनी, तुमच्या रक्तातील साखर १० मिली मोल/लिटर पेक्षा जास्त असावी. | 0.14 | 0.35 |
| 3 | तुमच्या A1C रक्त चाचणीचे परिणाम गेल्या वर्षभरातील तुमच्या रक्तातील साखरेची सरासरी पातळी दर्शवतात. | 0.24 | 0.43 |
| 4 | प्रतिकार प्रशिक्षण (पट्टा कवि वजन वापरणे) तुमचे स्नायू मजबूत करण्यास आणि रक्तातील साखर कमी करण्यात मदत करू शकते. | 0.58 | 0.49 |
| 5 | न्याहारी वगळणे आणि रात्रीचे जेवण जास्त प्रमाणात खाल्ल्याने रक्तातील साखरेचे प्रमाण जास्त व कमी होण्यास प्रतिबंध होईल. | 0.85 | 0.36 |
| 6 | तुमचा A1C कमी (७ % पेक्षा कमी) ठेवल्याने मधुमेहाची गुंतागुंत टाळण्यास मदत होईल. | 0.165 | 0.37 |
| 7 | तुमच्या भावनांची जाणीव ठेवणे आणि मदत व समर्थन मागणे तुम्हाला मधुमेह असण्यामुळे भारावून टाकण्यापासून प्रतिबंध करू शकते | 0.955 | 0.21 |
| 8 | तुमच्या रक्तातील साखरेचे व्यवस्थापनेची मदत करण्यासाठी व्यायाम हा एक चांगला मार्ग आहे. | 1 | 0.0 |
| 9 | तयार कवि प्रक्रिया केलेले अन्न हे दररोजसाठी आरोग्यदायी अन्न नविड असते. | 1 | 0.0 |
| 10 | तुमच्या कुटुंबाकडून आणि मित्रांकडून पाठबिा मळिवणे हा तुम्हाला तणावाचा सामना करण्यासाठी मदतीचा एक चांगला मार्ग आहे. | 1 | 0.0 |
| 11 | जर तुमचा मधुमेह व्यवस्थति नियंत्रति केला गेला नाही, तर तुमच्या रक्तवाहनि्या आणि निसा खराब होऊ शकतात. | 0.955 | 0.21 |
| 12 | तुम्हाला सर्दी कवि फ्लू असताना तुमच्या रक्तातील साखर नेहमीपेक्षा जास्त कवि कमी असू शकते. | 0.875 | 0.33 |
| 13 | व्यायामापूर्वी तुम्ही तुमच्या पायांना फोड, जखम कवि अल्सरसाठी तपासले पाहजि. | 0.335 | 0.47 |
| 14 | तंतुमय पदार्थ खाल्ल्याने तुमची रक्तातील साखर, एल. डी.एल (खराब) कोलेस्टेरॉल आणि रक्तदाब कमी करून मधुमेह नियंत्रति होण्यास मदत होते | 0.485 | 0.5 |
| 15 | तुम्ही तुमचा मधुमेह कसा व्यवस्थापति करता यावर नैराश्य परणिाम करत नाही | 0.5 | 0.5 |
| 16 | जर तुमची रक्तातील साखर खूप कमी असेल तर तुम्ही जलद-परणिामकारक कर्बोदके म्हणून चॉकलेट खावे. | 0.59 | 0.49 |
| 17 | जेव्हा तुमची हृदय गती लक्षणयति पातळीवर असते आणि तुम्हाला धाप लागते तेव्हा तुम्ही योग्य स्तरावर व्यायाम करत आहात | 0.25 | 0.43 |
| 18 | जर तुम्ही इन्सुलनि कवि तोंडाद्वारे काही मधुमेहाची औषधे घेत असाल (ग्लायब्युराइडसारख्या गोळ्या) तर तुमच्या रक्तातील साखर कमी होण्याची शक्यता जास्त असते. | 0.98 | 0.14 |
| 19 | टाइप २ मधुमेहामध्ये खराब झोप कवि स्लीप एपनयिा सामान्य आहे आणि त्यामुळे तुमचे आरोग्य बधिडू शकते. | 0.39 | 0.49 |
| 20 | मधुमेहासाठी आरोग्यदायी आहारामध्ये अधकि वनस्पतीजन्य पदार्थ खाणे समावषि्ट आहे. उदाहरणार्थ: फळे, भाज्या, संपूर्ण धान्य आणि शेंगा | 1 | 0 |
| Total | | 0.67 | 0.31 |

Cronbach's Alpha was used to assess the internal consistency of the questionnaire in its various domains. The overall scale, consisting of 20 items, demonstrated a high level of internal consistency with a Cronbach Alpha of 0.935, which slightly increased to 0.945 when the items were standardized. For individual domains, the Self-management domain (4 items) had a Cronbach Alpha of 0.975 (standardized=0.979), the long-term complications domain (4 items) showed an Alpha of 0.957 (standardized=0.966), the Being active domain (4 items) had 0.954 (standardized=0.967), the Healthy eating domain (4 items) achieved 0.975 (standardized=0.982), and the Psychosocial wellbeing domain (4 items) recorded 0.945 (standardized=0.964). These results indicate excellent internal consistency across all domains of the questionnaire, reflecting its reliability as a measurement tool.

ICC analysis provided additional reliability measures (Table 3). For single measures, the ICC was 0.985, with a 95% confidence interval of 0.980 to 0.989, and an F test value of 133.505 (df1=199, df2=200, p<0.001), indicating excellent reliability. For the average measures, the ICC was even higher at 0.993, with a confidence interval of 0.990 to 0.994 and the same F test results, reinforcing the high reliability of the average measures.

**Table 3.** ICC measures of the questionnaire

| | ICC | | | | | | |
|---|---|---|---|---|---|---|---|
| | Intraclass correlation | 95% confidence interval | | F Test with true value 0 | | | |
| | | Lower bound | Upper bound | Value | df1 | df2 | p |
| Single measures | 0.985 | 0.980 | 0.989 | 133.505 | 199 | 200 | <0.001 |
| Average measures | 0.993 | 0.990 | 0.994 | 133.505 | 199 | 200 | <0.001 |

## Discussion

The successful translation and validation of the Marathi version of the DATE-Q confirmed its cross-cultural equivalence to the original English version. This process adhered to established guidelines, ensuring that the translation preserved the original intent while taking into account linguistic and cultural nuances.[13,14] By maintaining high internal consistency and test-retest reliability, the Marathi version of the DATE-Q demonstrated its ability to assess the same constructs as the original tool, although it was applied in a different linguistic and cultural context. The careful attention to linguistic and cultural variations during the translation process was critical to ensuring that the Marathi version was both relevant and suitable for Marathi-speaking individuals. This cross-cultural equivalence is essential to confirm that the questionnaire is valid and applicable in different cultural settings, which is particularly important in a global health context where diabetes management needs to be tailored to local populations.

The study involved 200 participants, with a demographic breakdown of 69.5% male and 30.5% fe-

male respondents, and an average age of 61.68 years (SD=14.23). The variability in the age distribution was reflected in the HbA1c levels, with an average of 7.11 (SD=0.91). These demographic characteristics are representative of the general population involved in diabetes education initiatives, allowing for a meaningful interpretation of the findings.[18] Average HbA1c levels suggested that participants were generally managing their diabetes at a controlled level, consistent with findings from other studies on diabetes management.[19] The gender balance and the range of age provided context for understanding the applicability of the questionnaire, making the findings relevant to the target community.

The internal consistency of the Marathi DATE-Q was rigorously evaluated using Cronbach's alpha, which measures the degree to which items within each domain and across the overall scale reliably measure the same underlying construct. The general Cronbach alpha for the 20-item scale was 0.935, with a standardized value of 0.945. These values are significantly above the commonly accepted threshold of 0.70, indicating excellent internal consistency.[17] Each domain of the questionnaire-self-management, long-term complications, being active, healthy eating and psychosocial well-being-showed high Cronbach's alpha values, ranging from 0.954 to 0.979. This suggests that the translation and cultural adaptation process preserved the internal consistency of the original English version, confirming the robustness and reliability in assessing various aspects of diabetes education.

The reliability of the test-retest, assessed using the ICC, provided further evidence of the stability of the Marathi DATE-Q over time. The ICC of 0.993 for the mean measures indicated excellent stability, with minimal measurement error between the first and second administration of the questionnaire. This result aligns with similar studies, such as the Brazilian validation study by Felix et al., which reported good test-retest reliability (ICC=0.5) and acceptable internal consistency (Cronbach's alpha=0.6) for the Brazilian Portuguese version of DATE-Q.[9] This reinforces the reliability of Marathi DATE-Q as a tool for measuring diabetes-related knowledge related to diabetes in different linguistic and cultural contexts.

Responses to the Marathi DATE-Q also showed various patterns of agreement, as indicated by mean and standard deviation values for each item, highlighting its capacity to capture the diverse aspects of diabetes education and management. For example, the means ranged from 0.14 to 1.0, while the standard deviations ranged from 0.0 to 0.5, indicating a broad spectrum of responses between participants. This variability reflects differences in knowledge and understanding of diabetes education within the population, which is essential to identify gaps and tailoring interventions accordingly. A study by Heise et al. found that structured diabetes self-management education (DSME) programs are effective in increasing personal awareness of diabetes, consistent with the goals of DATE-Q in assessing diabetes-related knowledge.[20] Structured DSME programs positively impact individuals' understanding of their condition and improve self-management behaviors, underscoring the importance of reliable tools like Marathi DATE-Q in supporting such programs.

The validated Marathi DATE-Q has proven to be an important tool for both clinical practice and research. It allows healthcare professionals to assess the effectiveness of diabetes education programs in Marathi speaking populations and identify areas for improvement. This aligns with the findings of Gordon et al., who emphasized the need for valid and culturally sensitive tools to evaluate the outcomes of diabetes education outcomes.[21] The Marathi DATE-Q provides a reliable and culturally appropriate method to evaluate diabetes knowledge, ultimately supporting targeted interventions that can improve patient education and diabetes care in Marathi speaking communities. Future research should address certain limitations, such as incorporating a more balanced sample and comparing the Marathi DATE-Q with other validated tools. This would further establish the generalizability and provide insight into how it performs relative to other established measures in diabetes education. Additionally, expanding the use of Marathi DATE-Q across different regions and populations could help to further validate its cross-cultural applicability.

***Study limitations***

This study had several limitations that could impact the generalizability of the findings. First, the sample had a higher proportion of male participants (69.5%) compared to female participants (30.5%), which could potentially introduce gender bias. As a result, the findings may not fully capture the experiences and perceptions of the female participants, limiting the applicability of the Marathi DATE-Q to both genders equally.

Furthermore, the study did not include a comparison between Marathi DATE-Q and other established diabetes education measures, which would have improved the construct validity. Comparing the Marathi DATE-Q with other validated tools for assessing diabetes knowledge would provide a clearer understanding of how it performs relative to other existing instruments, strengthening its validation.

Another limitation is that Marathi is spoken in various regional dialects and the language can differ significantly between different areas. As the study focused on a specific demographic, there may be contextual variations in how the questionnaire is interpreted in the broader Marathi-speaking population. This could affect the generalizability of the findings in all regions where Marathi is spoken.

Finally, the baseline educational qualifications and socioeconomic criteria of the participants were not considered during recruitment. These factors could influence the understanding and responses to the questionnaire, potentially introducing variability that was not considered in this study. Future research should consider these factors to better understand how education and socioeconomic status impact responses to diabetes education assessments.

## Conclusion

The translation and cross-cultural adaptation of DATE-Q into Marathi were successfully completed in accordance with established guidelines and procedures. To ensure the cultural relevance and comprehensibility for the target population, ten items were culturally adapted. The translated version of the DATE-Q demonstrated excellent validity, reliability, and high internal consistency when applied to patients with diabetes and prediabetes participating in cardiac rehabilitation. The Marathi version of the DATE-Q has proven to be a reliable and valid tool for evaluating knowledge related to type 2 diabetes in this demographic.

Future research should focus on comparing Marathi DATE-Q with other diabetes education tools. This comparison would help validate its constructs more rigorously and improve its psychometric properties. Such research would be valuable for monitoring the effectiveness of ongoing diabetes education initiatives and assessing their impact on patient self-management.

## Declarations

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***Authors' contributions***

Conceptualization, V.J. and S.K.; Methodology, S.K.; Software, G.G. and V.J.; Validation, G.G., V.J. and S.K.; Formal Analysis, P.P.; Investigation, S.K. and V.J.; Resources, D.K. and M.K.; Data Curation, S.K.; Writing – Original Draft Preparation, S.K., V.J. and P.P.; Writing – Review & Editing, S.K., D.K. and M.K.; Visualization, V.J.; Supervision, G.G.; Project Administration, V.J. and S.K.; Funding Acquisition, V.J.

***Conflicts of interest***

The authors declare no conflict of interest.

***Data availability***

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

***Ethics approval***

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the MIT's MAEER Physiotherapy College, Talegaon Dabhade (approval number: EC/NEW/INST/2019/377/183).

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**ORIGINAL PAPER**

# Morphological and genetic identification of yeasts from skin and oral infection in children in the Basrah province

Fatima T. M. AL-Mosawi , Najwa M. J. A. Abu-Mejdad , Athraa A. A. Al-Hilfi

Biology Department, College of Science, Basrah University, Basrah, Iraq

**ABSTRACT**

**Introduction and aim.** Human fungus infections are widespread and can lead to a variety of diseases in children. The purpose of this study was to isolate and identify yeasts from various places in children, including skin (diaper area) and oral cavity, utilizing morphological and molecular approaches for precise categorization.

**Material and methods.** One hundred swabs were collected from children clinically diagnosed with fungal skin infections. The isolated yeast species were examined, purified, and morphologically. The sequences have been deposited in GenBank of Japan as new strains under accession numbers LC790886 to LC79098. including *Candida albicans, Pichia kudriavzevii, Magnusiomces capitatus, Nakaseomyces glabratus, Kluyveromyces marxianus, Candida tropicalis, Meyerozyma guiliermonolii, clavispora lusitaniae, Candida parapsilosis, Trichosporon ashii*. The isolates were cultured on Sabouraud Dextrose Agar with chloramphenicol, and species identification was carried out using CHROMagar Candida medium and lactophenol cotton blue staining. Molecular identification was performed using PCR amplification of the ITS1-5.8S-ITS2 rDNA region, followed by DNA sequencing.

**Results.** The presence of 10 yeast species, with *C. albicans* 56% representing the highest percentage of these, while the percentage of other yeasts was 44%. The *Candida* species was found to have the highest percentage of occurrence, 58% followed by the *C. tropicalis* species, 19%, which had a lower percentage of occurrence.

**Conclusion.** The phenotypical and genetic characteristics of yeast have been identified by the use of clinically isolated samples of children.

**Keywords.** *Candida,* newborns, oral candidiasis, skin infection

## Introduction

Cutaneous candidiasis is a widespread fungal infection that affects people of all ages, while oral candidiasis is a common infection that affects primarily people with weakened immune systems, such as newborns, infants, those taking antibiotics or corticosteroids, *Candida* can be the main cause of skin disease or develop as a result of other skin conditions such as atopic dermatitis, psoriasis, or diaper rash.[1,2] It can affect any area of the body, and frequent symptoms include interdigital candidiasis, cheilitis, diaper dermatitis, and intertrigo.[3,4]

The genus *Candida* contains many species, but *Candida albicans* is the most common cause of candidiasis in humans, accounting for more than 80% of cases. Other less common species include *Candida tropicalis, Candida parapsilosis,* and *Candida glabrata*.[5] The genus *Candida* has over 200 species, although only a tiny percentage of these are human opportunistic pathogens that infect people with compromised immune systems. Topical antifungal medications are an effective way to treat superficial Candida infections, which usually affect the skin or mucous membranes. Invasive fungal in-

**Corresponding author:** Fatima T. M. AL-Mosawi, e-mail: fatimaalmosawi567@gmail.com

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fections are frequently life-threatening, perhaps due to ineffective diagnostic techniques and inadequate first antifungal treatments.[6,7] More than 50% of surface *Candida* infections are caused by *C. albicans*, the most opportunistic fungal pathogen in humans.[8,9] It resides in the gastrointestinal and genitourinary tracts of approximately 70% of individuals as a benign commensal without inducing any symptoms of illness.[10] Children with oropharyngeal candidiasis can have acute or persistent symptoms, usually characterized by painful, erythematous, pseudomembranous plaques that can spread to the larynx and pharynx and make swallowing and eating difficult.[11]

Diaper dermatitis (DD) caused by candidiasis is a common problem, especially in newborns and infants. Diarrhea, which contributes to it, occurs in approximately one-third of affected children.[1] The affected area typically stays within the diaper zone, though severe cases can spread beyond it can extend beyond these limits.[5] Intertrigo, a fold skin condition, can develop due to either direct fungal infection or extension of the diaper rash. It can involve both major and minor skin creases.[12] Similar to DD, intertrigo manifests itself as reddened, scaly patches with potential blistering and swelling. Tiny bumps or pustules may also appear around the main lesion. In severe cases, ulceration and erosion can occur. Itching and/or pain are common.[5,12,13]

## Aim

The aim of the study was to isolate and identify yeasts from various places in children, including skin, diaper area, and oral cavity, utilizing morphological and molecular approaches for precise categorization.

## Material and methods

### *Collection of samples*

During October 2022 to August 2023, One hundred swabs were collected from children who were clinically diagnosed with different fungal skin infections attending Al-Fayhaa General Hospital and Basrah Women's and Children's Hospital, in AL-Basrah, Iraq. These specimens were brought to the Mycology Laboratory of the University of Basrah for further examination. Samples were cultured on SDA media (Himedia, India) that included 250 mg/L of chloramphenicol added to $SDA_C$ in order to prevent the contamination of samples with bacteria. After creating the samples, they were placed in an incubator at a temperature of 37°C for a period of time that ranged from two to thirty days. The cultures were then processed for inspection to determine the phenotypic and genetic characteristics of the organisms. The fungal isolates were examined macroscopically and microscopically using lactophenol cotton blue as a mounting material; this was done by preparing slides of each growing colony were prepared to observe under a light microscope with an objective lens magnification power of 40×. Observation of colony color and cell morphology on CHROM agar *Candida* (HiMedia, India) was used for yeast identification following the mycological literature.[14]

### *Examination and identification specimens*

Using Chrome agar *Candida* medium for the identification of yeast isolates.

### *Preparation Chrome agar Candida medium as follows*

Candida medium of Chrome agar (Himedia, India) 42.72 gm D.W 1000 ml (Table 1) .

**Table 1.** The color variation of *Candida spp*. in chrome agar candida medium [15]

| No. | *Candida* species | Color |
|---|---|---|
| 1 | *C. albicans* | light green |
| 2 | *C. tropicalis* | blue to metallic blue |
| 3 | *C. glabrata* | cream to white smooth |
| 4 | *C. krusei* | purple fuzzy |
| 5 | *C. parapsilosis* | white to cream |

### *Genetic diagnosis*

A Presto Mini gDNA yeast kit that Geneaid/Korea gave was utilized to extract DNA from isolated cultures. For the amplification of the ITS region, universal primers ITS1 (5-TCCGTAGGTGAACCTGCGG-3), ITS4 (5`TCCTCCGCTTATTGATAT GC-3`) are utilized. The thermal Cycler, manufactured by Bioneer Corporation in Korea, is utilized for this process. A total volume of 25 µL is composed of 5 µL of DNA Form, one microliter of F. Primer, one microliter of R. Primer, 1 microliters of Master Mix, 12.5 µL of nuclease-free water, and 5.5 µL of nuclease-free water.

PCR was carried out as follows: followed by 25 cycles at 94°C for thirty seconds, 56°C for 45 seconds and 72°C for one minute; the last extension was performed at 72°C for seven minutes.[16] The PCR result was observed by agarose gel electrophoresis, 2% agarose, 25 ml of TBE buffer, and 0.2 µL of green gel stain. Bioneer also used a 100 bp DNA ladder in Korea. Purific use and sequencing of the region ITS1-5.8S-ITS2 was performed by sending twenty microliters of the DNA of the PCR product for the ITS1-5.8S-ITS2 region to the Macrogen firm in South Korea.[17] Each yeast isolate that was discovered using the National Center for Biotechnology Information (NCBI) blast.

### *Ethical approval*

All subjects gave their informed consent to be included before participating in the study. The study was carried out according to the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Collage of Science in University of Basrah (1165 in Nov. 2022).

## Results

On the basis of the morphological and genetic analysis of yeast isolates, it was discovered that isolated species are related to eleven different species of yeast, as indicated in Table 2.

**Table 2.** Species of yeasts with percentage of occurrence

| No. | *Candida* species | Diaper rash (%) | Oral cavity (%) | Number of case | Occurrence % |
|---|---|---|---|---|---|
| 1 | *C. albicans* | 30 (51.7%) | 28 (48.2%) | 58 | **58%** |
| 2 | *C. tropicalis* | 14 (73.6%) | 5 (26.3%) | 19 | **19%** |
| 3 | *C. parapsilosis* | 0 | 1 (100%) | 1 | **1%** |
| 4 | *Clavispora lusitanaie* | 2 (50%) | 2 (50%) | 4 | **4%** |
| 5 | *Merozyma guiliermonolii* | 2 (100%) | 0 | 2 | **2%** |
| 6 | *Pichia kudriavezevii* | 3 (33.3%) | 6 (66.6%) | 9 | **9%** |
| 7 | *Trichosporon ashii* | 0 | 1 (100%) | 1 | **1%** |
| 8 | *Magunusiomyces capitatus* | 0 | 3 (100%) | 3 | **3%** |
| 9 | *Nakaseomces glabratus* | 1 (100%) | 0 | 1 | **1%** |
| 10 | *Kluyveromyces marxianus* | 0 | 2 (100%) | 2 | **2%** |
| | Total | 52 | 48 | 100 | **100%** |

### *C. albicans*

Colony characteristics: colonies on SDAc after 2 days at 37°C. Colonies appear white to cream-colored, glistening or somewhat waxy, soft, and usually smooth. Some strains may become wrinkled and have a mycelial border. Color of the colony on chrome agar *Candida*, this species produces distinctive light green colonies. Microscopy: Budding cells (sub)spherical, (3-8×2-7) µm. pseudomyclium present (Fig. 1). [18]

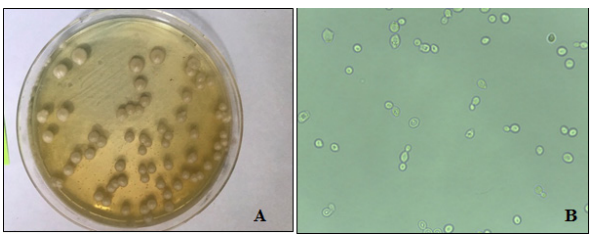


**Fig. 1.** A: Colonies of yeast *C. albicans*, B: yeast cells (B=13.5 µm)

### *C. tropicalis*

Colony characteristics: colonies on SDA after 2 days at 37°C. Colonies appear cream-colored, off-white to grey dull, soft, smooth, and creamy or wrinkled and tough. Color of the colony on chrome agar *Candida*, steel blue to dark gray colonies. Microscopy: ellipsoidal budding cells, characterized by the presence of long, branching components that either bear conidia individually or in small chains or clusters (Fig. 2).[19]

### *Clavispora lusitaniae*

Colony characteristics: colonies on SDA after 2 days at 37°C. These colonies are white to cream-colored, shimmering, and soft and smooth. On chrome agar *Candida*, colonies of this species have a coloration ranging from pink to lavender, and some of them develop a waxy texture on this medium. Microscopy: ellipsoidal budding cells, and often pseudomycelium often present. containing 4 smooth- walled, clavate heterothallic (Fig. 3).[20]

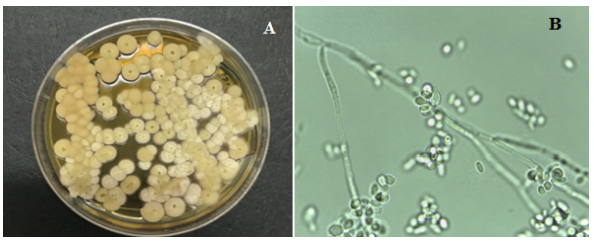


**Fig. 2.** A: Colonies of yeast *C. tropicalis*, B: yeast cells (B=13.5 µm)

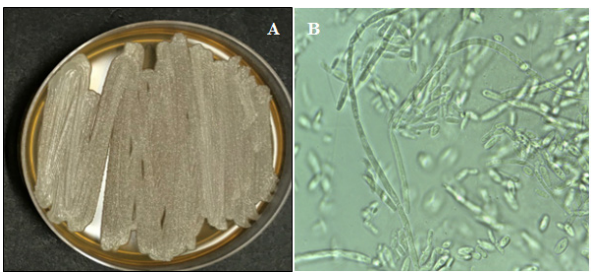


**Fig. 3.** A: Colonies of yeast *C. lusitaniae* , B: yeast cells (B=13.5 µm)

### *P. kudriavzevii*

Colony characteristics: colonies on SDA after 2 days at 37°C. colonies appear white to cream coloured, colonies appear wrinkled and flat smooth. Microscopy reveals that blastoconidia are usually elongated, measuring up to 2 5µm in length. Pseudohyphae are visible with multilateral blossoming arranged in a varied pattern. These cells often exhibit a morphology resembling a matchstick. Pink colonies with rough texture are observed on Candida chromium agar (Fig. 4).[21,22]

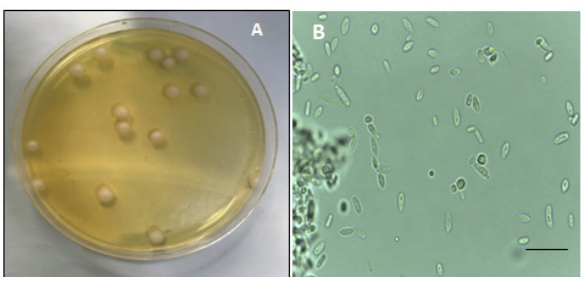


**Fig. 4.** A: Colonies of yeast *P. kudriavzevii*, B: yeast cells (B=13.5 µm)

### *T. asahii*

Colony characteristics: colonies on SDA after 2 days at 37°C. Colonies moderately expending, dry, pustular with white to cream color, farinose or cerebriform sur-

face with radial fissures, deep fissured marginal zone growth at 37°C. Microscopy shows that there are no lateral and budding cells, while arthroconidia are barrel-shaped. No appressoria is present (Fig. 5).[23]

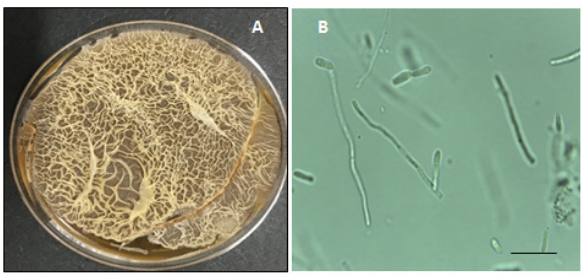


**Fig. 5.** A: Colonies of yeast *T. asahii*, B: yeast cells (B=13.5 µm)

***M. capitatus***

Colony characteristics: colonies on SDA after 2 days at 37°C. Colonies with moderate growth, whitish 24 Microscopy reveals conidiophores that are extensively branched at acute angles, measuring 180-500 µm long conidia cylindrical- clavate, hyaline, 1-celled, with a rounded apex and flat base, measuring (7–10×2.5–3.5) µm. In addition, rectangular arthroconidia are frequently observed. Endoconodia may be present at times (Fig. 6).

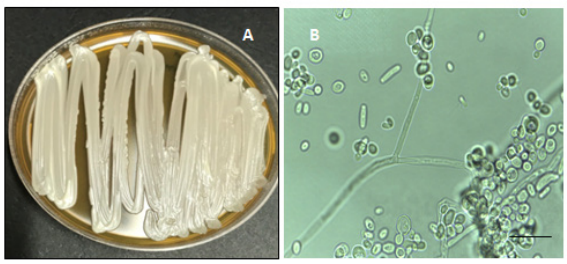


**Fig. 6.** A: Colonies of the yeast *M. capitatus*, B: yeast cells (B=13.5 µm)

***C. parapsilosis***

Colony characteristics: Colonies on SDA after 2 days at 37°C. The colonies are cream-colored to yellowish, shiny, and soft, typically smooth, but may be partially or fully wrinkled.[25] Microscopy: Consisting of branched chains of elongated cells with chains and clusters of spherical to ovoidal conidia forming at intervals along the hyphae (Fig. 7).

***M. guiliermonolii***

Colony characteristics: colonies on SDA after 2 days at 37°C. The colonies are white to cream-colored and butrous Microscopy shows budding cells that are subspherical to broadly ellipsoidal, measuring (3×6 and 2×4) µm. Pseudomycelium may be present, but hyphae are not generated. Colonies of *C. guilliermondii* appear pink to lavender on chrome agar *Candida* (Fig. 8).[26]

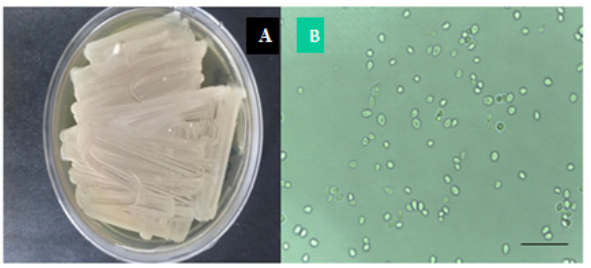


**Fig. 7.** A: Colonies of yeast *C. parapsilosis*, B: yeast cells (B=13.5 µm)

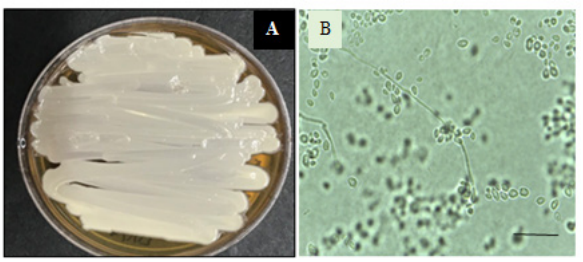


**Fig. 8.** A: Colonies of the yeast *M. guiliermonolii*, B: yeast cells (B=13.5 µm)

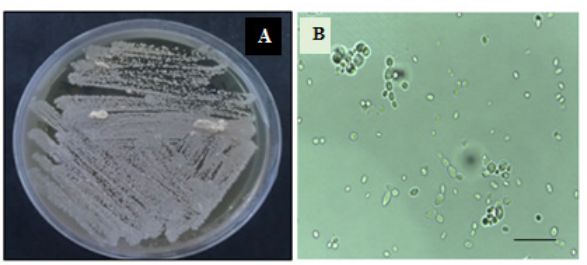


**Fig. 9.** A: Colonies of the yeast *N. glabratus*, B: yeast cells (B=13.5 µm)

***K. marxianus***

Colony characteristics: colonies on SDA after 2 days at 37°C. Colonies appear white to cream-colored butyrous. Microscopy: ellipsoidal cells of the budding cells ellipsoidal, (6–10×3–6) µm, hyphae absent, pseudomyclium absent or present pseudomyclium, containing 1-4 smooth-walled, ellipsoidal to reniform ascospores, homothalic (Fig. 10).[28]

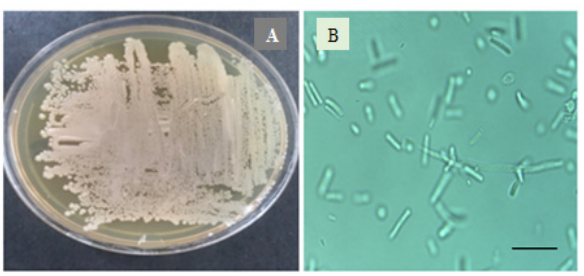


**Fig. 10**. A: Colonies of the yeast *K. marxianus*, B: yeast cells (B=13.5 µm)

***Molecular identification***

ITS1-5.8S-ITS2 rDNA is the gold standard for identifying yeast isolates. It is a fast reliability method compared to biochemical methods, as well as providing the formation of evolutionary relationships for the identification of yeast isolates.[29]

*ITS1-5.8s-ITS2 rDNA*

ITS1-5.8S-ITS2 rDNA of yeast isolates was displayed on agarose gel electrophoresis under UV trans-illuminator at the position 500 base pairs by comparing it with a standard DNA ladder (Fig. 11).

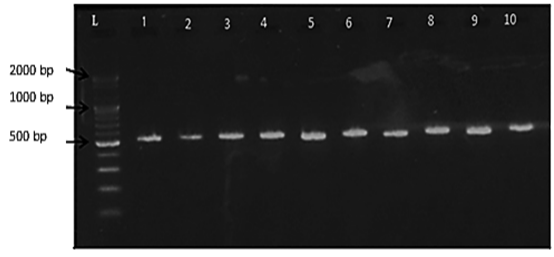


**Fig. 11.** Agarose gel electrophoresis 2% of PCR products for regions containing internal transcribed spacers (ITS1) and ITS2 (including 5.8S rDNA): 100 base pairs of the DNA ladder, lane L *C. albicans* (500 bp) *C. tropicalis* (500 bp), and *C. parapsilosis* (500 bp) are presented in lane 1, lane 2, and lane 3, respectively. Lane 4 has 500 bp of *C. lusitania*, lane 5 contains 500 bp of *M. guiliermonolii*, lane 6 includes 500 bp of *P. kudriavezevii,* and lane 7 contains 500 bp of *T. ashii* of the same species. Regarding yeast isolates, lane 8 contains *M. capitatus*, lane 9 contains *N. glabratus*, and lane 10 has *K marxianus*

## Discussion

***C. albicans***

Table 2 revealed that *C. albicans* exhibited the highest incidence at 58%, indicating its predominance among the identified fungal species. This finding aligns with established knowledge, as *C. albicans* is a common member of the human oral microbiome, colonizing the oral cavity in virtually all individuals.[30] In fact, it is frequently the most prevalent *Candida* species in healthy mouths. In particular, *C. albicans* colonization often begins within the first week of life, affecting both the skin and oral mucosa.[31,32]

The study observed the incidence (48.2%) of oral candidiasis in infants associated with the use of pacifiers. This practice can increase the risk of oral thrush and potentially hinder the efficacy of treatment unless the pacifier is meticulously cleaned after each use.[33]

Furthermore, (51.7%) of diaper dermatitis caused by *C. albicans* were documented. This is attributed to the presence of *C. albicans* in the fecal flora of infants. Diarrhea can increase the risk of infection, particularly in the diaper area. Prolonged diaper contact can further aggravate the severity of the infection.[13]

*C. tropicalis has* emerged as the second most prevalent cause of invasive candidiasis in children, following *C. albicans*. This is consistent with the established role of *C. tropicalis* as a commensal organism that resides on the skin and in the oral cavity.[34]

***C. tropicalis***

Table 2 indicated that *C. tropicalis* represented approximately 19 cases among all fungal infections observed in this study. In particular, this species exhibited a high incidence (73.6%) in cases of diaper rash.

Children, particularly newborns, are highly susceptible to *C. tropicalis* infections due to their immature immune systems. The neonatal intensive care unit (NICU) environment presents unique challenges, as the presence of medical devices can serve as potential entry points for this opportunistic fungus, facilitating the development of invasive candidiasis.

*C. tropicalis* was identified as the causative agent in five out of 19 cases of oral thrush (26.3%). Oral thrush, characterized by the presence of white or yellow patches on the tongue, inner cheeks, and oral mucosa, can cause discomfort and potentially lead to bleeding.[35]

***C. lusitaniae***

In this study, *C. lusitaniae* was found to be present at a rate of 4% of the total recorded species, which is a rather small percentage compared to *C. albicans* and *C. tropicalis*, where these fungi are generally harmless in healthy people, but they can cause cutaneous candidiasis in children when the skin is damaged, the conditions are warm and humid, or the immune system is weakened. *C. lusitaniae* is frequently found in healthcare settings, especially among children with hematologic malignancies and others receiving intensive care.[20,21]

***P. kudriavzevii***

*P. kudriavzevii* is the sexual form of *C. krusei*. A teleomorph is the stage of sexual reproduction of an organism, where *P. kudriavzevii* reproduces sexually by combining haploid cells. The nonsexual phase of a fungus is referred to as an "anamorph".[36] *P. kudriazvevii* may be commensal but can also exhibit pathogenicity. Some may classify *P. kudriavzevii* as an "opportunistic pathogen." *P. kudriavzevii* specifically infects individuals with weakened immune systems.[37] During this study, a species characterized as a rare species was detected, as approximately 9 isolates were diagnosed in the oral cavity and diaper area in infants, the percentage was 66.6% in the oral cavity and 33.3% in the diaper area. The infection is attributed to recurrent diarrhea in children. In

some cases, the infection results from contact with animals or through the environment when they bite sources of this. The species or infection occurs as a result of some systemic diseases that cause a weakened immune system in children, such as cancerous diseases, because this species is opportunistic.[38-40]

***T. asahii***

This species appeared with an incidence of 1%, since it was diagnosed in the oral cavity, and this species colonizes human skin, gastrointestinal tract, and mucosal surfaces as part of the human microbiota.[41,42] Acquiring infection in children is the result of weak immunity, especially in young newborns and children with hereditary blood diseases.[43]

***M. capitatus***

This study showed that the incidence of this species is low compared to the total number of other isolates, as it was diagnosed in three cases, all in the oral cavity. This species lives symbiotically in the intestines, skin, and also in the respiratory system. The infection occurs in children due to weak immunity, and the disease is caused by the presence of cancerous diseases or antibiotics.[44]

***C. parapsilosis***

This species was diagnosed in the oral cavity of samples from hospitalized children, where its presence rate was 1% of the complex of isolated species. This species is usually found on the skin naturally in healthy people, but in the current study, the cause of infection in children was perhaps acquired from the hospital environment. From the central care unit, the infection occurred as a result of weak immunity in children, especially those who have systemic diseases such as cancer, as this species is opportunistic.[45,46]

***M. guiliermonolii***

This species was identified among other fungal species in this study, as about two isolates from diaper rash were diagnosed. This species is found in the mucous membrane and skin and is associated with people who suffer from serious diseases or who have undergone surgeries in the digestive system, heart, and blood vessels. It is also infected with children who suffer from the disease. Immunodeficiency and recent studies have shown a high resistance of this species to antibiotics in recent years.[47]

***N. glabratus***

This species appeared at a rate of 1% in the current study in the diaper area, as this species is considered a common species in the hospital environment, especially in children with HIV and cancer, those who have diabetes, and those who take chemical doses.[48]

***K. marxianus***

It is one of the diseases associated with immunodeficiency and blood diseases. Two isolates from the oral cavity in children during this study. This is due to its association with dairy products, as they can metabolize lactose.[49-51]

## Conclusion

In this study, ten different pathogenic yeast species were isolated that cause infection in children in the Basra province. The phenotypical and genetic characteristics of the condition have been identified through the use of clinically isolated samples from children. Using PCR technology, pathogenic isolates were identified from the face, mouth, and diaper area; *C. albicans* recorded the highest incidence rate of 58%, followed by *C. tropicalis* at 19%, while *C. parapsilosis*, *T. ashii,* and *N. glabratus* recorded 1%. It has virulence characteristics that allow it to cause infection and it can develop at a temperature of 37°C. In addition to infections that are acquired in hospitals.

## Declarations

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The study was funded by the authors.

***Author contributions***

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

***Conflicts of interest***

There is no conflict of interest.

***Data availability***

The data used to support the results of this study can be found in (1165), but their availability is restricted because they were used under license for this work and are therefore not publicly available. However, the Basrah Health Department has granted the authors permission to make the data available on reasonable request.

***Ethics approval***

Before participating in the trial, each participant gave his informed consent. In November 2022, the Ethics Committee of 1165 accepted the protocol and the study was carried out in accordance with the Declaration of Helsinki.

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**ORIGINAL PAPER**

# Risk factors for ischemic stroke in the elderly in Morocco – a case-control study

Hafid Arzoug [1,2], Chouaf Loubna [2,3], Baba Med Amine [1], Amsdar Lahoucine [4], Nawal Adali [1,2,3,5]

[1] NICE research team Neuroscience Innovation Cognition and Ethics, Faculty of Medicine and Pharmacy of Agadir, Ibn Zohr University, Agadir, Morocco
[2] REGNE Research Laboratory "Rein Endocrinology Gastroenterology Neurosciences and Ethics"; Faculty of Medicine and Pharmacy of Agadir, Ibn Zohr University, Agadir, Morocco
[3] Neurology Department, Souss Massa University Hospital, Agadir, Morocco
[4] Biotechnology and Medicine Laboratory, National School of Applied Sciences, Ibn Zohr University, Agadir, Morocco
[5] Faculty of Medicine and Pharmacy of Agadir, Ibn Zohr University, Agadir, Morocco

**ABSTRACT**

**Introduction and aim.** Ischemic strokes occur most frequently in the elderly (more than 80%). This study aimed to determine the risk factors for ischemic stroke in elderly subjects in the Souss Massa region of southern Morocco.

**Material and methods.** Two hundred and thirteen cases and four hundred and thirty-two controls of the same age (76.51±2 years vs. 73.90±2 years) and sex were collected. Cases were selected from patients with ischemic stroke hospitalized in the neurology department of the regional hospital Souss Massa. All risk factors have been documented from interviews and review of patient medical records.

**Results.** Multivariate regression analysis showed that low-income families (odds ratios [OR]=4.19, 95% confidence intervals [95% CI] 2.886–6.09, p<0.001), residence area (urban OR=9.408, 95% CI 4.7133–18.778, p<0.001), high blood pressure (OR=62.984, 95% CI 26.7374–148.367, p<0.001), diabetes (OR=18.138, 95% CI 5.2320–62.880, p<0.001), are risk factors for ischemic stroke. On the contrary, health insurance (OR=0.295, 95% CI 0.1513–0.577, p<0.001) and marital status (in couple OR=0.448, 95% CI 0.284–0.708, p<0.001) are protective factors.

**Conclusion.** In addition to traditional risk factors of ischemic strokes such as high blood pressure and diabetes, low-income family as well as lack of health insurance and marital status (living with a partner) require particular attention. Integrating these new factors into public health strategies could significantly reduce the risk of stroke in the elderly.

**Keywords.** aged, ischemic stroke, Morocco, risk factors

## Introduction

Stroke is a growing problem in developing countries, yet it receives very little attention.[1] Ischemic strokes account for more than 80% of cases in people 65 and over, and these patients often have less effective treatments and less favorable outcomes compared to younger patients.[2] Around 86% of stroke deaths worldwide occur in low and middle-income countries.[3]

**Corresponding author:** Hafid Arzoug, e-mail: hafid.arzoug@edu.uiz.ac.ma

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In Africa, more than half of all stroke patients suffer from hypertension, reflecting trends observed in the Middle East and Eastern Mediterranean regions.[4] In the Arab world, patients often have a lower socioeconomic status, a higher prevalence of diabetes and a higher body mass index than in non-Arab countries, with the incidence of hypertension and the percentage of diabetes exceeding 50%.[5] In the same way, patients with ischemic stroke living in Arab countries had a lower average socioeconomic status, a much higher prevalence of diabetes mellitus, and a higher body mass index than patients in non-Arab countries.[6]

In Morocco, the overall prevalence of ischemic stroke is 284 per 100,000 inhabitants, with a higher incidence in men (306 per 100,000) than in women (278 per 100,000). This prevalence increases significantly with age, reaching 3,409 per 100,000 people over 75 years old.[7] In addition, rural areas (323 per 100,000) and low-income households (346 per 100,000) also have higher prevalence rates.[2] These factors merit research and strategic preventive and curative measures aimed at reducing the risk of ischemic stroke in the elderly population.

## Aim

The aim of this study was to determine the risk factors for ischemic stroke in aged subjects in the Souss Massa region of southern Morocco.

## Material and methods

### *Study design*

The present study is a retrospective data analysis, a case-control study carried out in the center of the neurology care unit of the university hospital of the Souss Massa region in Morocco. This establishment is the reference hospital at the regional level. The cases and controls belonged to the same base population and had the same reference date.

### *Case definition and selection of patients*

The definition of ischemic stroke was a focal neurological deficit of any duration with evidence of cerebral ischemia on the brain CT scanner or magnetic resonance imaging (MRI).

The diagnosis of ischemic stroke was established on the basis of clinical, biological, and imaging data in all patients included in this study.

### *Matching*

Controls are similar to cases in terms of age (>60 years) and gender. Each case is matched to two controls (2 controls per case) who meet the eligibility criteria. Confounding data was avoidedby matching controls and cases by age and sex.

### *Inclusion and exclusion criteria for cases*

Participants aged 60 and over, according to the World Health Organization (WHO) definition of aging, which generally begins at this age and is characterized by a progressive decline in physical and mental capacities, as well as an increased risk of disease.[8] Patients 60 and over, presenting with a constituted ischemic stroke, admitted to the neurology care unit of the University Hospital Center Souss Massa.

The study excluded patients with hemorrhagic stroke or transient ischemic attack (TIA), defined as transient neurological dysfunction without evidence of infarction on brain imaging.[9]

### *Selection of controls*

The control group was classified as patients in the same age group, aged 60 and older, hospitalized in trauma or visceral surgery units of university hospital center Souss Massa.

### *Eligibility criteria for controls*

Controls were selected based on the absence of the disease in the past, as well as the absence of the disease at the time of the study. The controls come from the same target population as the cases and have a potential risk of exposure similar to that of the cases.

### *Data collection*

A data collection form was completed for each respondent who had given written consent, comprising a first section devoted to sociodemographic characteristics. The second part included questions on risk factors associated with the disease: medical history, personal and family history of brain disease, associated comorbidities, and lifestyle (eating habits and toxic habits). Data was collected for controls in the same way as for cases, with information on sociodemographic variables and risk factors associated with ischemic stroke.

### *Risk factors for ischemic stroke*

All traditional risk factors have been documented from interviews and review of patient medical records; incomplete records were rejected. The analysis concerns the diagnostic workup performed on admission, vital parameters, and laboratory tests. Patients diagnosed with blood pressure (BP) (>140/90 mmHg) or with antihypertensive medication before or at the time of stroke. Patients diagnosed with diabetes or taking anti-diabetic medication, or with fasting blood glucose levels above 120 mg/dl. Patients were considered smokers if they smoked or if there was the notion of smoking during the year preceding the stroke. Hyperlipidemia was defined based on history and lipid-lowering treatment, or a total cholesterol level greater than 200 mg/dL or a low-density lipoprotein level greater than 120 mg/dL.

The notion of obesity was raised or diagnosed based on body mass index (BMI) calculations in certain cases where weight and height were recorded. A previous diagnosis of vascular risk factors or cerebrovascular damage is retained if confirmed by specialists during the patient's hospitalization.

We evaluated from medical records the notion of heart disease; family history of stroke; personal history of unspecified brain injury; alcohol consumption or report of alcoholism; use of oral contraception was mentioned based on patient history, given that the study population was comprised of elderly patients. For COVID-19, human immunodeficiency virus (HIV) and hepatitis C (HCV), serology, and syphilitic serology; we noted patients who had already contracted the disease at least once before suffering from ischemic disease. Patients were interviewed about their diets: diabetic, low salt, both, or no diet at all.

***Sample size and calculation method***

In this study, the desired statistical power was 80% with a confidence level of 95% confidence intervals, the control/case ratio is two (2), and alpha risk α is 5%.

There was one case for every two controls, because the prevalence of exposure is not very high in the target population and these controls are easy to obtain.

The sample size is calculated according to the open Epi standards of the epidemiological statistics software, the result obtained was as follows:

Sample size two hundred and thirteen (213) cases aged 60 and over. For the controls, two controls/1 case±10 are set, for a total of 460 two (432) controls. An increase of 10 controls was added to the sample size obtained to compensate for patients who did not wish to participate in the study.

***Statistical analysis***

Data will be entered and analyzed using JAMOVI 2.3.21 software. Categorical variables are expressed as headcount and percentage, while quantitative variables are expressed as mean and standard deviation and median (25% quartile, 75 per cent quartile). The Chi2 test was used to compare groups (Chi2) or the Fisher exact test according to the application conditions of each. The results will be obtained in the form of univariate and multivariate analysis. The degree of association between risk factors and disease will be measured by odds ratios (OR) and 95% confidence intervals (95% CI). All independent variables with a $p<0.25$ in the univariate analysis were taken into account in the multivariate logistic regression analysis. $p<0.05$ were considered to indicate statistical significance.

***Ethical considerations***

The study has been approved by the Ethics Committee for biomedical research of the Mohammed V Faculty of Medicine and Pharmacy in Rabat (N/R: Folder Number 03/23), and informed consent was obtained from each subject. In this study, the ethical standards of the institutional and/or national research committee were applied in all procedures carried out with human participants. The confidentiality and anonymity criteria were respected as charted in the Declaration of Helsinki and its subsequent amendments.

## Results

***Socio-demographic***

This study included two hundred thirteen (213) cases experiencing ischemic stroke for the first time and four hundred and thirty-two (432) age- and sex-matched controls. The mean age was 76.51± 2 years for cases (median 68.0 years) of patients with ischemis stroke and 73.90±2 years (median 68 years) for controls. The male/female ratio was 0.6 in each group.

There is a statistically significant difference between the case and control groups regarding sociodemographic characteristics: sex ($p=0.006$), marital status ($p<0.001$), professional occupation ($p<0.001$), residential location ($p<0.001$), health coverage ($p<0.001$), and living conditions ($p=0.008$) (Table 1).

***Comorbidities, medical history, and lifestyle factors (diet and substance use)***

Significant differences were observed between the case and control groups in terms of associated comorbidities, medical history, and lifestyle (dietary habits and substance use): history of hypertension ($p<0.001$), diabetes ($p<0.001$), hypercholesterolemia ($p<0.001$), other cardiac diseases ($p<0.001$), smoking ($p<0.001$), adherence to a dietary regimen ($p<0.001$), regular physical exercise ($p=0.001$), previous infection COVID-19 infection ($p<0.001$), HCV ($p<0.001$), HIV serology ($p<0.001$) and adherence to diet ($p=0.001$) (Table 2).

***Sociodemographic factors, comorbidities, and lifestyle associated with ischemic stroke in the aged: univariate logistic regression***

According to the univariate logistic regression analysis, the following factors were significantly associated with ischemic stroke in the aged: low income (OR=13.97; 95% CI: 5.87–33.26; $p<0.001$), urban residence (OR=9.41; 95% CI: 4.71–18.78; $p<0.001$), health insurance (OR=0.30; 95% CI: 0.15–0.58; $p<0.001$), marital status (married) (OR=0.45; 95% CI: 0.28–0.71; $p<0.001$), living condition: not alone, (OR=0.36; 95% CI: 0.16–0.79; $p<0.011$), hypertension (OR=39.75; 95% CI: 24.88-63.53; $p<0.001$), diabetes (OR=18.14; 95% CI: 12.26–28.29; $p<0.001$), dyslipidemia (OR=4.48; 95% CI: 2.95–6.82; $p<0.001$), cardiac disease (OR=3.03; 95% CI: 1.97–4.66; $p<0.001$), smoking (OR=2.28; 95% CI: 1.91–2.73; $p<0.001$), alcohol use (OR=0.87; 95% CI: 0.50–1.52; $p<0.001$), lack of dietary regimen (OR=0.15; 95%

CI: 0.07–0.32; p<0.001) and regular physical exercise (OR=4.88; 95% CI: 3.06–7.79; p<0.001) (Table 3).

**Table 1.** Socio-demographic characteristics of the study sample*

| Variables | Case, n (%) | Control, n (%) | p |
|---|---|---|---|
| Age | | | |
| 60–70 | 55 (8.5) | 120 (18.6) | |
| >70 | 158 (24.5) | 312 (48.4) | 0.599 |
| Sex | | | |
| Male | 97 (15) | 148 (22.9) | |
| Female | 116 (18) | 284 (44) | 0.006 |
| Marital status of the patient | | | |
| With partner or without partner | 170 (26) | 388 (26.2) | |
| Marital status of the patient | | | <0.001 |
| Married | 121 (26.5) | 389 (60.3) | |
| Single | 2(0.3) | 0.00 | <0.001 |
| Divorced | 5 (0.8) | 11 (1.7) | |
| Widowed | 35 (5.4) | 32 (5) | |
| Level of education of patient | | | |
| Illiterate | 180 (27.9) | 373 (57.8) | |
| With instruction | 33 (5.1) | 59 (9.1) | 0.531 |
| Illiterate patient | | | |
| Yes | 182 (22.2) | 376 (58.3) | |
| No | 31 (4.8) | 56 (8.7) | 0.578 |
| Professional occupation | | | |
| Employed | 12 (1.9) | 1 (0.2) | |
| Self-employed | 27 (4.2) | 296 (45.9) | <0.001 |
| Inactive (unemployed) | 143 (22.2) | 132 (20.5) | |
| Retired | 31 (4.8) | 3 (0.5) | |
| Professional occupation DOT | | | |
| classification | 12 (1.9) | 0 | |
| Manual | 31 (4.8) | 291 (45.1) | <0.001 |
| Non manual | 170 (26.4) | 141 (26.4) | |
| Inactive | | | |
| Socioeconomic level (family income) | 4 (0.6) | 0 | |
| Rich | 58 (9.0) | 367 (57) | <0.001 |
| Middle class | 150 (23.3) | 65 (10.1) | |
| Poor | | | |
| High income family | 5 (0.8) | 8 (1.2) | 0.674 |
| Yes | 208 (32.2) | 424 (65.7) | |
| No | | | |
| Middle income family | 52 (8.1) | 79 (12.2) | |
| Yes | 161 (25) | 353 (54.7) | |
| No | | | |
| Low-income family | 164 (25.5) | 194 (30.1) | 0.069 |
| Yes | 48 (7.5) | 238 (37) | |
| No | | | |
| Residential location (rural or urban) | 136 (21.1) | 86 (13.4) | <0.001 |
| Yes | 76 (11.8) | 346 (53.7) | |
| No | | | |
| Health coverage (insurance) | 79 (12.2) | 237 (36.7) | <0.001 |
| Without insurance | 134 (20.8) | 195 (30.2) | |
| With insurance | | | |
| Living conditions | 8 (1.2) | 42 (6.5) | 0.008 |
| Alone | 202 (31.7) | 390 (60.6) | |
| Not alone | | | |

* Chi2 test (Fisher's exact test)

***Factors associated with ischemic stroke in the aged: multivariate logistic regression***

According to multivariate logistic regression analysis, the following factors were significantly associated with ischemic stroke in the aged: low income (OR=13.97; 95% CI: 5.87–33.26; p<0.001), urban residence (OR=9.41; 95% CI: 4.71–18.78; p<0.001), health coverage (OR=0.30; 95% CI: 0.15–0.58; p<0.001), hypertension (OR=62.98; 95% CI: 26.74–148.37; p<0.001), diabetes (OR=18.14; 95% CI: 12.26–28.29; p<0.001) and marital status (married) (OR=0.45; 95% CI: 0.28–0.71; p<0.001) (Table 4).

**Table 2.** Characteristics related to associated comorbidities, medical history and lifestyle (eating habits and toxic habits)*

| Variables | Case | Control | p |
|---|---|---|---|
| Past history of hypertension (comorbid diseases) | | | |
| Yes/No | 145 (22.5)–64 (9.9) | 64 (9.9)–368 (57.1) | <0.001 |
| Diabetes | | | |
| Yes/No | 73 (11.3)–140 (21.7) | 45 (7.0)–387 (60.0) | <0.001 |
| Past history of hypercholesterolemia (comorbid diseases) | | | |
| Yes/No | 57 (8.9)–157 (24) | 47 (7.3)–385 (59.9) | <0.001 |
| Past history of other cardiac diseases | | | |
| Yes/No | 1 (0.2)–31 (4.8) | 8 (1.2)–16 (2.5) | <0.0001 |
| Notion of smoking | | | |
| Yes/No | 20 (3.1)–192 (29.8) | 46 (7. 1)–386 (59.9) | 0.419 |
| Alcoholism | | | |
| Yes/No | 50 (7.8)–14 (2.2) | 48 (7.5) –12 (1.9) | 0.633 |
| Regular physical exercise (life style behaviors) | | | |
| Yes/No | 5 (0.8)–208 (32.2) | 0–432 (67) | 0.399 |
| Syphilis serology | | | |
| Yes/No | 207 (32.1)–6 (0.9) | – | <0.001 |
| Previous notion of COVID-19 status | | | |
| Yes/No | 0–213 (33) | 432 (67) | <0.001 |
| Serology HCV status | | | |
| Yes/No | 0–213 (33) | 0–432 (67) | <0.001 |
| HIV serology status | | | |
| Yes/No | 0–213 (33) | 0–432 (67) | <0.001 |
| History of stroke family | | | |
| Yes/No | 29 (4.5)–184 (28.5) | 39 (6)–393 (60.9) | 0.074 |
| Psychoaffective history | | | |
| Yes/No | 209 (32.4) | 3 (0.5)–429 (66.5) | 0.172 |
| Past history of others brain diseases | | | |
| Yes/No | 8 (1.2)–201 (31.4) | 12 (1.9)–420 (65.5) | 0.474 |
| History of smoking (toxic habits) | | | |
| Occasional smoker | 1 (0.2) | 8 (1.2) | <0.001 |
| Daily smoker | 31 (4.8) | 16 (2.5) | |
| Ex-smoker | 60 (9.4) | 11 (17.3) | |
| Never smoked | 149 (23.2) | 321 (50.1) | |
| Alcoholism | | | |
| Yes/No | 50 (7.8)–14 (2.2) | 48 (7.5)–12 (1.9) | 0.633 |
| Followed diet | | | |
| Diabetic diet | 4 (0.6) | 13 (2) | <0.001 |
| Hyposode diet | 209 (32.4) | 419 (65) | |
| Diabetic diet and hyposode diet | 5 (0.8) | 0 | |
| No diet | 208 (32.2) | 432 (67) | |

* Chi2 test (Fisher's exact test)

## Discussion

Ischemic stroke is a multifactorial disease governed by modifiable and non-modifiable risk factors. Other less well-documented risk factors include geographical location and socio-economic status.[10] Identifying these risk factors in the elderly is crucial to reducing the incidence of disability and the costs associated with stroke.

This study of elderly people in the Souss Massa region of Morocco confirms that the main modifiable risk factors for ischemic stroke are hypertension, diabetes, smoking, and heart disease.

**Table 3.** Sociodemographic, comorbidity and lifestyle, dietary habits, toxicity, and ischemic stroke associated with ischemic stroke (univariate logistic regression)

| Variables | OR | 95% CI | | p |
|---|---|---|---|---|
| Education level patient: with instruction – illiterate | 0.86 | 0.544 | 1.37 | 0.531 |
| Professional occupation: | 1.47 | 0.57 | 4.25 | 0.978 |
| Manual – no manual | 1.30 | 0.87 | 3.87 | 0.981 |
| Inactive – no manual | | | | |
| High-income family: no – yes | 1.27 | 0.41 | 3.94 | 0.674 |
| Middle-income family: no – yes | 1.44 | 0.91 | 2.15 | 0.070 |
| Low-income family: nes – no | 13.97 | 5.87 | 33.25 | <0.001 |
| Residence area: urban –rural | 9.40 | 4.71 | 18.77 | <0.001 |
| Health insurance: with assurance – without assurance | 0.29 | 0.15 | 0.57 | <0.001 |
| Living condition: not alone – alone | 0.36 | 0.16 | 0.79 | 0.011 |
| Marital status: no partner – partner | 0.44 | 0.28 | 0.70 | <0.001 |
| Stroke history in the family | 1.59 | 0.95 | 2.65 | 0.076 |
| Arterial hypertension | | | | |
| Yes/ No | 39.75 | 24.87 | 63.52 | <0.001 |
| Diabetes | | | | |
| Yes/No | 18.14 | 12.26 | 8.28 | <0.001 |
| Dyslipidemia | | | | |
| Yes/No | 4.48 | 2.95 | 6.81 | <0.001 |
| Cardiac disease | | | | |
| Yes/No | 3.03 | 1.974 | 4.66 | <0.001 |
| History of cerebral disease | | | | |
| Yes/No | 1.39 | 0.56 | 3.46 | 0.475 |
| Stroke history in family | | | | |
| Yes/No | 0.07 | 0.95 | 2.65 | 0.076 |
| History of personnel disease cerebral | 1.39 | 0.56 | 3.46 | 0.475 |
| Psycho-affective history | | | | |
| Yes/No | 2.73 | 0.60 | 12.34 | 0.190 |
| BMI class: obesity – overweight | 1.02 | 0.68 | 1.53 | 0.918 |
| History of hormonal contraception | | | | |
| Yes/No | 2.11 | 1.05 | 4.23 | 0.093 |
| Notion of smoking | | | | |
| Yes/No | 2.28 | 1.90 | 2.73 | <0.001 |
| History of alcoholism | | | | |
| Yes/No | 0.87 | 0.503 | 1.52 | <0.001 |
| Diet followed | | | | |
| Yes/No | 0.14 | 0.0678 | 0.31 | <0.001 |
| Regular exercise | | | | |
| Yes/No | 4.88 | 3.06 | 7.78 | <0.001 |

**Table 4.** Factors associated with ischemic stroke (multivariate logistic regression)

| **Variables** | **OR** | **95% CI** | | **p** |
|---|---|---|---|---|
| Low-income family (yes) | 13.97 | 5.87 | 33.25 | <0.001 |
| Residence area (urban) | 9.40 | 4.71 | 18.77 | <0.001 |
| Health insurance (with assurance) | 0.29 | 0.15 | 0.57 | <0.001 |
| High blood pressure (HBP) (yes) | 62.98 | 26.73 | 148.36 | <0.001 |
| Diabètes (yes) | 18.13 | 5.23 | 62.88 | <0.001 |
| Marital status (couple) | 0.44 | 0.28 | 0.70 | <0.001 |

In this study, the main modifiable risk factors for ischemic stroke in elderly people in the Souss Massa region of Morocco were: hypertension (OR=62.984), diabetes (OR=18.138) and dyslipidemia (11.3%). Smoking and alcoholism were not considered risk factors for ischemic stroke in the study population. This may be due to the age and socioeconomic status of the study population. On the other hand, and in the same vein, studies have reported that smoking and alcoholism are risk factors for ischemic stroke.[11,12]

In this study, hypertension was significantly associated with stroke, as in other studies of this kind.[13,14] In addition, heart disease and ischemic stroke share modifiable risk factors such as hypertension, lipid abnormalities, smoking, sedentary lifestyle, obesity, and diabetes.[15]

In the same vein, a systematic review of ischemic stroke in Morocco reports that hypertension (31–65.4%), diabetes (12–41.8%), smoking (4–41.8%), and heart disease (7–44.3%) are the four main risk factors for ischemic stroke. In fact, another study reports that this was due to the westernization of Moroccan behavior and eating habits.[16]

This study reports that certain risk factors were not included. On the other hand, one study reported that associated heart disease (2.5–22%), dyslipidemia (0–61.8%), obesity (10.7–26.1%), previous stroke (5–26.6%), alcoholism, oral contraceptives (6.6–12.2%), and migraine (6.5%) were all risk factors.[17] Similarly, more than half of ischemic stroke patients in Africa suffered from hypertension.[18] These results are consistent with studies carried out in the Middle East between 1980 and 2000, and with data from Eastern Mediterranean countries where hypertension and diabetes are prevalent at rates in excess of 50%.[19]

These results underline the importance of adopting prevention strategies targeted at these risk factors, and promoting healthy lifestyles among this vulnerable population, in order to reduce the incidence and burden of the disease.

In another sense, the results of this study showed that living conditions, marital status and place of residence were risk factors for ischemic stroke in the study population. These results are in line with those reported in other studies, and at odds with other countries in North Africa and the Middle East.[6,21,20] In fact, around 80% of strokes could be prevented by simple lifestyle changes. Further research into the role and interaction between living conditions, marital status and place of residence will improve our understanding of ischemic stroke and help develop more effective prevention programs for high-risk groups.[10]

Our study indicates that, for the population studied, living in an urban environment offers protection against ischemic stroke. In fact, the region's population is divided into 1,505,896 urban dwellers and 1,170,951 rural dwellers. Although the region remains predominantly rural, it is nevertheless undergoing strong urbanization, reflected in an urban growth rate of 3.2%, compared with a decrease of -0.5% in rural areas.[20] In fact, studies report that the high frequency of this risk factor is due to rapid urbanization (60.3% of the population) and lifestyle changes in the Moroccan population that allow rapid access to healthcare.[21] In addition, one study indicates that living in rural areas is associated with an increased risk of stroke and death. Therefore, future efforts should not only focus on controlling

known vascular risk factors, but also examine other determinants of health in rural areas.[7]

Living environment influences the risk of ischemic stroke due to factors such as access to care, lifestyle habits, and socioeconomic conditions.[22–24] In another sense, this study reports no association between ischemic stroke and a family history of ischemic stroke. Similarly, two other studies confirm these results, indicating that the correlation between this risk factor and the disease is not representative.[25,26] With regard to marital status, the study shows that living with a partner and having social security coverage are protective factors against ischemic stroke among elderly people in the Souss Massa region. According to the present study, living as a couple attenuates the increased risk of stroke. Although our results do not suggest a significant gender difference in stroke risk after marital transition. In addition, another study showed the same result in young subjects, and that living alone without being married was one of the independent risk factors for ischemic stroke.[27] On the other hand, one study reported that an increased risk of stroke was observed in men and women who had undergone a matrimonial transition (married or unmarried), Participants in matrimonial transition and living with children had an increased risk of stroke and in particular among those with an average level of education.[28] In terms of social security coverage, the incidence of multidimensional poverty in the study area is 8.2%, the national average, and higher in rural areas (13.7%) than in urban areas (2.1%). Despite significant progress, Morocco's social protection system faces a number of challenges, including unequal access to health coverage, financial difficulties and disparities between different insurance schemes. In addition, the lack of access to high-performance diagnostics and care has given rise to some discontent.[29] in the same sense that uninsured patients were less likely to take appropriate control medication prior to stroke, to use an ambulance to get to the emergency room, or to arrive promptly after the onset of symptoms. As a result, these uninsured patients had a higher in-hospital mortality rate than privately insured patients, with a relative risk (OR) of 1.33 for those under 65 (95% CI 1.22–1.45) and 1.54 for those 65 and over (95% CI 1.34–1.75).[30] Similarly, the disproportionate increase in stroke incidence in low and middle-income countries has been attributed to environmental factors.[5] Furthermore, in a cohort study of a population of six million with universal access to medical and hospital services, stroke risk factors were widespread but less well controlled in rural residents.[5]

### *Study limitations*

The present study represents the first investigation in Morocco which attempted to explore the risk factors of cerebral ischemia with a case-control design, in line with the findings of a systematic literature review in Morocco and which explained the cross-sectional nature of the majority of included studies.[17] Nevertheless, some limitations were raised in relation to the methodological and empirical aspect of the study. In this respect, the case-control study opted for a hospital reference population. In addition, risk factors, particularly those related to atrial fibrillation and carotid artery stenosis, were not specifically studied, due to a traceability constraint. The study was limited to the presence or absence of any notion of heart disease in the patient's medical record.

## Conclusion

In addition to traditional risk factors for ischemic stroke, such as high blood pressure and diabetes, this study revealed that living in a low-income family is also a risk factor for this disease. On the other hand, having social security coverage and living in urban areas and in couples are protective factors for the elderly. Integrating these elements into public health strategies and future research could significantly improve the quality of life of elderly people affected by ischemic stroke.

## Declarations

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There is no funding for this study.

### *Author contributions*

Conceptualization, A.H., C.L. and N.A, Methodology, A.H., M.A.B. and A.N.; Software, A.L.; Validation, A.H., A.N. and M.A.B.; Formal Analysis, A.H. and A.N.; Investigation, A.H. and C.L.; Resources, None; Data Curation, A.H. and A.L.; Writing - Original Draft Preparation, A.H.; Writing - Review & Editing, A.H., C.L., A.L., M.A.B. and N.A.; Visualization, A.H., C.L. and A.L; Supervision, A.N.; Project Administration, A.H. and N.A.

### *Conflicts of interest*

The authors declared no conflicts of interest for this paper.

### *Data availability*

Data available on request from the authors.

### *Ethics approval*

The ethical approval was granted by the Ethics Committee for Biomedical Research of the Faculty of Medicine and Pharmacy Mohammed V in Rabat, Morocco (Reference: CERB 03/23).

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**ORIGINAL PAPER**

# D-dimer as a potential biomarker in chronic obstructive pulmonary disease

Anish V. Patel [1], Mayurika S. Tyagi [2], Adreena Mittal [1], Sumit Chandra [1], Hina Kausar [1], Sivanesan Dhandayuthapani [3]

[1] Department of Pathology, Santosh Medical College & Hospital, Santosh Deemed to be University, Ghaziabad, Delhi, Uttar Pradesh, India
[2] Department of Immunohematology and Blood Transfusion, Santosh Medical College & Hospital, Santosh Deemed to be University, Ghaziabad, Delhi, Uttar Pradesh, India
[3] Central Research Facility, Santosh Deemed to be University, Ghaziabad, Delhi, Uttar Pradesh, India

**ABSTRACT**

**Introduction and aim.** Despite signs of drop in tuberculosis in the middle of the twentieth century, up to 75% of men were smokers at that time, which contributed to the epidemic of chronic obstructive pulmonary disease (COPD) in the latter half of the century. The present study was conducted with the main focus of establishing a relation between D-dimer and lung function in patients with COPD.

**Material and methods.** A hospital-based observational cross-sectional study involved 108 subjects, divided into 54 cases (COPD patients) and 54 healthy controls (41-80 years old). The dry volume spirometer was used to assess the lung health of the study population. D-dimer assay was performed on peripheral blood drawn from study subjects using the second generation latex-enhanced immunoturbidimetric assay on the Diagon Fully Automatic COAG XL Coagulation Analyzer.

**Results.** Spirometry tests revealed COPD patients showing reduced lung function (42.59% with normal, 51.85% with mild, and 5.56% with moderate degree of forced expiratory volume in 1 second and forced expiratory volume in 1 second/ forced vital capacity). Patients with COPD under different age groups and both the genders showed an elevated level ($p<0.05$) of D-dimer in correlation with the spirometry measurements.

**Conclusion.** The D-dimer is promising plasma biomarker which demonstrated a strong correlation with the spirometry measurements and different morphological categories in patients with COPD. The D-dimer could serve as a reliable biomarker for validating and confirming the various morphological classifications among individuals with COPD.

**Keywords.** chronic obstructive pulmonary disease, D-dimer, inflammatory markers, smoking, spirometry

## Introduction

In India, chronic obstructive pulmonary disease (COPD) is considered the second most common cause of death with disability adjusted life years. In addition, 7% of Indian adults over 30 years of age have had COPD.[1] Over the years, the smoking epidemic has been rapidly declining in western nations, but things are different in other parts of the world. COPD was one of the leading causes of illness and death worldwide in the 2020s, with a predicted increase in morbidity.[1]

COPD is a progressive lung disease characterized by persistent airflow limitation. The pathophysiology

**Corresponding author:** Mayurika S. Tyagi, e-mail: somyamayuri@hotmail.com

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of COPD involves chronic inflammation of the airways and lung tissue, primarily due to long-term exposure to harmful particles like cigarette smoke (Fig. 1). This leads to structural changes in the lungs, including airway remodeling, mucus hypersecretion, and destruction of alveolar walls (emphysema), which alter gas exchange. Inflammation in COPD is primarily driven by immune cells such as neutrophils, macrophages, and T lymphocytes, which release pro-inflammatory cytokines and proteases. These mediators contribute to airway narrowing, fibrosis, and alveolar destruction. The imbalance between proteases and antiproteases (e.g., α1-antitrypsin deficiency) accelerates lung tissue damage. Additionally, oxidative stress plays a crucial role in exacerbating inflammation and tissue injury. As the disease progresses, patients experience increased dyspnea, reduced lung function, and frequent exacerbations, significantly affecting quality of life.[2,3]

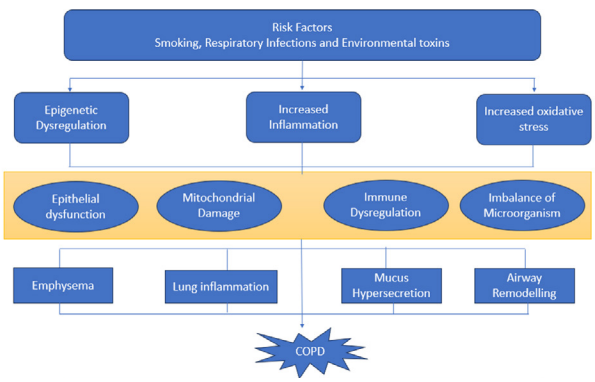


**Fig. 1.** Pathophysiology of COPD

Most of morbidity associated with COPD worldwide usually begins with persistent respiratory symptoms, such as progressive dyspnea, sputum production, coughing, wheezing, and tightness in the chest.[4] With some daily fluctuation, symptoms manifest differently from asthma. In addition, periods of clinical stability are sometimes interspersed with abrupt worsening, also known as acute exacerbations. However, some patients also reported non-respiratory symptoms such as anxiety, depression, anorexia, exhaustion, and weight loss.[5,6]

A D-dimer is a protein fragment that the body makes when a blood clot dissolves in the body. The D-dimer is normally undetectable or detectable only at a very low level unless the body forms and breaks down significant blood clots. The presence of D-dimers in the blood is a reliable clue that clotting has begun. The study evaluated that patients with COPD are prone to clinical exacerbations that may be associated with a powerful prothrombotic stimulus. Prothrombotic markers are also significantly altered in the state of COPD and exacerbation, which could predispose to venous thromboembolism in these patients, thus modifying the severity of the disease.[7]

COPD is a curable and preventable. However, it also has some important extrapulmonary side effects that may influence a patient's condition with nonreversible airflow limitation as the hallmark of its pulmonary component.[8] The restriction of airflow is typically progressive and linked to an aberrant inflammatory reaction of the lungs to harmful particles or gases.

## Aim

The purpose of this study is to assess the clinical value of specific biomarkers in chronic diseases by evaluating their ability to diagnose and predict disease outcomes. As precision medicine gains momentum, it is crucial to identify biomarkers that accurately reflect disease progression and treatment response. Additionally, exploring the biological pathways linked to these biomarkers can offer valuable insight into disease development and possible therapeutic targets. The current study specifically examines the biomarker with significant translational potential, focusing on their ability to predict disease progression and response to treatment. The novelty of this study stems from its robust methodological approach, the diverse functionality of patients, and the focus on practical application, which makes it relevant to both researchers and clinicians. The results have the potential to influence future guidelines, contributing to more personalized and effective treatment of COPD.

## Material and methods

A hospital observational cross-sectional study conducted in the Central Clinical Laboratory of Pathology in a tertiary care hospital at NCR-Delhi. Men and women in all age groups attending the outpatient and inpatient department of the Department of Medicine from January 2023 to January 2024 in a tertiary care hospital with a confirmed diagnosis of COPD were involved in the present study with proper informed written consent along with healthy persons with the same age and sex. The study was carried out after receiving approval from the Institutional Ethics Committee (IEC) Lr. No.: SU/2022/3108[37]

Sample size calculated by using the formula:

$$n = [Np(1 - p)]/[(d2/Z12 - \alpha/2*(N - 1) + p*(1 - p)]$$

Where,

- N: Population size (for finite population correction factor) 60
- *p:* Hypothesized % frequency of outcome factor in the population 16.67%+/-3.2
- *d:* Confidence limits as % of (absolute +/-%) 3.2%

With 95% confidence interval. The study group consists of a total of 108 subjects, including 54 cases and 54 controls.

Inclusion and exclusion of cases: known cases of COPD and those who have given their informed consent were included in the study. Whereas patients with

comorbid conditions such as sepsis, pulmonary embolism, venous thromboembolism, patients on anticoagulant therapy, known cases of malignancy, and patients who refuse to sign the written informed consent form were excluded from the current study.

The relevant patient history, including the time of diagnosis of COPD, past history, and treatment, was obtained from the patients' outpatient and inpatient department files according to the standard proforma. The dry volume spirometer was used to assess the lung health of the study population by a set of well-trained residents and technicians. The dry volume spirometer, which was used in other large population-based studies, such as the European Community Respiratory Health Survey (ECRHS).[9] The calibration / quality check of the spirometers was carried out daily basis following the American Thoracic Society standardization of spirometry.[10] Furthermore, the D-dimer assay was performed on peripheral blood drawn from study subjects under aseptic precautions in a 3.2% sodium citrate vacutainer tube with a blood to anticoagulant ratio of 9: 1. Following the manufacturer's instructions, the D dimer assay was done using the second generation latex-enhanced immunoturbidimetric assay, which is Dia-D dimer kit (Catalog No. 32120) from Diagon, on the Diagon Fully Automatic COAG XL Coagulation Analyzer. It is a fully automatic, quantitative assay, using agglutination and photometric analysis to measure the D-dimer levels. In the presence of D-dimer antigens, antibody-coated latex beads will cause agglutination. In the presence of D-dimer antigens, agglutination occurs because of antibody-coated latex beads which are directly proportional to the degree of light absorption, as measured by the integrated absorbance reader of the COAG XL analyzer (Budapest, Hungary).

### *Statistical analysis*

Data were collected and entered in MS Excel 2021. Different statistical analyzes were performed using SPSS trial software (IBM, Armonk, NY, USA).

## Results

The study consists of a total of 108 subjects, including 54 cases and 54 controls (Table 1).

**Table 1.** Distribution by age of study subjects

| Age distribution | Control | | COPD patients | |
|---|---|---|---|---|
| | Frequency | Percentage | Frequency | Percentage |
| 41–50 | 23 | 42.59 | 26 | 48.15 |
| 51–60 | 16 | 29.63 | 13 | 24.07 |
| 61–70 | 9 | 16.67 | 8 | 14.81 |
| 71–80 | 6 | 11.11 | 8 | 12.96 |

The control and study subjects ranged from 41 to 80 years of age with maximum subjects in the 41 to 50 years age group (42.59% controls and 49.15% cases) followed by the 51 to 60-year age group. (Table 1). The male to female ratio in the control group was 1:1 (27 males and 27 females) and for the COPD patient group was 1.07:1 (28 males and 26 females).

There was no significant difference in the number of male and female participants between the two study groups.

The distribution of control and COPD patients according to their occupations showed that among study subjects, 37.04% of the control group and 31.48% of the COPD group were farmers. Additionally, 35.19% of the control and COPD groups were manual workers, while 27.78% of the control group and 33.33% of the COPD group were skilled professionals (Table 2).

**Table 2.** Occupational distribution of the study subjects

| Occupation | Control | | COPD patients | |
|---|---|---|---|---|
| | Frequency | Percentage | Frequency | Percentage |
| Farmer | 20 | 37.04 | 17 | 31.48 |
| Manual | 19 | 35.19 | 19 | 35.19 |
| Skilled/Professional | 15 | 27.78 | 18 | 33.33 |

The degrees of forced expiratory volume in 1 second (FEV1) of the study groups are presented in Table 3. It is clearly recorded that 94.44% of control subjects with normal and only 5.56% with mild degree of FEV1 and FEV1/forced vital capacity (FEV1/FVC) were recorded. However, in patients with COPD only 42.59% with normal, 51.85% with mild, and 5.56% with moderate degree of FEV1 and FEV1/FVC were recorded. No patient found with sever degree of FEV1 and FEV1/FVC was found (Table 3). The results observed in COPD patients were significant ($p<0.05$) on comparison with the normal subjects.

**Table 3.** Degree of 'FEV1' and "FEV1/FVC ratio" of the study subjects

| Category | Category | Control | | COPD patients | |
|---|---|---|---|---|---|
| | | Frequency | Percentage | Frequency | Percentage |
| GOLD I (>80%) | FEV1/FVC (>0.7) | 51 | 94.44 | 23 | 42.59 |
| GOLD II (50 to 80%) | FEV1/FVC (<0.7) | 3 | 5.56 | 28 | 51.85 |
| GOLD III (30 to 50%) | FEV1/FVC (<0.6) | 0 | 0 | 3 | 5.56 |
| GOLD IV (<30%) | FEV1/FVC (<0.5) | 0 | 0 | 0 | 0 |

The results recorded from the control and COPD patients with respect to forced expiratory flow (FEF) 25% to 75% as tabulated in Table 4 the FEV1 and FEV1/FVC observed in the control and COPD patients in the current study. The results showed that the statistical significance was $p<0.05$.

Table 5 showed the various morphological types observed among patients with COPD. Approximately 61.11% of the patients were classified as chronic bronchitis, whereas 33.33% were classified as emphysema

and only 5.56% were with small airway disease. The results were highly significant (p<0.005) among the various categories of morphological types observed in patients with COPD.

**Table 4.** FEF over the middle half of the FVC. FEF 25% to 75% of the study subjects

| Category | Control | | COPD patients | |
|---|---|---|---|---|
| | Frequency | Percentage | Frequency | Percentage |
| GOLD I (>79%) | 51 | 94.44 | 23 | 42.59 |
| GOLD II (60 to 79%) | 3 | 5.56 | 28 | 51.85 |
| GOLD III (40 to 59%) | 0 | 0 | 3 | 5.56 |
| GOLD IV (<40%) | 0 | 0 | 0 | 0 |

**Table 5.** Morphological types of COPD cases among study subjects

| Cases | Frequency | Percentage |
|---|---|---|
| Chronic bronchitis | 33 | 61.11 |
| Emphysema | 18 | 33.33 |
| Small airway disease | 3 | 5.56 |

The D-dimer levels determined in the COPD patients have been classified according to morphological variations and tabulated in Table 6. The results showed that the majority of COPD under the chronic bronchitis, emphysema, and Small Airway disease are found to have elevated levels of D-dimer, which is significant (p<0.05) between the other morphologically varied categories. On comparison using ANOVA, the values are significant (p<0.05)

**Table 6.** Level of D-dimer amongst the various morphological cases of COPD

| D-dimer level | Chronic bronchitis | Emphysema | Small airway disease | Total |
|---|---|---|---|---|
| <0.5 µg/mL | 3 | 6 | 1 | 10 |
| 0.5–3.9 µg/mL | 18 | 12 | 2 | 32 |
| >4 µg/mL | 12 | 0 | 0 | 12 |
| Total | 33 | 18 | 3 | 54 |

The D-dimer levels determined in the COPD patients have been compared with the different age groups of the patients and are tabulated in Table 7. The results showed that COPD under different age groups showed an elevated level of D-dimer, which is significant (p<0.05) on compared to the age group against the control subjects. On comparison, the values are significant (p<0.05)

**Table 7.** Comparison of age group with D-dimer

| Age in years | Control | COPD | p |
|---|---|---|---|
| 41–50 | 0.24±0.12 | 1.36±1.24 | <0.0001 |
| 51–60 | 0.27±0.12 | 2.52±1.31 | <0.0001 |
| 61–70 | 0.23±0.14 | 3.86±0.93 | <0.0001 |
| 71–80 | 0.24±0.13 | 5.17±0.92 | <0.0001 |

The levels of D-dimer determined in study subjects have been compared with the gender of the control patients and tabulated in Table 8. The results showed that COPD under both the genders showed an elevated level of D-dimer, which is significant (p<0.05) on compared to the age group against the control subjects. In comparison, the values are significant (p<0.05).

**Table 8.** D-dimer of male vs. female between control and COPD patients

| D-dimer value (µg/mL) | | | |
|---|---|---|---|
| Gender | Control | COPD patients | p |
| Male | 0.24±0.13 | 2.64±1.96 | <0.0001 |
| Female | 0.25±0.12 | 2.36±1.60 | <0.0001 |

Table 9 shows the positive correlation with elevated D-dimer against the spirometry measurements. In correlation coefficient analysis, it was determined that the levels of elevated D-dimer showed a positive correlation with the spirometry measurements. Values are statistically significant (p<0.05).

**Table 9.** Correlation coefficient of D-dimer of COPD cases against spirometry values

| D-dimer level | | FEV1 | Degrees of FEV1/FVC | FEF 25% to 75% | Correlation coefficient | p |
|---|---|---|---|---|---|---|
| <0.5 µg/mL | 0.232 | 23 | 23 | 23 | 0.792 | 0.032 |
| 0.5–3.9 µg/mL | 2.05 | 28 | 28 | 28 | | |
| >4 µg/mL | 5.75 | 3 | 3 | 3 | | |

## Discussion

In the current study, the highest prevalence of COPD among the group was determined to be in the age group 40–50 (48.15%) and followed by 50-60 (24.07%), respectively, with the least number observed in the age group 70–80 (12.96%). Although earlier studies have reported that COPD is most commonly affects males than the female, in our current study we have observed that the prevalence of COPD in both men and female are almost equal, which might be one of key findings of the current research.[11] Similar to the other studies, in the current study also the GOLD criteria grade II followed by grade III, 28% and 23% were observed respectively.[12-14]

COPD is a heterogeneous disease with varying pathophysiological mechanisms in different GOLD stages, which may influence biomarkers differently. The D dimer, as a marker of fibrinolysis and inflammation, is likely to vary in clinical utility across stages, particularly as the disease progresses from mild (GOLD-1) to more severe stages (GOLD-4). Limiting the study to only GOLD-1 patients would compromise the ability to assess D-dimer as a reliable biomarker across the full spectrum of severity of COPD. By excluding higher stages, key insights related to systemic inflammation, comorbidities, and disease complications, which are more pronounced in moderate to severe COPD, could be missed or lead to a false negative result. Therefore,

including patients in the full GOLD spectrum ensures a more comprehensive and accurate evaluation of the potential as a biomarker in COPD.[15,16]

In the present study, we confirmed that the D-dimer level was higher in COPD compared to healthy individuals. This was in contrast to studies by Maclay et al. and Arregui et al., where there was no significant difference in D-dimer compared to normal level.[17,18] Comparison of D-dimer values with respect to the Spirometer findings of our current study is also compared to the earlier studies where a significant correlation has been established.[19,20]

COPD is a common, debilitating and life-threatening respiratory disease and healthcare costs for many years worldwide, including India. 'GOLD' is best explained as 'a common, preventable, and treatable disease characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases.' Exacerbations and comorbidities contribute to the overall severity in individual patients.[21]

The D-dimer has been increasingly studied as a potential biomarker to predict mortality and survival in various diseases, including COPD. In the context of COPD, D-dimer levels reflect fibrinolysis and systemic inflammation, both of which are involved in disease progression and exacerbations. Elevated D-dimer levels in patients with COPD can indicate an increased thrombotic risk, endothelial dysfunction, and inflammatory state, all of which are associated with worse outcomes.[22]

A recent study has suggested that elevated levels of D-dimer correlate with higher mortality in patients with COPD, likely due to the increased risk of cardiovascular events, deep vein thrombosis, and pulmonary embolism, which are common comorbidities in COPD.[23] Additionally, D-dimer has been shown to correlate with exacerbations and disease severity, further supporting its role as a prognostic tool.[24] However, while elevated D-dimer is associated with poor outcomes, it is not specific to COPD and can be influenced by other factors such as infection, cancer, and systemic inflammation, making its use as a solitary biomarker challenging. The referenced study supports the utility of D-dimer as an indicator of mortality risk in patients with COPD, particularly in the context of exacerbations or comorbidities.[25] However, its predictive value is enhanced when combined with other clinical markers or diagnostic tools, such as lung function tests or biomarkers of systemic inflammation, to improve accuracy and clinical applicability.[25]

COPD occurs in most of the morbidity all over the world and usually manifests with chronic respiratory symptoms including progressive dyspnea, sputum production, coughing, wheezing and tightness of the chest.[4] The symptoms show different to asthma with a little variation day by day.[26] Furthermore, durations of clinical stability are most often punctuated with sudden episodes of deterioration, called acute exacerbations. On the other hand, several patients would also experience nonrespiratory symptoms,including fatigue, anorexia, weight loss, mood down, and anxiety.[5-6] Moreover, periods of clinical stability in COPD are often disrupted by abrupt episodes of worsening, referred to as acute exacerbations. In addition, numerous patients also encounter nonrespiratory symptoms, including lethargy, loss of appetite, reduced body weight, depression, and anxiety.[5-6]

COPD is termed the second leading cause of mortality with disability adjusted life years in India. Furthermore, the prevalence rate of COPD among the Indian population above the age of 30 years is also reported to be 7%.[27] In western countries, the smoking epidemic was on a rapid decline, where the situation on the other side of the world is different. COPD was determined to be one of the leading causes of morbidity globally and was expected to be the third leading cause of death worldwide during the 2020s.[1] In many Western nations, the smoking crisis has decreased rapidly, while the condition in other parts of the world remains quite different. Chronic obstructive lung disease (COPD) has been identified as a growing contributor to global morbidity and is projected to become the third major cause of death globally during the 2020s.[1]

COPD symptoms appear before the age of 40, and are usually preceded by a minimum period of a decade of smoking habits or any other harmful airway exposure as well. Symptoms of COPD usually do not appear before the age of 40 years and are often preceded by a minimum of 10 years of smoking or other harmful exposures to the airways. Chronic dyspnea is determined to be one of the main symptoms of COPD.[16] During the initial days, it was only possible to recognize by means of exercise, as and when the disease progressed, and dyspnea might also be present with minimal exertion, even at rest. Increased sputum or phlegm with or without cough could be one of the first symptoms of COPD, which is persistent in approximately 30% of patients.[28] Along with dyspnea, another symptom that accompany COPD is the wheezing or tightness in the chest.[29] However, the intensity of symptoms might vary differently, but the patient will never completely resolve them, at best. On the other side of the scale, the patient may experience different episodes of worsening symptoms called as "Acute Exacerbations of COPD (AECOPD)".[30] The severity of symptoms can fluctuate, but the patient will likely never experience complete resolution, even under the best circumstances. On the contrary, the patient may encounter periodic episodes of symptom aggravation, referred to as "Acute Exacerbations of COPD (AECOPD)".[30]

In addition to poor lung function, patients with COPD may also have other medical concerns, such as muscle loss, fatigue and the development of cachexia, which are identified as some of the common findings in advanced COPD. To add a few more, anxiety and depression are some of the other closely related conditions to COPD, especially during the progressive stage of the disease. Furthermore, cardiovascular diseases, diabetes mellitus, and lung cancer are some of the few examples of common comorbidities associated with COPD. Overall, the total disease burden in patients with COPD is formidable. It is not only determined by lung function but also requires additional diagnosis, one of which may be the determination of the levels of D-dimers.

## Conclusion

Measurements of FEV1, FEV1/FVC ratio and FEF 25% to 75%, showed a significant difference with a p-value of less than 0.05 when comparing control subjects with patients with COPD. The morphological variations observed in patients with COPD include Chronic Bronchitis, Emphysema, and small bowel disease. In particular, the D-dimer, the promising plasma biomarker identified in this study, demonstrated a strong correlation with these different morphological categories in patients with COPD. This suggests that the D-dimer could serve as a reliable biomarker for validating and confirming the various morphological classifications among individuals with COPD.

The correlation between biomarkers and disease severity is well established in the study, however causality remains unproven, which might be due to a smaller sample size could be considered as a limitation of the present investigation. Therefore, prospective cohort studies or mechanistic investigations would strengthen these findings and could address the limitation identified in our study. Furthermore, conducting larger, multicenter studies will also be necessary to validate the findings across diverse populations to establish predictive values to track the changes based on biomarkers.

## Declarations

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***Author contributions***

Conceptualization, A.P. and M.T.; Methodology, A.P., A.M. and M.T.; Software, A.P. and M.T.; Data Validation, A.P., A.M, M.T. and S.D.; Formal Analysis, A.P., M.T., S.C. and H.K.; Investigation, A.P, M.T., S.C. and H.K; Writing – Original Draft, A.P.; Writing – Review & Editing, A.P. and M.T.; Visualization, A.P., A.M. and M.T.; Supervision, A.M. and M.T.; Project Administration, A.M. and M.T.

***Conflicts of interest***

The authors declare no competing interests.

***Data availability***

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

***Ethical approval***

The approval of the ethics committee was obtained before the initiation of the study (meeting date; 23/12/2022, decision number: SU/2022/3108[37]).

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**ORIGINAL PAPER**

# Cytopathological diagnoses obtained in endobronchial ultrasound-guided transbronchial needle aspiration – a single-center one-year analysis

Paweł Zając [1,2], Wojciech Kądziołka [1,2], Ewa Kaznowska [1,2]

[1] Faculty of Medicine, Univeristy of Rzeszów, Rzeszów, Poland
[2] Subcarpatian Lung Disease Center, Rzeszów, Poland

**ABSTRACT**

**Introduction and aim.** Endobronchial ultrasound-guided fine needle aspiration (EBUS-FNA) is a widely adopted technique that replaces mediastinoscopy for the diagnosis of mediastinal lesions, significantly improving patient safety. This study assesses its diagnostic effectiveness and compares procedural quality with the existing literature, in order to identify characteristics of the patient population referred to the center.

**Material and methods.** During a year-long retrospective analysis, data from 312 EBUS-FNA procedures were collected, resulting in a final study group of 274 patients. For patients initially without a definitive diagnosis, reinterventions were conducted, typically with additional EBUS or tissue biopsy, followed by precise statistical analyses and calculations.

**Results.** The sensitivity of the EBUS examination to detect sarcoidosis, non-small cell lung cancer, small cell lung cancer, and lymphoproliferative disorders was determined to be 87.36%, 87.23%, 91.30% and 20%, respectively, based on false negative findings. Among patients who received a final diagnosis (n=237), a significant majority, i.e. 206 individuals or 86.92%, obtained it based on the first intervention.

**Conclusion.** EBUS-TBNA is an effective method to diagnose the cause of mediastinal lymphadenopathy, allowing for a definitive diagnosis in a significant majority of patients in the first intervention and showing high sensitivity in detecting metastatic malignant lymph node involvement and sarcoidosis.

**Keywords.** EBUS, lymphadenopathy, sarcoidosis

## Introduction

Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is an effective and recognized bronchoscopic technique for the diagnosis of mediastinal lesions. Since its widespread adoption, it has replaced mediastinoscopy as a diagnostic tool for mediastinal lymphadenopathy, mediastinal tumors, and pulmonary hilum lesions, significantly improving patient safety and showing to be more effective tool.[1-4] The obtained material often reveals malignant primary or metastatic tumor cells, epithelioid cell granulomas consistent with tuberculosis or sarcoidosis diagnosis, as well as cells present in silicotic and reactive nodes. In this study, our objective was to analyze the results from our center to determine diagnostic effectiveness, comparing the quality of our procedures with those reported in publications. Furthermore, based on a one-year patient sample, we sought to identify the specific characteristics of the patient population referred to our center.

**Corresponding author:** Paweł Zając, e-mail: zajac.pawel.32@gmail.com

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## Aim

In particular, to our knowledge, this is the first study to evaluate the diagnostic performance of EBUS-TBNA in our region, providing novel insights into the characteristics and diagnostic outcomes of this specific population. Our findings contribute valuable regional data that can improve a better understanding of EBUS-TBNA between diverse patient groups.

## Material and methods

This study was approved by the Bioethics Committee of the University of Rzeszów (KB 08/2025).

In a retrospective analysis covering a 12-month period (from June 2021 to June 2022), we collected data on patients referred to our clinic for EBUS-TBNA due to mediastinal lymphadenopathy or hilar tumors. Data were obtained from the hospital computer system, anonymized, and compiled in a spreadsheet. During this period, 312 EBUS-TBNA examinations were performed in our clinic. We excluded 38 records related to a second or subsequent diagnostic intervention, resulting in a final study group consisting of 274 patients undergoing their initial EBUS examination.

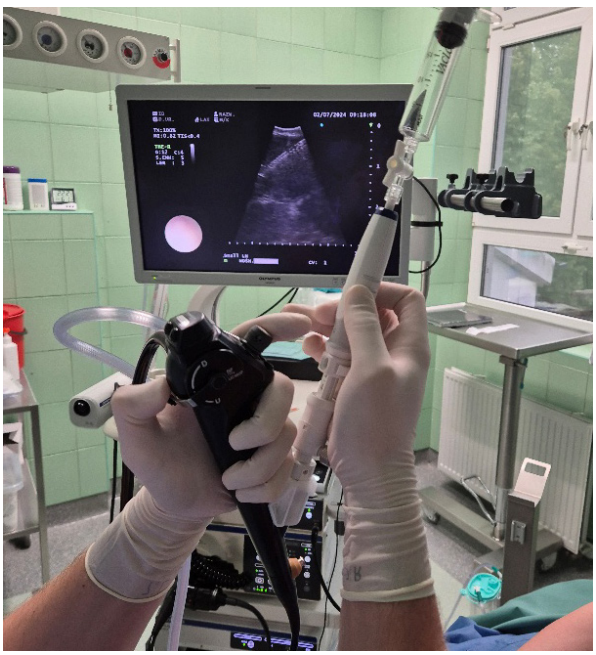

**Fig. 1.** The EBUS examination is performed using an OLYMPUS fiberoscope model. BF-UC190F and OLYMPUS single use 22 gauge aspiration needle, on the screen behind the endoscopist you can see the needle passing through station 7 mediastinal lymph node

Patients in our center receive local anesthesia with lidocaine solutions, followed by general anesthesia, usually with fentanyl and propofol. EBUS examination is performed using an OLYMPUS fiberoscope model: BF-UC190F and OLYMPUS 22 gauge single use aspiration needle (Fig. 1.). During a single examination, material is aspirated multiple times from available nodal or tumor lesions (Fig. 2), usually 3-5 times, depending on technical conditions, the amount of aspirated material, and the examination's purpose.

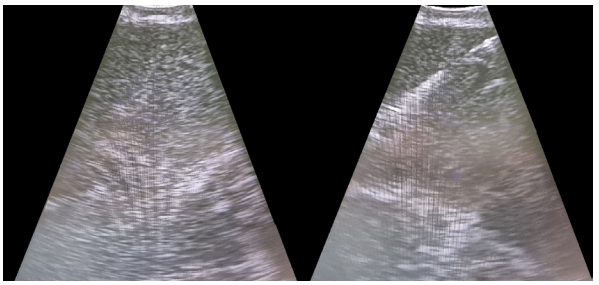

**Fig. 2.** Ultrasound image of the enlarged mediastinal lymph node found during examination (on the left) and the needle that passed through the lesion during biopsy (on the right)

The pathology department receives 10% buffered formalin, slides with smears fixed with CytoFix, and EBUS needle bronchial washings from the EBUS needle preserved with 96% ethanol. The cytological examination results based on this material were recorded in the spreadsheet. All material collected during this examination and considered by this study was acquired by needle aspiration. We refrained from performing an endobronchial forceps biopsy, bronchoalveolar lavage, or brush bronchial biopsy. Below are example micrographs of histopathological specimens, including those conventionally stained with hematoxylin and eosin, as well as samples stained during immunohistochemical analyses specific to the given diagnosis.

Lung adenocarcinoma: sheets of atypical cells, some with micropapillae, with marked nuclear pleomorphism; typical lung immunoprofile positive for TTF-1 and negative for p40 (Fig. 3).

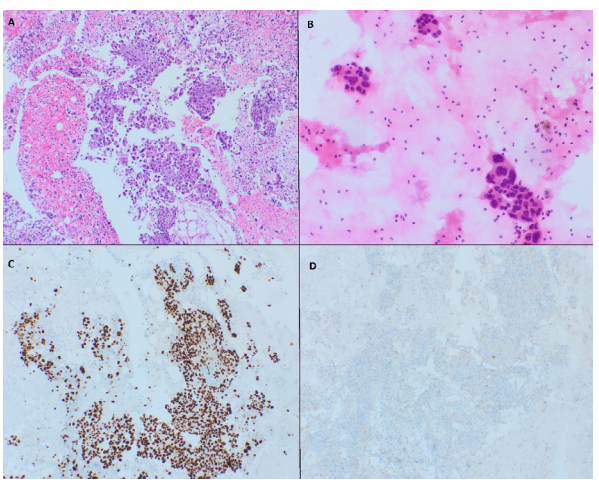


**Fig. 3.** Lung adenocarcinoma A: hematoxylin and eosin (H&E), 200×, B: H&E, 400×, C: TTF1, 100×, D: p40, 100×

Lung squamous cell carcinoma: multilayered sheets of cells with well-defined cell borders and intercellular bridges, some of them with keratinizing cells with a pyknotic nucleus (Fig. 4).

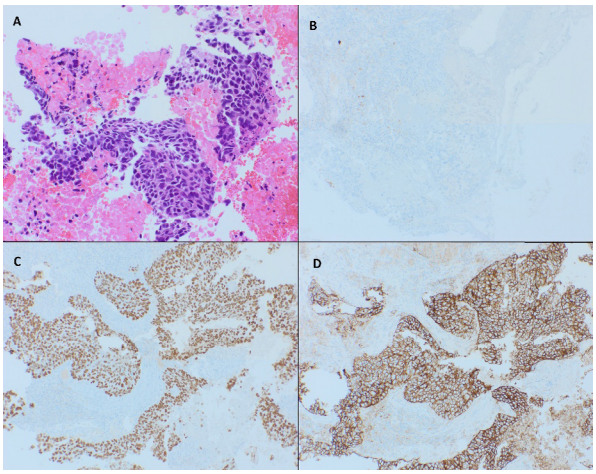

**Fig. 4.** Squamous cell carcinoma A: H&E, 200×, B: TTF1, 200×, C: p40, 200×, D: PD-L1, 200×

Small cell lung cancer: small cells with scant cytoplasm, with nuclear molding, and dark, hyperchromatic nuclei without nucleoli; positive for TTF-1 and the neuroendocrine marker synaptophysin (Fig. 5).

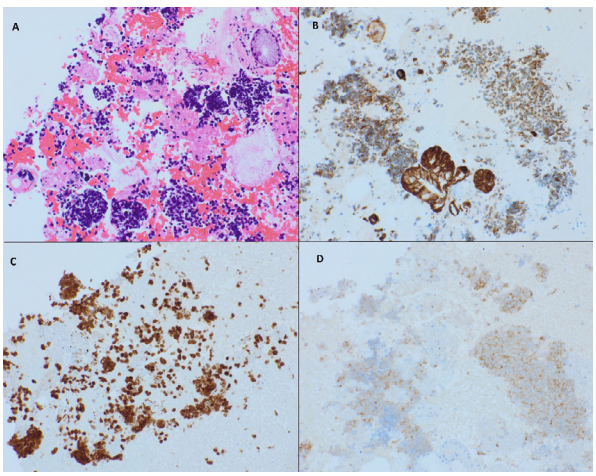

**Fig. 5.** Small cell lung cancer A: H&E, 200×, B: pancytokeratin, 200×, C: TTF1, 200×, D: synaptophysin, 200×

Metastatic prostatic adenocarcinoma: cellular cribriform aggregates of small, uniform cells with well-defined centrally located nucleoli and generally positive NKX 3.1 and PSA (Fig. 6).

Sarcoidosis: non-crotizing granulomas composed of epithelioid histiocytes, giant multinuclear cells, and small amounts of lymphocytes in the background (Fig. 7).

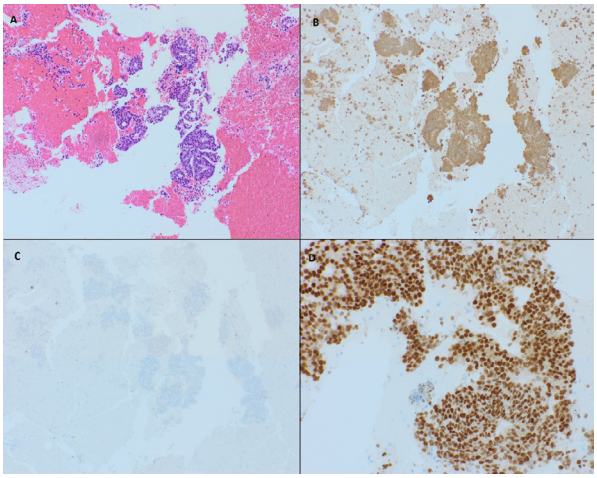

**Fig. 6.** Metastatic prostatic adenocarcinoma A: H&E, 100×, B: PSA, 100×, C: TTF1, 100×, D: NKX3.1, 200×

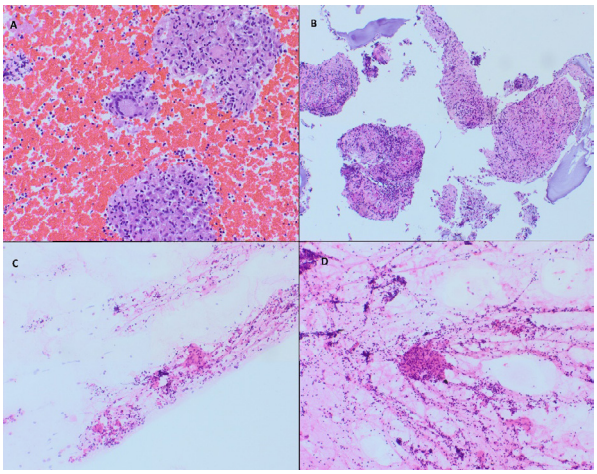

**Fig. 7.** Sarcoidosis A: H&E, 200×, B: H&E, 100×, C: H&E, 40×, D: H&E, 100×

Pneumoconiotic lymph nodes: three-dimensional clusters of lymph node tissue with deposits of dark, coal pigment (Fig. 8).

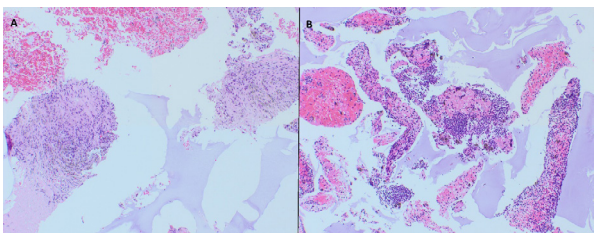

**Fig. 8.** Pneumoconiotic lymph nodes A, B: H&E, 100×

For patients without a definitive diagnosis (initially negative group), a reintervention is usually performed, either with another EBUS examination or, in justified cases, tissue biopsy for histopathological material. Typically, a tissue biopsy is taken from the lung parenchyma, lymph node biopsy in the neck and supraclavicular region, or mediastinoscopy.

For these patients (initially negative group), the hospital's computer system was searched for cytological and histopathological results of subsequent interventions or entry into outpatient clinics with respect to their further treatment. Based on these constructed assumptions, further subgroup divisions and calculations were performed. All calculations were carried out with precision to two decimal places.

## Results

The study group consisted of 181 males and 93 females. Based on the material obtained in the initial EBUS examination, diagnoses were established: non-small cell lung cancer (n=82), small cell lung cancer (n=21), lymphoproliferative disorders (n=1), sarcoidosis (n=76). A total of 180 received a definitive diagnosis (initially positive group). The material from the remaining 94 patients contained fragments of lymph nodes: normal, reactive or silicotic, occasional epithelioid granulomas, inflammatory cells, and other findings considered benign or not sufficiently expressed to provide a definitive diagnosis (initially negative group). In every examination, the pathologist has found lymph node tissue or tumor tissue, therefore, there were no cases of nondiagnostic material. Table 1 presents the detailed distribution of diagnoses in the study group.

**Table 1.** Detailed distribution of diagnoses in the study group (n=274) after the initial procedure

| Diagnoses | n |
|---|---|
| Sarcoidosis | 76 |
| Adenocarcinoma | 33 |
| Squamous-cell carcinoma | 24 |
| NSCLC NOS | 23 |
| Large cell carcinoma | 2 |
| Small cell carcinoma | 21 |
| Neoplastic lymphatic growth | 1 |
| Normal or non-specific lymph nodes | 94 |

Patients with normal or nonspecific findings were offered diagnostic reinterventions, often preceded by additional imaging studies. In 6 patients, complete regression of the lesions was observed on chest CT images, and further interventions were abandoned. Despite the proposal, 37 patients did not present for reintervention or follow-up visits, leading to the exclusion of these records from further analysis. Among the remaining 51 patients, ongoing evaluation revealed: persistent absence of definitive disease (n=20), disease progression (false negative results group, n=31). The results of secondary interventions/evaluations in the initially negative group are presented in Table 2.

Based on the results of false negative findings, the sensitivity of the examination to detect sarcoidosis, non-small cell lung cancer small cell lung cancer and lymphoproliferative disorders can be determined, respectively, at levels of 87.36%, 87.23%, 91.30%, and 20%. Table 3 presents a compilation of the final diagnoses in the study group, taking into account the results of the initial EBUS examination and follow-up.

**Table 2.** Secondary intervention / evaluation results in the initially negative group (n=94)

| Secondary interventions/evaluations | n |
|---|---|
| Other benign lesions (verified negative) | 20 |
| Sarcoidosis | 11 |
| Adenocarcinoma | 3 |
| Squamous-cell carcinoma | 3 |
| NSCLC NOS | 6 |
| Small cell carcinoma | 2 |
| Hodgkin Lymphoma | 3 |
| Other lymphatic growth | 1 |
| Tuberculosis | 2 |
| Radiological regression | 6 |
| Lost to follow-up | 37 |

**Table 3.** Final diagnoses in the study group, considering the results of the initial EBUS examination and the follow-up data

| Diagnosis | | n | Percentage |
|---|---|---|---|
| Patients with definite diagnosis | | 237 | 100% |
| Non-small cell carcinoma (total) | | 94 | 39.66% |
| | Not otherwise specified | 29 | 12.24% |
| | Adenocarcinoma | 36 | 15.19% |
| | Squamous-cell carcinoma | 27 | 11.39% |
| | Large cell carcinoma | 2 | 0.84% |
| Small cell carcinoma | | 23 | 9.70% |
| Neoplastic lymphatic growth (total) | | 5 | 2.11% |
| | Hodgkin lymphoma | 3 | 1.27% |
| | Other lymphatic growth | 2 | 0.84% |
| Benign lesions (total) | | 115 | 48.52% |
| | Sacroidosis | 87 | 36.71% |
| | Tuberculosis | 2 | 0.84% |
| | Other benign lesions | 20 | 10.97% |

Based on this, it can be stated that among patients who received a final diagnosis (n=237), a significant majority, that is, 206 individuals or 86.92%, obtained it based on the first intervention.

## Discussion

The EBUS-TBNA procedure is widely recognized and used globally in numerous centers as a diagnostic tool for mediastinal disorders, primarily for the diagnosis of mediastinal lymphadenopathy. Its widespread adoption has almost replaced mediastinoscopy, which, although still useful and necessary in many cases, is not performed as frequently. The shift from a surgical procedure burdened with, especially in inexperienced hands,

a relatively high risk of bleeding complications to a minimal-risk bronchoscopic procedure was an obvious step in the entire field of cancer diagnostics.

Over the years, EBUS has been thoroughly examined and has shown high sensitivity and specificity. In the 2014 ESTS guidelines, the reported sensitivity of EBUS-TBNA in lung cancer staging ranges from 87% to 93%, with nearly 100% specificity.[5] Furthermore, Crombag et al. demonstrated a sensitivity of 82% in diagnosing sarcoidosis.[6] The good safety profile and low learning curve for EBUS-TBNA training have encouraged many physicians to implement this method in their centers. However, it is crucial for the endoscopists performing the procedure to verify its effectiveness over the years. In centers where young physicians are trained, this is particularly important, as the excellent test parameters reported in clinical studies are achieved by experienced bronchoscopists. For this reason, we conducted an analysis in our center, where resident physicians are also trained to perform EBUS-TBNA. We can consider the results obtained satisfactory, with an average sensitivity of 89% to detect lung cancer metastases and 87% to detect sarcoidosis, supporting the continuation of our current examination and training doctrine. In a similar study, Murthi et al. aimed to evaluate the effectiveness of EBUS-TBNA in the hospital, staffed by pulmonologists with and without formal interventional lung training. EBUS-TBNA for all pathologies had a precision of 81.2% (CI 95% 73.8–87.4) and a sensitivity of 55.1% (CI 95% 41.5-67.3).[7]

One issue we need to address in this discussion is the low sensitivity to detect lymphomas (20%). Almost all patients required histopathological material for diagnosis, which is noteworthy. Erer OF et al. reported in their study that none of the cases of Hodgkin lymphoma was diagnosed using EBUS-TBNA, but there are also studies where sensitivity can reach close to 91%.[8-9] The wide range of reported results may be due to the needle sizes used, the availability of flow cytometry, and criteria for diagnosis. In many pathology departments, it is accepted that the diagnosis of lymphomas can only be based on the architectural assessment of the node and the cytological assessment is limited to raising suspicions. This suspicion was raised in 2 of our negative biopsies, so if suspicion is considered a sufficient indicator of test quality, the sensitivity could be calculated at 60%. However, this is still a low sensitivity in detecting lymphomas compared to some reports, so it remains a matter that requires further attention from our part. It is a case to be made that the implementation of flow cytometry can improve the diagnostic yield of EBUS-TBNA in these cases.

An obvious limitation of the study is its retrospective nature. The analysis of records from more than two years ago is not an optimal way to determine the parameters of a diagnostic test. Designing and conducting a prospective study with histopathological verification for all subjects would be an optimal approach, providing objectively the most accurate numerical values for the test parameters. Certainly, such a form would allow one to determine the specificity of the test, which we did not undertake in this analysis. The construction of our study, by accepting cytological diagnoses of tumors as certain and noncaseating epithelioid cell granulomas without necrosis as significant for the diagnosis of sarcoidosis, and the lack of secondary intervention in these patients, precluded the existence of false positive test results. Therefore, instead of accepting a specificity of 100%, it was better for us to refrain from determining this indicator with these limitations.

The study population in our center, representing a regional population of around 2 million people, in terms of cytopathological diagnoses, seems quite typical. Sangorini et al. described a similar cross section of patients in their analysis, with nearly 53% having cancer diagnoses, 1% lymphomas and 39% benign lesions, including sarcoidal ones.[10] Zhang et al. in their study presented a population with a comparable distribution: cancer – 43%, sarcoidosis – 42%, reactive nodes – 13% and tuberculosis – 1.5%.[11] On the other hand, Usluer et al. reported a smaller percentage of cancer and sarcoidosis diagnoses in favor of reactive nodes (39%).[12] Cetinkaya and his team, as the EBUS-TBNA diagnoses included tuberculosis – 35%, sarcoidosis – 35%, carcinoma - 25%, and lymphoma – 5%.[13] This large percentage of tuberculosis patients may be due to culture and other microbiological tests performed simultaneously. Nevertheless, it is uncommon to see such significant proportion of tuberculous disease reported, especially considering not even one percent in our study group. Ortakoylu et al. described a more standard cross section of results, consisting of 31% cancer, 36% sarcoidosis, 14% tuberculosis, 16% reactive/normal nodes.[14] The variability in results is small over the available studies, and only a significant proportion of sarcoidosis diagnoses juxtaposed with an exceptionally low percentage of tuberculosis may seem intriguing in our region, encouraging further research on granulomatous diseases in our center.

## Conclusion

EBUS-TBNA is an effective method for diagnosing the cause of mediastinal lymphadenopathy, allowing a definitive diagnosis in 86.92% of patients in the first intervention and demonstrating high sensitivity to detect metastatic malignant lymph node involvement (approximately 89%).

## Declarations

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*Author contributions*

Conceptualization, P.Z.; Methodology, P.Z.; Software, P.Z.; Validation, W.K. and E.K.; Formal Analysis, P.Z.; Investigation, P.Z. and W.K.; Resources, E.K.; Data Curation, P.Z. and E.K.; Writing – Original Draft Preparation, P.Z.; Writing – Review & Editing, P.Z. and E.K.; Visualization, P.Z.; Supervision, E.K.; Project Administration, E.K.

*Conflicts of interest*

The author(s) declare no conflict of interest.

*Data availability*

The data sets generated and/or analyzed during the current study are not publicly available due to their nature as medical records collected from a hospital information system. However, anonymized records derived directly from these data supporting the findings of this study are available from the corresponding author [PZ] upon reasonable request.

*Ethics approval*

This study was approved by the Bioethics Committee of the University of Rzeszów (KB 08/2025).

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**ORIGINAL PAPER**

# Plasma KIM-1 and interleukin-18 are superior biomarkers for diagnosing and stratifying risk in type 1 acute cardiorenal syndrome

Redha Dawud Abd Alredha [1], Hassan Ali Farman [1], Krarr Haider Haddawi [2]

[1] Department of Medical Laboratory Techniques, College of Health and Medical Techniques, Kufa, Al_Furat Al_Awsat Technical University, Al-Kufa, Iraq
[2] Department of Clinical Biochemistry, College of Medicine, University of Al-Qadisiyah, Al Diwaniyah, Iraq

**ABSTRACT**

**Introduction and aim.** Acute cardiorenal syndrome (CRS) is a condition in which acute cardiac dysfunction leads to acute kidney injury (AKI), resulting in high morbidity and mortality rates. This study aimed to assess the diagnostic and prognostic value of plasma kidney injury molecule 1 (KIM-1) and interleukin-18 (IL-18) levels in acute CRS compared to acute heart failure (AHF) and healthy controls.

**Material and methods.** A case-control study was conducted with 90 participants divided into three groups: control (n=30), AHF (n=30), and acute CRS (n=30). Renal function parameters (serum creatinine, blood urea nitrogen, estimated glomerular filtration rate) and plasma biomarkers (KIM-1, IL-18) were measured. A receiver operating characteristic curve analysis was used to evaluate diagnostic performance and logistic regression was used to identify predictors of disease outcomes.

**Results.** Plasma KIM-1 and IL-18 levels were significantly higher in the acute CRS group than in the AHF and control groups. KIM-1 demonstrated superior diagnostic accuracy (the area under the curve (AUC)=1.000) with 100% sensitivity and specificity, while IL-18 also performed well (AUC=0.96, sensitivity=96%, specificity=97%). ROC analysis identified plasma KIM-1 and IL-18 cut-off values of >72.78 pg/mL and >254.8 pg/mL, respectively, which may be used as thresholds for early diagnosis and risk stratification. Logistic regression analysis revealed that plasma KIM-1 was a significant predictor of adverse outcomes (OR=3.5, 95% CI 1.50–8.49, p=0.003), while IL-18 also contributed to risk stratification (OR=1.06, 95% CI 1.04–1.125, p=0.03). These adverse outcomes included progression to kidney disease. However, these findings require validation in an independent cohort to confirm reproducibility and generalizability.

**Conclusion.** KIM-1 and IL-18 are highly effective biomarkers for diagnosing and stratifying the risk of acute CRS, outperforming traditional markers of renal function. Their clinical integration could enable early detection and personalized treatment, thus improving patient outcomes. However, more studies with larger cohorts, serial measurements, and independent validation are warranted.

**Keywords.** acute cardiorenal syndrome, AHF, AKI, IL-18, KIM-1

## Introduction

Acute cardiorenal syndrome (CRS) represents a complex and bidirectional interplay between the heart and kidneys, in which acute cardiac dysfunction precipitates acute kidney injury (AKI) or exacerbates preexisting renal dysfunction.[1] Type 1 CRS, characterized by acute de-

**Corresponding author:** Redha Dawud Abd Alredha, e-mail: redhadawud@gmail.com

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compensated heart failure leading to AKI, is associated with a high morbidity and mortality burden, affecting more than 30% of hospitalized patients with acute heart failure (AHF).[2,3] The intricate pathophysiology of CRS involves hemodynamic alterations, neurohormonal activation, systemic inflammation and oxidative stress, culminating in structural kidney damage and functional impairment.[4,5] Early identification and stratification of patients at risk of CRS are critical for improving clinical outcomes; however, this remains a challenge due to the overlapping presentation of cardiac and renal dysfunction and the limitations of traditional diagnostic tools.[6]

Recent advances in precision medicine underscore the limitations of conventional renal markers, such as serum creatinine and estimated glomerular filtration rate (eGFR). In addition to these markers, emerging biomarkers such as neutrophil gelatinase-associated lipocalin (NGAL), cystatin C, and soluble ST2 are being investigated for early detection and risk stratification in cardiorenal syndrome.[7,8] For example, a study by Erdil highlights the role of advanced nanobiomaterials and innovative signaling approaches in cardiovascular disease.[9] Consequently, there has been growing interest in identifying new biomarkers that can provide more precise insights into the underlying pathophysiology of CRS.[6] Plasma biomarkers, such as kidney injury molecule-1 (KIM-1) and interleukin-18 (IL-18), have emerged as promising candidates in this context.[10] KIM-1 is a transmembrane glycoprotein expressed in renal proximal tubular cells in response to ischemic or toxic injury and has been validated as a sensitive marker of tubular injury in AKI.[11,12] Similarly, IL-18, a proinflammatory cytokine, plays a key role in mediating renal inflammation and apoptosis, making it a potential biomarker for detecting inflammatory kidney injury.[13,14]

Recent studies have highlighted the utility of KIM-1 and IL-18 in various renal pathologies, including AKI and chronic kidney disease (CKD), but their roles in CRS remain underexplored.[15] Given the dynamic interplay between cardiac and renal dysfunction in CRS, these biomarkers may offer valuable insights into both the structural and inflammatory aspects of kidney injury, thus complementing traditional renal function parameters. Furthermore, the integration of biomarkers into clinical practice has the potential to enhance risk stratification, allowing timely therapeutic interventions, and improving patient outcomes.[16,17] However, our study is novel in that it focuses specifically on the diagnostic and prognostic utility of plasma KIM-1 and IL-18 in type 1 CRS, where acute heart failure leads to acute kidney injury.

## Aim

In this study, our aim was to evaluate the clinical utility of plasma levels of KIM-1 and IL-18 as diagnostic and prognostic biomarkers in patients with acute CRS. Specifically, we investigated their correlation with renal function parameters, the ability to differentiate CRS from AHF, and the predictive value of adverse outcomes. By comparing these biomarkers across the control, AHF, and CRS groups, we sought to provide novel information on their relevance to the pathophysiology and management of CRS. Furthermore, this study contributes to the growing body of evidence supporting biomarker-based approaches in cardiorenal syndromes, paving the way for more personalized and effective patient care.

## Material and methods

### *Study design and participants*

A case-control study was conducted between February 2023 and July 2024 at the (Al-Sadr Teaching Hospital in Najaf, Iraq), following approval from the Institutional Review Board (approval number: 34328). Written informed consent was obtained from all participants prior to inclusion, in accordance with the principles of the Declaration of Helsinki.[18] The sample size (n) was determined using the following formula to compare the two proportions in case-control studies.[19]

$$n = \left\{ \left( Z_{\{\frac{\alpha}{2}\}} + Z_{\{\beta\}} \right)^2 \cdot \left[ p_{1(1-p_1)} + p_{2(1-p_2)} \right] \right\} / \{ (p_1 - p_2)^2 \}$$

- $Z_{\{\frac{\alpha}{2}\}} = 1.96$ for a 95% confidence level,
- $Z_{\{\beta\}} = 0.84$ for 80% power,
- $p_1 = 0.70$ (proportion of a specific marker in the CRS-1 group based on previous studies),
- $p_2 = 0.30$ (proportion in controls).

Ninety participants were divided into three groups: (i) control (n=30), (ii) AHF (n=30), and (iii) acute CRS (n=30). A sample size of 30 subjects per group was chosen based on previous biomarker studies in similar clinical settings. Although this is a preliminary investigation, the sample size provided sufficient power to detect statistically significant differences; however, future studies with larger cohorts are recommended. The inclusion criteria for the AHF and acute CRS groups were clinical, radiological, and laboratory evidence of acute cardiac dysfunction with or without concomitant AKI. Subjects were excluded if they had chronic dependence on dialysis, severe systemic illness (e.g., advanced liver disease or active malignancy), or recent exposure to nephrotoxic agents. Patients with AHF and CRS were recruited based on established diagnostic criteria, including the European Society of Cardiology guidelines for AHF and definition of type 1 CRS as acute cardiac dysfunction leading to renal impairment.[20,21] The classification of AKI among patients with acute CRS was performed using Kidney Disease: Improving Global

Outcomes (KDIGO) criteria, which define AKI based on an increase in serum creatinine of <0.3 mg / dL in 48 hours, an increase to ≥1.5 times the baseline within 7 days, or a reduction in urine output of <0.5 mL/kg/hour for at least 6 hours. Further stratification was performed using the same criteria, which categorize AKI severity based on serum creatinine changes and urine output over time.[22]

***Data collection***

Demographic details (age and sex) and relevant medical history were documented. Anthropometric measurements included body mass index (BMI), calculated as weight (kg) divided by the square of height (m).[23] Disease severity was assessed using clinical and echocardiographic parameters. Functional status in the AHF and CRS groups was classified using the New York Heart Association (NYHA) classification.[24] The duration of hospitalization was recorded in days for each patient. The left ventricular ejection fraction (EF) was assessed using two-dimensional transthoracic echocardiography (TTE) following the guidelines of the American Society of Echocardiography guidelines.[25]

***Sample collection***

Venous blood samples (5–7 mL) were collected from each participant by standard phlebotomy into serum separator tubes and plasma tubes of ethylenediaminetetraacetic acid. Samples were drawn under fasting conditions (if clinically feasible) or at a standardized time point immediately after admission. The tubes were centrifuged at 3,000×g for 10 min at 4°C and serum and plasma aliquots were stored at -80° C until the test.

***Biochemical measurements***

Renal function parameters, including serum creatinine and blood urea nitrogen (BUN), were measured using an automated chemistry analyzer (Beckman Coulter AU5800; Cat No. A18504, Beckman Coulter Inc., USA). eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) formula:[26]

MDRD-2 (abbreviated) equation: GFR (expressed in ml/min/1.73 $m^2$)=$186\times[Pcr]^{-1.154}\times[age]^{-0.203}\times$[0.742 if the patient is female]

Plasma biomarkers, including KIM-1 and IL-18, were quantified using enzyme-linked immunosorbent assay (ELISA) kits. KIM-1 levels were measured using the human KIM-1 quantikine ELISA Kit (Cat No. DY1757-05, USA). This assay had a sensitivity of 0.1 pg/ml and an intra-assay coefficient of variation (CV)<6%. IL-18 levels were assessed using a human IL-18 high-sensitivity ELISA Kit (Cat No. BMS267-2, USA), which had a detection limit of 1.0 pg/ml and an inter-assay CV<8%. All biomarker measurements were performed in duplicate to ensure precision following the manufacturer's protocol.

***Statistical analysis***

GraphPad Prism 9 (GraphPad Software, Inc. Boston, MA, USA) was used to detect the effect of different groups (patients and controls) on study parameters. A t-test was used to compare the means. The chi-square test was used to compare the percentages (0.05 and 0.01 probability). Estimation of the correlation coefficient and multiple linear regression between variables. The sensitivity and secrecy of parameters in the patient and control groups for biomarkers were determined using receiver operating characteristic (ROC) curve analysis to maximize sensitivity and specificity. The Youden index was used to identify the optimal thresholds for each parameter.[27]

## Results

***Demographic characteristics of study groups***

Table 1 shows the baseline characteristics of the control, AHF and acute CRS groups. No significant differences were observed in age (p=0.26), sex distribution (p=0.25) or BMI (p=0.16) between the groups, ensuring demographic comparability. The prevalence was higher in the AHF (73%) and Acute CRS (83%) groups than in the controls, but this difference was not statistically significant (p=0.5). The duration of hospitalization (p<0.001) and NYHA classification (p<0.001) showed significant differences, with acute CRS patients experiencing longer hospital stays and more severe functional impairment (70% in NYHA class IV). EF also decreased progressively between groups, with a significant decrease from control (60.77±4.30%) to AHF (41.70±5.08%) and acute CRS (34.73±2.49%) (P> 0.001). These findings reflect the increasing clinical severity from AHF to acute CRS, highlighting the class EF and NYHA as key indicators of disease progression.

***Distribution and severity of AKI stages in patients with acute CRS***

Table 2 and Figure 1 illustrate the distribution of AKI stages according to KDIGO criteria among patients with acute CRS. Most of the patients (76%) were classified as stage II AKI, while only 24% were classified as stage I. This finding highlights the prevalence of severe renal impairment in this cohort. Figure 1 provides a clear visual representation of this distribution, highlighting the significant proportion of stage II cases. These findings underscore the critical severity of kidney dysfunction in patients with acute CRS and have important implications for clinical management and prognosis.

**Table 1.** Baseline demographic and anthropometric characteristics of the control, AHF, and acute CRS groups[a]

| Characteristic | Control n=30 | AHF n=30 | Acute CRS n=30 | p |
|---|---|---|---|---|
| Age (years) | | | | |
| Mean±SD | 65.5±5.6 | 62.9±5.6 | 64.6±7.09 | 0.26 O[NS] |
| Range | 55–75 | 55–73 | 55–75 | |
| Sex | | | | |
| Male, n (%) | 14 (46%) | 19 (63%) | 13 (43%) | 0.25 F[NS] |
| Female, n (%) | 16 (54%) | 11 (37%) | 17 (57%) | |
| BMI (kg/m$^2$) | | | | |
| Mean±SD | 25.7±1.26 | 28.4±0.84 | 28.9±1.54 | 0.16 O[NS] |
| Range | 23.6–28.4 | 27.1–29.7 | 25.4–30.2 | |
| Smoking Status | | | | |
| Non-Smoker, n (%) | - | 8 (27%) | 5 (17%) | 0.5 F[NS] |
| Smoker, n (%) | - | 22 (73%) | 25 (83%) | |
| Duration of hospitalization | | | | |
| ≤ 7 days, n (%) | - | 19 (63%) | 0 (0.0 %) | <0.001 F[***] |
| >7 days, n (%) | - | 11 (37%) | 30 (100 %) | |
| NYHH classification | | | | |
| Class I, n (%) | - | 0 (0.0%) | 0 (0.0 %) | <0.001 F[***] |
| Class II, n (%) | - | 8 (27%) | 0 (0.0%) | |
| Class III, n (%) | - | 22 (73%) | 9 (30 %) | |
| Class IV, n (%) | - | 0 (0.0%) | 21 (70 %) | |
| Ejection fraction % | | | | |
| Mean±SD | 60.77±4.30 | 41.70±5.08 | 34.73±2.49 | <0.001 O *** |
| Range | 59.16–62.37 | 39.80–43.60 | 33.80–35.66 | |

[a] n number of cases, SD – standard deviation, O one-way ANOVA, NS – not significant (p <0.05), F – Fisher's exact test

**Table 2.** Distribution of AKI stages (KDIGO criteria) among acute CRS patients

| | AKI stagE (KDIGO) | |
|---|---|---|
| | Stage I, n (%) | Stage II, n (%) |
| AcutE CRS | 7 (24%) | 23 (76 %) |

These findings are illustrated in Figure 1, which shows the proportional representation of the stages of AKI in patients with acute CRS. The dominance of stage II AKI is evident, highlighting the need for effective strategies to manage and monitor renal dysfunction in this high-risk group.

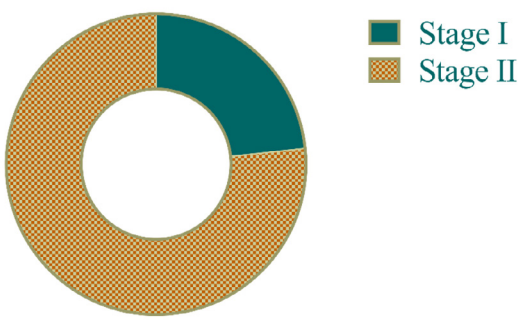


**Fig. 1.** Proportional representation of AKI stages (KDIGO criteria) in acute CRS patients

***Renal function parameters among study groups***

Table 3 and Figure 2 summarize renal function parameters, demonstrating progressive renal dysfunction from the control to the AHF and acute CRS groups. Blood urea levels were significantly elevated in acute CRS (41.2±18.1 mg/dL) compared to control (20.9±5.4 mg/dL) and AHF (25.4±9.3 mg/dL). Baseline and current serum creatinine levels followed a similar trend, with significant differences between all groups, highlighting the worsening of renal impairment in acute CRS (current serum creatinine: 2.64±0.53 mg/dL).

**Table 3.** Renal function markers in acute heart failure and cardio-renal syndrome patients a control comparison

| Characteristic | Control | AHF | Acute CRS | p |
|---|---|---|---|---|
| BUN (mg/dL) | | | | |
| Mean±SD | 9.7±2.5 A | 11.8±4.3 A | 19.2±8.4 B | <0.001 O *** |
| Range | 4.6–14.4 | 6.2–26.3 | 10.7–37.5 | |
| Baseline serum creatinine (mg/dL) | | | | |
| Mean±SD | 0.85±0.08 A | 0.9±0.06 B | 1.14±0.08 B | <0.001 O *** |
| Range | 0.72–0.99 | 0.89–1.09 | 1.02–1.26 | |
| Current serum creatinine (mg/dL) | | | | |
| Mean±SD | 0.84±0.09 A | 1.14±0.06 B | 2.64±0.53 C | <0.001 O *** |
| Range | 0.7–1.0 | 1.0 –1.23 | 1.65–3.4 | |
| eGFR (mL/min/1.73 m$^2$) | | | | |
| Mean±SD | 78±13.9 A | 69.3±10.3 B | 55.1±9.6 C | <0.001 O *** |
| Range | 54.9–112 | 51.6–86 | 41.7–73 | |

[a] n number of cases, SD – standard deviation, O one-way ANOVA, *** – significant at p <0.001, capital letters A, B, and C were used to indicate the level of significance following Tukey's multiple comparison test, similar letters indicate no significant difference, while different letters indicate significant differences, * the conversion factor (CF) to convert blood urea (BU) to blood urea nitrogen is 2.14

eGFR showed a significant decline, with the mean eGFR decreasing progressively from control (78±13.9 mL/min/1.73 m$^2$) to AHF (69.3±10.3 mL/min/1.73 m$^2$) and acute CRS (55.1±9.6 mL/min/1.73 m$^2$). Figure 2 visually represents this reduction in eGFR, highlighting the marked decline in renal function in acute CRS.

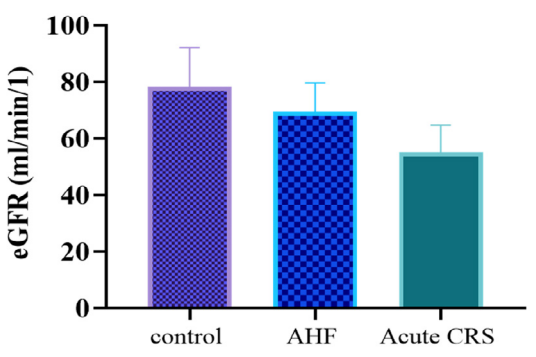


**Fig. 2.** Comparison of mean eGFR among study groups

### *Plasma biomarkers in study groups*

Table 4 highlights the significant differences in plasma biomarkers (KIM-1 and IL-18) between the control, AHF and acute CRS groups. Plasma KIM-1 levels were markedly elevated in acute CRS (432.8±55.2 pg/mL) compared to AHF (63.8±27.2 pg/mL) and control (50.6±10.5 pg/mL), with, indicating severe kidney tubular injury in acute CRS. Similarly, plasma levels of IL-18 were significantly higher in the acute CRS (415±52 pg/mL) than in the AHF (258±41.6 pg/mL) and control (105.2±24.5 pg/mL) groups, reflecting increased inflammation associated with renal dysfunction.

**Table 4.** Comparison of plasma KIM-1 and IL-18 among the control, AHF, and acute CRS groups[a]

| Characteristic | | Control | AHF | Acute CRS | p |
|---|---|---|---|---|---|
| Plasma KIM-1 (pg/mL) | | | | | |
| | Mean±SD | 50.6±10.5 A | 63.8±27.2 B | 432.8±55.2 C | <0.001 O *** |
| | Range | 22.1–72.8 | 18.5–125 | 310–562 | |
| Plasma IL-18 (pg/mL) | | | | | |
| | Mean±SD | 105.2±24.5 A | 258±41.6 B | 415±52 C | <0.001 O *** |
| | Range | 58–154 | 179–342 | 307–514 | |

[a] n number of cases, SD – standard deviation, O one-way ANOVA, *** – significant at p<0.001, capital letters A, B, and C were used to indicate the level of significance following Tukey's multiple comparison test, similar letters indicate no significant difference, whereas different letters indicate significant differences

### *Correlation analysis of biomarkers in cardiorenal syndrome*

Correlation between plasma biomarkers (IL-18 and KIM-1) and renal function parameters (current creatinine and eGFR). Plasma levels of IL-18 showed a strong positive correlation with current creatinine levels (r=0.841, p<0.0001, Fig. 3A) and a significant negative correlation with eGFR (r=-0.604, p<0.0001, Fig. 3B). Similarly, plasma KIM-1 showed a robust positive correlation with current creatinine level (r=0.917, p<0.0001, Fig. 4A) and moderate correlations with eGFR (r=-0.594, p>0.0001, Fig. 4B). These findings suggest that plasma IL-18 and KIM-1 are closely associated with markers of renal dysfunction, highlighting their potential utility as reliable biomarkers for assessing the severity and progression of kidney injury. The negative correlation with eGFR further supports its relevance in reflecting a decline in renal function.

### *Diagnostic efficacy of biomarker in the studied groups*

Figures 5A and 5B demonstrate the diagnostic utility of plasma levels of KIM-1 and IL-18 in identifying acute CRS. Plasma KIM-1 showed outstanding diagnostic performance with a cut-off value of >72.78 pg/mL, achieving 100% sensitivity and specificity with an area

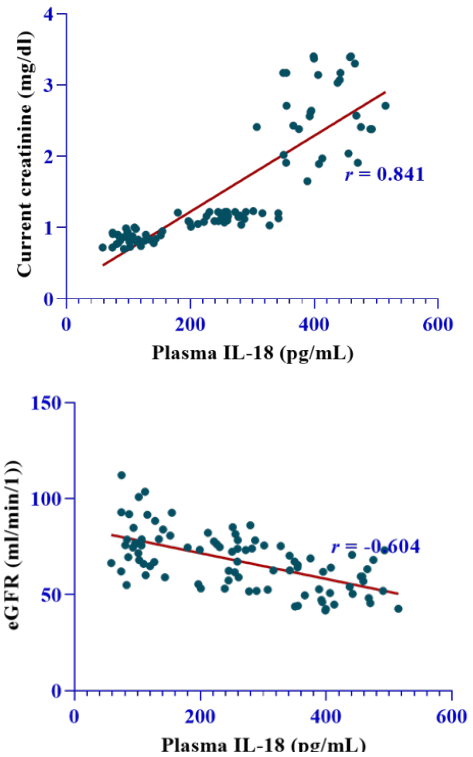


**Fig. 3.** A: Correlation between current creatinine and plasma IL-18, B: Correlation between eGFR and plasma IL-18

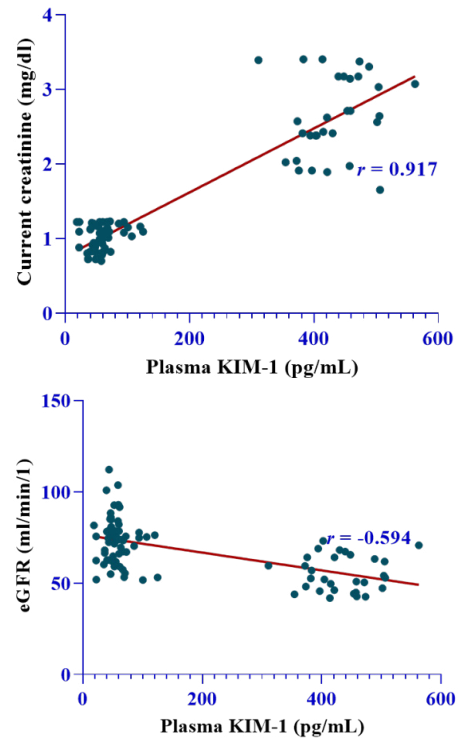


**Fig. 4.** A: Correlation between current creatinine and plasma KIM-1, B: The correlation between eGFR and plasma KIM-1 (all correlation coefficients (r) are statistically significant at p<0.0001, the strong positive correlations suggest that higher KIM-1 levels closely with increases in creatinine levels, indicating possible deterioration in renal function)

under the curve (AUC) of (1.000, p<0.001), as shown in Figure 5A. Similarly, plasma IL-18 showed a high diagnostic capability with a cut-off value of >254.8 pg/mL, yielding a sensitivity of 96% and specificity of 97%, with an AUC of (0.996, p<0.001), as depicted in Figure 5B.

These results highlight the exceptional diagnostic power of plasma KIM-1 and IL-18 as biomarkers of acute CRS, with plasma KIM-1 showing perfect discrimination. Both biomarkers demonstrated a robust potential for early detection and risk stratification in patients with acute CRS, supporting their clinical utility in improving outcomes through targeted interventions.

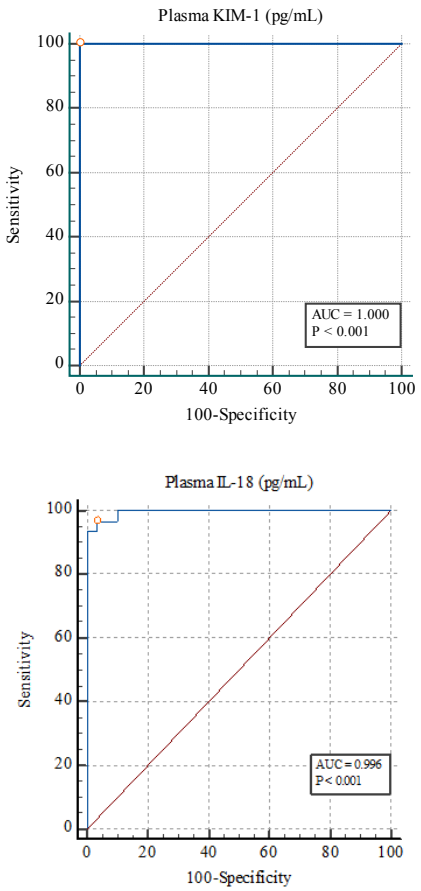


**Fig. 5.** A: The ROC curve for plasma KIM-1, B: The ROC curve for plasma IL-18

***Statistical analysis of plasma KIM-1 and IL-18***

Table 5 presents the logistic regression analysis of the plasma biomarkers KIM-1 and IL-18 as predictors of disease outcome, with model 1 achieving high explanatory power (R2=0.81, (p<0.0001). Plasma KIM-1 level demonstrated a significant positive association with disease outcome (B=1.27, OR=3.5), highlighting its strong predictive potential. Similarly, plasma IL-18 showed a significant, although weaker, association (B=0.06, OR=1.06) The model ROC AUC of 0.971 (95%CI: 0.911–0.995) reflects excellent discriminative ability, further corroborated by high classification accuracy for negative cases (93.33%), positive cases (90%), and overall accuracy (91.11%). These results emphasize the clinical utility of plasma KIM-1 and IL-18 levels as significant and complementary predictors of disease outcomes, and plasma KIM-1 emerging as a stronger predictor in the logistic model.

**Table 5.** Logistic regression analysis of biomarkers to predict disease outcome: evaluation of plasma KIM-1 and IL-18 as significant predictors, model 1 (R2=0.81) (p<0.0001)[a]

| Variables | B (coef) | Wald | Odds ratio | 95% CI for Odds Ratio | p |
|---|---|---|---|---|---|
| Plasma KIM-1 (pg/mL) | 1.27 | 8.3 | 3.5 | 1.50 to 8.49 | 0.003** |
| Plasma IL-18 (pg/mL) | 0.06 | 4.45 | 1.06 | 1.04 to 1.125 | 0.03* |
| ROC AUC | 0.971 (95% CI: 0.911 to 0.995) | | | | |
| Classification accuracy | Negative cases: 93.33%, Positive cases: 90%, Overall: 91.11% | | | | |

[a] B (coef) regression coefficient, CI – confidence interval, dependent variable current creatinine

## Discussion

This study evaluated the clinical utility of plasma biomarkers, kidney function parameters, and disease severity indices to distinguish CRS from AHF and healthy controls. Additionally, we explored the prognostic potential of these biomarkers for predicting disease outcomes. Our findings demonstrated that plasma levels of KIM-1 and IL-18 are significantly associated with renal dysfunction and systemic inflammation. Integrating these biomarkers into clinical practice could revolutionize the management of acute CRS.

The progressive decline in renal function parameters, such as serum creatinine, BUN, and eGFR, is observed to reinforce the pathophysiological continuum from AHF to acute CRS. Acute CRS epitomizes a bidirectional interplay, in which cardiac dysfunction precipitates renal hypoperfusion, resulting in structural kidney damage and impaired filtration capacity.[28] Our study corroborates previous research that indicates that eGFR and serum creatinine level are critical markers of renal dysfunction, with progressive deterioration being a hallmark of CRS.[29,30]

However, although these traditional parameters are instrumental in reflecting established kidney injury, their diagnostic sensitivity in the early stages of CRS remains suboptimal.[6] This limitation arises from their inherent nonspecificity and delayed elevation following renal injury.[31] For example, the serum creatinine level may not rise until significant renal impairment has occurred, thus delaying timely diagnosis and intervention.[32]

Our findings are further aligned with those of other studies that highlight BUN as an adjunctive marker of renal dysfunction. Elevated BUN levels in acute CRS patients not only signify renal impairment, but also reflect

metabolic derangements associated with CRS.[33] The increase in BUN is indicative of reduced renal perfusion and neurohormonal activation, which contribute to the accumulation of uremic toxins.[34] The concurrent decline in eGFR and the increase in serum creatinine underscore the progressive deterioration of glomerular function, a critical aspect of the pathophysiology of CRS.

This study underscores the clinical utility of plasma KIM-1 and IL-18 as biomarkers for the early detection of renal injury and inflammation in acute CRS. KIM-1, a type I transmembrane glycoprotein expressed in proximal tubular cells, is markedly up-regulated after ischemic or toxic injury, and serves as a sensitive and specific indicator of tubular damage.[11,12] Elevated levels of plasma KIM-1 likely reflect tubular injury due to renal ischemia, as KIM-1 is up-regulated in response to tubular cell damage.[11] In our study, elevated plasma KIM-1 levels were significantly associated with acute CRS, demonstrating superior diagnostic accuracy (AUC=1.0), while our ROC analysis demonstrated exceptionally high AUC values for plasma KIM-1 (1.000) and IL-18 (0.996), these results should be interpreted with caution. The near-perfect discrimination may partly reflect overfitting due to our limited sample size and the absence of an independent validation cohort. Future studies are warranted to validate these findings in larger, multicenter cohorts. This finding aligns with that of Liu et al., who reported that KIM-1 is a robust biomarker for AKI with high sensitivity and specificity.[35]

IL-18, a pro-inflammatory cytokine, plays a crucial role in the immune response to renal injury by activating the inflammasome pathway, leading to tubular apoptosis and exacerbating kidney damage.[13,36] The elevated IL-18 in our study are consistent with previous evidence linking IL-18 to ischemic AKI and its role as a biomarker of systemic inflammation.[14,37] The positive correlation of IL-18 with serum creatinine and the negative correlation with eGFR in our study further substantiates its diagnostic relevance.

Our logistic regression analysis confirmed that KIM-1 and IL-18 were independent predictors of adverse clinical outcomes in patients with CRS. KIM-1 demonstrated the highest predictive strength with an odds ratio of 3.5, indicating a strong association between elevated KIM-1 levels and unfavorable outcomes. This finding is consistent with that of Zhang et al., who identified KIM-1 as a significant predictor of progression to CKD and adverse outcomes in patients with AKI. IL-18 has also emerged as an independent predictor, albeit with a weaker association, which may reflect its role as a systemic inflammatory marker rather than a direct indicator of tubular damage.[37,38] This distinction highlights the multifaceted nature of CRS, in which both structural and inflammatory processes interact to determine patient outcomes.

The high area under the ROC (AUC=0.971) and the classification accuracy (91.11%) achieved by our predictive model suggest that incorporating KIM-1 and IL-18 into clinical workflows could substantially enhance the precision of CRS diagnosis and prognosis. This aligns with the contemporary shift in nephrology and cardiology towards adopting biomarker-based approaches for the early detection of high-risk patients.[39]

The robust correlations among the parameters of KIM-1, IL-18, and renal function offer significant pathophysiological information about CRS. The strong positive correlation with serum creatinine and a negative correlation with eGFR underscores its close association with tubular injury and impaired filtration capacity. Similarly, IL-18's significant correlations with both renal function and systemic inflammation highlight its dual role in mediating kidney damage and orchestrating immune responses.[40] The inverse relationship between eGFR and both biomarkers emphasizes the intricate interplay between declining filtration capacity and progressive renal damage in CRS. These findings suggest that KIM-1 and IL-18 could serve not only as diagnostic tools, but also as surrogate markers to monitor disease progression and therapeutic efficacy. This dual functionality aligns with the principles of precision medicine, advocating personalized therapeutic strategies based on individual biomarker profiles.[41,42] Recent studies have also investigated emerging biomarkers such as NGAL, cystatin C, and soluble ST2 for CRS.[43,44] Although our study focused on KIM-1 and IL-18, future comparative analyzes are needed to determine their relative performance and potential for integration into clinical practice.

These findings have profound clinical implications. Early detection of acute CRS using biomarkers such as KIM-1 and IL-18 can facilitate timely therapeutic interventions, including optimizing volume status, managing neurohormonal activation, and mitigating inflammation. Moreover, these biomarkers can inform personalized treatment strategies by stratifying patients according to disease severity. For example, KIM-1's sensitivity to tubular injury can identify patients who may benefit the most from renal protective therapies, while IL-18 can aid in monitoring the efficacy of anti-inflammatory treatments. Integrating these biomarkers into clinical practice aligns with evolving paradigms in precision medicine, which prioritize individualized risk stratification and therapeutic approaches in the management of complex syndromes such as CRS.[45,46] Furthermore, the use of KIM-1 and IL-18 can improve the early identification of high-risk patients, potentially improving clinical outcomes and reducing the healthcare costs associated with delayed diagnosis and treatment. Our results are in line with recent reports that highlight the promise of biomarker-guided approaches for early

detection and personalized management in renal dysfunction.[47] Despite the promising diagnostic and prognostic potential of plasma KIM-1 and IL-18, several practical barriers remain. These include the cost-effectiveness of routine biomarker measurement, the need for standardized assay protocols in clinical laboratories, and regulatory hurdles that can affect widespread clinical adoption. Addressing these issues will be critical to a successful translation into daily clinical practice.

***Study limitations and strengths***

One of the main strengths of this study was the comprehensive evaluation of traditional renal biomarkers (serum creatinine) and a new marker of tubular injury (KIM-1) in an acute cardiorenal population. By correlating baseline and current creatinine levels with KIM-1, our findings provide more insight into the pathophysiology of AKI in the setting of heart failure. Furthermore, the use of standardized analytical techniques and well-defined clinical groups strengthened the internal validity of our results. Despite these compelling findings, this study has several limitations. The case-control design restricts our ability to draw causal inferences regarding biomarker dynamics and disease progression. Longitudinal studies are essential to validate the temporal relationships between KIM-1 and IL-18 levels and clinical outcomes in CRS. A major limitation of our study is the small sample size (n=30 per group), which can increase the risk of overfitting and limit the generalizability of our findings. Future studies with larger, multicenter cohorts are needed to confirm these results.

***Future directions***

Future research should explore the interaction between cardiac biomarkers (eg, NT-proBNP) and renal biomarkers to provide a more holistic understanding of the pathophysiology of CRS. Investigating the combined prognostic value of these biomarkers could reveal the complex interdependencies between cardiac and renal dysfunctions. Future research should include an independent validation cohort to verify the diagnostic and prognostic performance of these biomarkers. Moreover, evaluating the cost effectiveness and feasibility of integrating KIM-1 and IL-18 into routine clinical workflows is crucial to their widespread adoption. Although our findings are promising, future studies involving larger and more heterogeneous cohorts over extended time frames are essential to validate the diagnostic and prognostic functions of these biomarkers and to ensure their effective integration into clinical practice. Furthermore, our study represents a single time-point analysis. Future research should include serial measurements of plasma KIM-1 and IL-18 to better elucidate their temporal dynamics during disease progression and recovery.

## Conclusion

This study highlights the clinical utility of plasma KIM-1 and IL-18 levels as diagnostic and prognostic biomarkers for acute CRS. Their strong association with renal dysfunction and adverse disease outcomes underscores their potential to enhance risk stratification, facilitate early diagnosis, and inform personalized treatment strategies. These findings contribute to the growing evidence supporting biomarker-based approaches in the management of cardiorenal syndromes, paving the way for more precise and effective care in this high-risk patient population.

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## Declarations

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***Author contributions***

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

***Conflicts of interest***

The author declare that they have no competing interests.

***Data availability***

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

***Ethics approval***

Ethical clearance was granted by the Institutional Review Board of the Medical Laboratory Techniques, College of Health and Medical Techniques at Al-Furat Al-Awsat Technical University, Al-Kufa, Iraq (Approval Number: 34328).

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**ORIGINAL PAPER**

# Radiological evaluation of the normal patella position using the Insall-Salvati ratio

Beryl Shitandi Ominde [1], Mamerhi Taniyohwo Enaohwo [2], Onajite Gloria Erabocha [2], Ogheneyole Jeremiah [2], Joyce E Ikubor [3], Orovwoghene Faithful Omoro [2], Patrick Sunday Igbigbi [2]

[1] Department of Biomedical Sciences, Aga Khan University, Nairobi, Kenya
[2] Department of Human Anatomy and Cell Biology, Delta State University, Abraka, Nigeria
[3] Department of Radiology, Delta State University Teaching Hospital, Oghara, Nigeria

**ABSTRACT**

**Introduction and aim.** Normal patellofemoral relationship relies on the patella's location which is vital in the knee's stability and biomechanics. This research aimed to determine the normal Insall Salvati Ratio (ISR) and the cut-off values that will be useful for diagnosing patella alta and baja in Delta State in Nigeria.

**Material and methods.** This retrospective cross-sectional study assessed the Insall-Salvati Ratio by dividing the patella tendon length by the patella length. These lengths were measured on lateral knee radiographs of 300 patients (149 males, 151 females) aged 20 years and older using digital calipers calibrated in cm.

**Results.** With exception to the ISR, the measured variables showed sexual dimorphism and a significant weak negative association with age ($p<0.05$). Based on the international ISR cut-off values (<0.8 and >1.2), the prevalence of patella baja and alta was 15 (5%) and 64 (21.3%) respectively using the calculated cut-off values (<0.73 and >1.41). A lower prevalence of 6 (2%) and 9 (3%) were recorded correspondingly.

**Conclusion.** The normal ISR cut-offs provided by this study will aid radiologists and orthopedic specialists in Delta State, Nigeria to precisely diagnose patella alta and baja and ensure proper restoration of the knee's stability and biomechanics as well as minimizing complications.

**Keywords.** alta, baja, height, insall-salvati ratio, length, patella

## Introduction

Among all joints, the knee is the most prone to injuries with a high tendency of natural degeneration.[1] The chief orthopedic complaint among physically active adults and adolescents is anterior knee pain.[2,3] The complex knee joint contains the patellofemoral joint which is the articulation between the femoral trochlea and a sesamoid bone called the patella.[4] This bone is an essential part of the knee's extensor mechanism, together with the patella retinaculum, quadriceps muscle and tendon, tibial tubercle and patella ligament.[5,6] The patellofemoral joint's stability depends on the congruence of articulating surfaces, patella tendon (PT)/quadriceps tendon and joint capsule. Patellofemoral pain occurs in 25% of all knee problems.[4,7] It has a multifactorial pathogenesis such as malalignment and maltracking which pre-

**Corresponding author:** Beryl S. Ominde, e-mail: berylominde@gmail.com

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dispose to osteoarthritis.[3] Patellofemoral malalignment can be managed by strengthening exercises, taping and orthoses.[7]

Normal patellofemoral relationship relies on the patella's location which is vital in the knee's stability and biomechanics.[8,9] Abnormal patella height is linked with patella instability, extensor mechanism disorders, patella arthritis, meniscal tears, patella chondromalacia, and anterior cruciate ligament (ACL) injuries.[1,10] Patella baja and patella alta are the most prevalent patella pathologies physicians and radiologist's encounter.[3]

Patella alta, typically described as an abnormally high riding patella is likely seen in patients with Osgood-Schlatter disease, patella chondromalacia, Sinding-Larsen-Johansson disease, patella ligament rupture, recurrent lateral patellar dislocation or subluxation, and patella tendonitis.[2,10] Patella alta with no instability may be regarded as a normal variant.[11] Patella baja is a low riding patella which can be congenital or acquired.[12] Congenital causes could be due to shortened PT, distal patella location within the femoral trochlear, and reduced distance from inferior patella pole to proximal tibial articular surface.[6] It may also signify neuromuscular conditions and achondroplasia.[2] Acquired patella baja can be due to patella fracture, and quadriceps tendon rupture subsequent to trauma or surgeries like high tibial osteotomy, retrograde femoral nailing, and anterior cruciate ligament (ACL) reconstruction.[6,8] Patella baja, further predisposes to lateral meniscal tears.[1]

Computed tomography (CT), conventional radiography, and magnetic resonance imaging (MRI) can be utilized in characterizing patella maltracking, and malalignment.[1] Conventional radiography is routinely used due to its availability and affordability, although, it has inherent magnification and limited visualization of the joint cartilage.[11] Three-dimensional visualization of the patella-trochlear cartilage overlap is clear on MRI hence, useful in assessing the patella ratios with no risk of exposure to radiation.[4,9] Nonetheless, MRI is expensive, not widely available, and also challenging to standardize the flexion angles of knee in a non- weight bearing position.[11]

Several indices from radiological measurements may be employed in patella height evaluation namely: Insall-Salvati, Blackburne-Pell, modified Insall-Salvati, Caton-Deschamps, and patellotrochlear index.[1,11] Insall-Salvati ratio (ISR) is regarded as a simple, precise, reproducible and reliable index applicable in all flexion degrees of the knee and uses identifiable patella and tibial landmarks.[10,13] It represents the proportion of the patella tendon's length (PTL) to patella's length, introduced by Insall and Salvati[14] from lateral radiographs with knees flexed at 30 degrees.[2] The maximum distance from the distal patella's pole to the tibial tuberosity defines the PTL while the patella's supero-inferior diagonal dimension is the patella length.[1] An ISR of <0.8 suggests patella baja while >1.2 implies patella alta.[14] There is an acceptable correlation between MRI and radiographic Insall –Salvati ratios.[1] The limitations of the ISR encompass the variant shape of the patella's inferior pole, inability to assess the tibial tuberosity, and the fact that measurements don't change after the tibial tubercle distalization.[11,15]

The ISR and the metric parameters of the patella vary in different study cohorts based on factors that impact knee morphology such as genetics, ethnicity, environmental and geographical factors.[2,3,5,8,16]

The patella's position is influenced by lifestyle and socio-cultural practices such as sitting on the floor, kneeling, yoga and squatting.[2,3] The ISR thresholds have previously been established based on previous investigations among Western populations that have distinct lifestyle and cultural patterns like chair sitting. However, these thresholds may not be applicable in other different genetic, racial, cultural and environmental settings.[3,4,8] This highlights the need for population specific cut-offs for diagnosing abnormal patella position.[2,3] Common Nigerian activities involve squatting, kneeling and floor-sitting which affect the patella height and largely the morphology of the knee. There is potential for patient misdiagnosis in Delta State Nigeria owing to scarcity of research to establish the thresholds for normal patella height in the region.

## Aim

This study aimed at establishing the ISR thresholds for diagnosing patella baja and alta in Delta State, Nigeria. By developing population-specific diagnostic criteria, the research aimed at improving the accuracy of radiological diagnosis and classification of abnormal patella position. The cut-off values will also aid orthopedic surgeons in monitoring post-operative changes following procedures such as ACL reconstruction and high tibial osteotomy, where patella position is critical to long-term knee function.

## Material and methods

This investigation was retrospectively conducted in a single radiological unit and the observational cross-sectional design was used. Lateral radiographic views of knees from 300 patients (149 males and 151 females) seen at the Radiology Department of a Tertiary Hospital in Delta State, Nigeria, from 2nd January, 2017 to 30th December, 2022 were assessed. The convenience sampling technique allowed the inclusion of all available radiographs taken within the specified time frame which fulfilled the selection criteria. The adopted sampling procedure provided a sufficient sample size although the findings can't be generalized to the whole Nigerian population.

The study therefore included patients of both sex groups aged 20 years and older. The exclusion of children was based on the presence of cartilaginous growth plates around joints due to incomplete ossification of bones.[2,17] The study excluded radiographs with evidence of previous knee surgery or knee pathologies namely: fractures, osteoarthritis, inflammatory arthritis, congenital anomalies, tumors, and dislocation. As this was a retrospective investigation, patients with clinical knee symptoms such as pain and abnormal gait could not be excluded. Technically inadequate lateral views with knee rotation or flexion less than 30 degrees were excluded. We did not calculate an estimated sample size. However, from the 794 lateral knee radiographs identified in the database, 300 were utilized, exceeding the sample size used in previous similar studies.[4,8] This was considered sufficient to generate reliable data for this study.

The protocol used was authorized by the Hospital's ethical board (DELSUTH/HREC/2024/004/0742). This board did not deem it necessary to obtain patient consent owing to the retrospective design of the study involving no contact with the patients. The study adhered to the ethical guidelines outlined in the Declaration of Helsinki. According to the center's radiography techniques, only the knee with suspected pathology was imaged to minimize exposure to radioactive rays. Therefore, bilateral measurements could not be obtained. To tense the PT, visualize the PT's tibial insertion and ensure imaging consistency, the lateral knee radiographs were acquired at a 30 degrees semiflexed knee position. The images were acquired 100 cm from the film and rays focused perpendicular to the joint.

Using a desktop computer, the selected images in the picture archiving communications systems were examined and the indicated age and gender of the patients were recorded. Using a digital ruler calibrated in centimeters (cm), a single investigator measured each metric variable three times, and the averages were documented to minimize inter- and intra-observer variability. To ensure reliability, repeatability of measurements was assessed through consistency checks during initial trials. The maximum supero-inferior distance from the proximal to distal patella poles diagonally was the patella length while the patella's tendon length was the maximum distance from the patella's inferior pole to tuberosity of tibia (Fig. 1).[1] The ratio of these two lengths; PTL to the patella length was calculated as the Insall-Salvati ratio.[14]

Based on the international ISR thresholds, patella height was classified as patella baja (ISR <0.8), normal (ISR 0.8-1.2) and patella alta (ISR >1.2).[14] Furthermore, we established cut-off values at two standard deviations above and below the mean ISR as described by Di et al.[3] Based on these, the prevalence of patella baja and alta were determined.

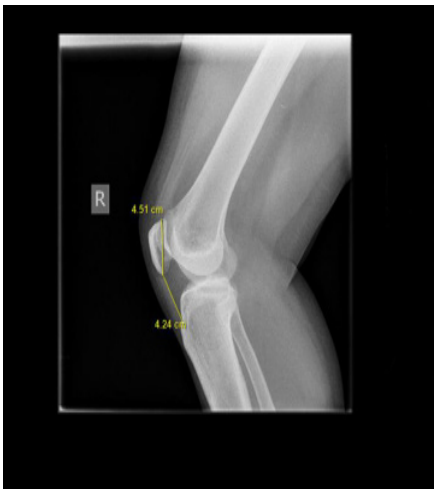


**Fig. 1.** Right lateral knee radiograph showing the patella tendon length (4.24 cm) and patella length (4.51cm)

Data gathered was explored utilizing Version 27.0 of the statistical package of social sciences (SPSS) software (Inc. Chicago, IL, USA). Descriptive statistics were presented in tables. The independent t-test compared the quantitative variables in the two sex groups while the analysis of variance (ANOVA) assessed how these variables differed among the age groups. The Pearson's correlation test was used to investigate the inter-variable associations. Significance level was fixed at $p<0.05$.

## Results

The study assessed the ISR using 300 radiographs comprising 149 (49.7%) male patients and 151 (50.3%) female patients. The mean age and age-range of the patients was 43.82±15.81 years and 20-80 years respectively. The average age of males and females was 48.02±14.79 years and 39.57±15.72 years respectively. The age-group with the highest frequency of patients was the 21–30 years while oldest age group (71–80 years) had the least frequency (10, 3.3%) (Table 1).

**Table 1.** Patient distribution based on age-groups

| Age group (years) | N (%) | | |
|---|---|---|---|
| | Males | Females | Total Population |
| <20 | 9 (6) | 7 (4.6) | 16 (5.3) |
| 21–30 | 47 (31.5) | 17 (11.3) | 64 (21.3) |
| 31–40 | 35 (23.5) | 24 (15.9) | 59 (19.7) |
| 41–50 | 18 (12.1) | 32 (21.2) | 50 (16.7) |
| 51–60 | 19 (12.8) | 36 (23.8) | 55 (18.3) |
| 61–70 | 15 (10.1) | 31 (20.5) | 46 (15.3) |
| 71–80 | 6 (4) | 4 (2.6) | 10 (3.3) |
| Total | 149 (100) | 151 (100) | 300 (100) |

The descriptive statistics following the analysis of the lengths and ISR are depicted in Table 2. The patella and its tendon were significantly longer in males than

females and varied across age-groups (p<0.05). However, the ISR lacked sexual dimorphism and age-related variances (p=0.583, 0.690) (Tables 2 and Table 3).

**Table 2.** Descriptive statistics of the metric variables and their gender based comparison*

| | | PTL (cm) | PL (cm) | ISR |
|---|---|---|---|---|
| **Male** | Min | 3.07 | 3.22 | 0.6 |
| | Max | 7.87 | 6.18 | 1.9 |
| | Mean±SD | 5.00±0.77 | 4.75±0.47 | 1.06±0.19 |
| **Female** | Min | 3.20 | 3.53 | 0.7 |
| | Max | 5.88 | 5.81 | 1.5 |
| | Mean±SD | 4.58±0.55 | 4.29±0.39 Eo | 1.07±0.15 |
| **Total Population** | Min | 3.20 | 3.22 | 0.59 |
| | Max | 7.87 | 6.18 | 1.86 |
| | Mean±SD | 4.79 ±0.70 | 4.52 ±0.49 | 1.07 ± 0.17 |
| **p** | | 0.001 | 0.001 | 0.583 |

* PTL – patella tendon length, PL – patella length, ISR – Insall-Salvati ratio, SD – standard deviation

**Table 3.** Differences in the variables based on age group*

| Variables | Age (years) | | | | | | | p |
|---|---|---|---|---|---|---|---|---|
| | <20 | 21–30 | 31–40 | 41–50 | 51–60 | 61–70 | 71–80 | |
| PTL | 5.10±0.81 | 5.00±0.76 | 4.83±0.64 | 4.63±0.77 | 4.74±0.67 | 4.60±0.48 | 4.55±0.70 | 0.010 |
| PL | 4.56±0.38 | 4.68±0.56 | 4.58±0.43 | 4.40±0.51 | 4.45±0.41 | 4.42±0.50 | 4.55±0.52 | 0.025 |
| ISR | 1.13±0.21 | 1.08±0.20 | 1.06±0.17 | 1.06±0.17 | 1.07±0.15 | 1.06±0.16 | 1.01±0.14 | 0.690 |

* PTL – patella tendon length, PL – patella length, ISR – Insall-Salvati ratio

The PTL and the patella length showed a significant weak negative correlation with age and a significant weak positive association with one another (p<0.05). The ISR had a significant strong positive relationship with PTL and weak negative association with patella length respectively (p<0.05). The ISR lacked a significant association with age (p=0.117) (Table 4).

**Table 4.** Correlation between the quantitative variables*

| | | Age (years) | PTL (cm) | PL (cm) | ISR |
|---|---|---|---|---|---|
| **Age (years)** | r | 1 | -0.219* | -0.167* | -0.091 |
| | P | | 0.001 | 0.004 | 0.117 |
| **PTL (cm)** | r | -0.219* | 1 | 0.210* | 0.753* |
| | P | 0.001 | | 0.001 | 0.001 |
| **PL (cm)** | r | -0.167* | 0.210* | 1 | -.474* |
| | P | 0.004 | 0.001 | | .001 |
| **ISR** | r | -0.091 | 0.753* | -0.474* | 1 |
| | P | 0.117 | 0.001 | 0.001 | |

* PTL – patella tendon length, PL – patella length, ISR – Insall-Salvati ratio, P- probability value, r – Pearson's correlation coefficient, *p considered significant at <0.05

Table 5 shows the percentage of patella variants classified based on cutoffs by Install and Salvati 1971 with ISR <0.8 classified as patella baja and ISR >1.2 classified as patella alta. Based on these cut-offs, the frequency of patella baja and alta was 15 (5%) and 64 (21.3%) respectively. From the calculated thresholds herein, the occurrence of patella baja and alta was 6 (2%) and 9 (3%) correspondingly. The prevalence of patella baja was equal in both males (2%) and females (2%) while patella alta had a higher prevalence in males (4%) than in females (2%) (Table 5).

**Table 5.** Frequency of patella variants

| | | N (%) | | |
|---|---|---|---|---|
| | | Male | Female | Total |
| Based on Insall and Salvati | Patella baja | 10 (6.7) | 5 (3.3) .e | 15 (5) |
| | Patella alta | 31 (20.8) | 33 (21.9) | 64 (21.3) |
| | Normal | 108 (72.5) | 113 (74.8) | 221 (73.7) |
| | Total | 149 (100) | 151 (100) | 300 (100) |
| Based on current study | Patella baja | 3 (2) | 3 (2) | 6 (2) |
| | Patella alta | 6 (4) | 3 (2) | 9 (3) |
| | Normal | 140 (94) | 145 (96) | 285 (95) |
| | Total | 149 (100) | 151 (100) | 300 (100) |

The midline of the graph in Figure 2 represents the mean ISR (1.07) in this study while the dotted lines show the normal range cut-off values within the 2 SD range (0.73, 1.41). The patella height was classified as patella baja and alta when the ISR was below 0.73 and above 1.41 respectively (Fig. 2).

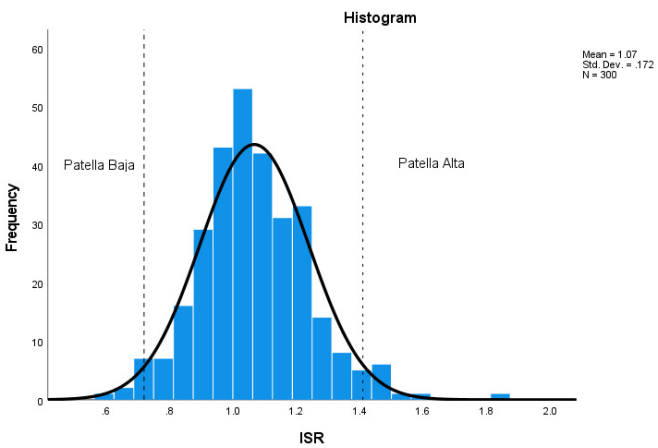


**Fig. 2.** Distribution of the ISR in the study population

The mean variables, ISR cut-offs and frequency of patella baja and alta in diverse population groups are compared on Table 6.

## Discussion

The mean PTL and patella length in this study were higher than the findings by Di et al.[3] The Indians evaluated

**Table 6.** Comparison of cut-offs and prevalence of patella baja and alta in different populations*

| Author | Country | n | Modality | Gender | Patella tendon length (cm) | Patella length (cm) | Insall-Salvati ratio | ISR Cut-offs | | Prevalence (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Baja | Alta | Baja | Alta |
| Althani et al.[2] | United Arab Emirate (Emirati) | 400 | Radiographs | Males | | | 1.22 | <0.86 | >1.54 | | |
| | | | | Females | | | 1.18 | <0.80 | >1.20 | 4 | 38 |
| | | | | Total | | | 1.20 | | | | |
| Rhatomy et al.[4] | Indonesia | 100 | MRI | Males | | | 1.09 | | | | |
| | | | | Females | | | 1.09 | | | | |
| | | | | Total | | | 1.09 | | | | |
| Di et al.[3] | Vietnam | 455 | MRI | Males | 4.21 | 4.19 | 1.01 | <0.72 | >1.32 | 2.4 | 0.9 |
| | | | | Females | 3.88 | 3.76 | 1.03 | | | | |
| | | | | Total | 4.06 | 4.00 | 1.02 | | | | |
| Kumar and Ram[8] | South India | 200 | Radiographs | Males | | | 1.41 | | | | |
| | | | | Females | | | 1.28 | | | | |
| | | | | Total | | | 1.34 | | | | |
| Upadhyay et al.[17] | India | 400 | Radiographs | Males | 5.16 | 4.63 | 1.12 | <0.70 | >1.50 | 1 | 2.8 |
| | | | | Females | 4.14 | 3.80 | 1.17 | <0.80 | >1.20 | 3 | 19 |
| | | | | Total | 4.80 | 4.22 | 1.14 | | | | |
| Present Study | Nigeria | 300 | Radiographs | Males | 5.00 | 4.75 | 1.06 | <0.73 | >1.41 | 2 | 3 |
| | | | | Females | 4.58 | 4.29 | 1.07 | <0.80 | >1.20 | 5 | 21.3 |
| | | | | Total | 4.79 | 4.52 | 1.07 | | | | |

* MRI – magnetic resonance imaging, ISR – Insall-Salvati ratio

by Upadhyay et al.[17] had longer PTL and shorter patella length than the Nigerians herein. The ISR was higher than the reports by Uduoka and Bienonwu,[5] Capkin et al.[13] and Di et al.[3] and lower than the observations by several other authors (Table 6).[2,4,8,17] Variations in the PTL have been linked to individual differences in height.[18] Taller people have longer patella tendons than shorter people. Long PTs predispose to high riding patella (patella alta).[11] The variant PTL is influenced by its migration rate. As bones grow, tendons and ligaments migrate at varying rates depending on the different insertions. The migration is greater when the insertion to the bone is nearer. Migration is caused by periosteal growth and stretching caused by inherent epiphyseal plate activity. As the PT migrates, its deeper layer lengthens at its insertion point while the superficial fibers slide over the tibial tuberosity's surface.[17] Awareness of the patella's morphometry is necessary in the design of implants and minimizing surgical complications.[18]

The variability in morphometry could have resulted from discrepant imaging modalities, landmarks used and knee positioning, thus highlighting the importance of standardized imaging protocol.[3,11] With the improved soft tissue contrast offered by MRI, the surface of the tendon measured affects the length recorded since it is significantly shorter posteriorly than anteriorly.[11] The tendon's central fascicles inserting on the patella's most inferior pole are shorter than the lateral and medial tendon fascicles.[3,11]

The experience of the researchers to recognize the tendon's insertion at the tibial tuberosity may explain the dissimilarities in measurements.[10] Additionally, diversity could stem from variant knee positioning whereby in MRI, the knee is imaged in passive extension while in lateral radiograph, different protocols with variable flexion degrees are used.[13,18] Although some studies report consistency of the ISR in the all the flexion degrees.[1,2] Shape variations of the patella's inferior pole such as blunt, intermediate or pointed also affects the measurements.[11,15] This can be avoided by calculating the modified Insall-Salvati ratio involving the measurement from the lower limit of the patella's articular surface to the PT's insertion and the extent of the patella's articular surface.[8,9] Sampling biasness of normal subjects versus symptomatic knees and age differences contributes to the diversities observed.[3,18] In China, there is rare over-exercising among the young population who also have a low hospital visit rate due to knee pain or discomfort.[16]

The ISR discrepancies have been ascribed to diverse ethnic and cultural practices.[3,10] Compared to western cultures, the patella position is higher in Asians and does not exhibit any differences from Africans.[2,5] Unlike the Western and African cultures, Asians have unique lifestyle activities involving knee flexion namely; praying, squatting, and sitting cross-legged. These continuously stretch the PT and lengthen the patella hence causing a high ISR.[2,8,17] Clinicians and radiologists need to apply the population-specific ISR for accurate diagnosis of patella instability, malalignment and PT pathologies.

The patella and PT were significantly longer in males than females. This corresponded with Upadhyay et al.[17] Sex bias in sampling could explain the variant degree of sexual dimorphism of patella variables in different studies. Males are involved in many sporting activities and are more likely to have knee complaints or injuries earlier than females whose knee pain is experienced later, mainly caused by degenerative arthri-

tis.[3,18] Intense physical activities in males are responsible for their larger skeletal morphology and muscle mass.[3] The larger patella height in males could be ascribed to a higher circulating testosterone in men than women which is responsible for more periosteal bone thickening.[19] Appreciating the sexual dimorphism of patella variables improves the understanding of sex related patellofemoral problems and their surgical management.

Consistent with several researchers, the ISR lacked sexual dimorphism.[3-5,8] Conversely, Upadhyay et al.[17] documented significantly higher ISR in females while Althani et al.[2] documented significantly larger ISR in males. The sex differences in the distribution of stresses within the PT and in the prevalence of tendinopathy may explain the ISR's sexual dimorphism.[15] The ISR lacked significant association with age. Therefore, clinicians in Delta State can estimate the patella position by applying the normative ISR reference values obtained regardless of the gender or age.

Using the international ISR cut-offs (<0.8 and >1.20) by Insall and Salvati,[14] the prevalence of patella baja and alta (5% and 21.3%) were higher than the prevalence obtained (2% and 3%) with the calculated cut-offs (<0.73 and >1.41). Althani *et al*.,[2] observed higher rates of patella alta using the Insall and Salvati's threshold in the Emirati population of Middle East, therefore recommending an extension of the cutoffs to <0.86 and 1.54. In Vietnam the cut-off documented was <0.72 and >1.32.[3] Among central Indians, the cut-off for patella alta was >1.5; 10% more than the internationally recommended threshold of >1.2, hence, proposing the normal range among Indian squatters to be 0.7 to 1.5.[17] Therefore, ISR should be specific for each ethnic or population group for accurate evaluation of patella position (Table 6).[3] Although this study establishes normative ISR values for the local population, further validation in multi-ethnic cohorts is necessary to generalize findings.

Patella alta is more common than patella baja in most populations but vice versa is true among Asians.[2,3,17] This inconsistency shows ethnic diversity in patella position.[3] Nevertheless, the race bias in the use of the international ISR threshold from western culture in other populations contributes greatly to the disparities in the frequency of patella baja and alta.[16] The occurrence of patella baja was equal in both males and females while patella alta had a slightly higher frequency in males than females. Conversely, using modified Insall-Salvati ratio, a cadaveric study by Wambua *et al*.[15] revealed more patella alta cases among black Kenyan females and concluded that this predisposes them to patella tendinopathy. Females have higher occurrence of patella alta than males due to their weaker hamstrings and higher strength ratio of quadriceps to hamstrings.[18] Females are more prone to patella instability due to ligamentous laxity and larger Q angle caused by the wider pelvis. The Q angle is a known indicator of patellofemoral stability although in individuals of the same height, it shows no sexual dimorphism.[15]

The findings herein are important to radiologists and orthopedic surgeons in evaluating knee conditions, planning of treatment, and monitor the postoperative prognosis.[3,10] In patella alta, performing patella tenodesis and tibial tubercle distalization normalizes the patella height.[11] On the contrary, Patella baja may occur inadvertently after some surgical knee procedures. The prevalence of total knee arthroplasty (TKA) has increased in the recent past and these predispose to postoperative patella infera due to subsequent PT shortening by scarring. Additionally, elevation of femorotibial joint line in TKA may also change the patella height leading to restricted range of motion, pain and crepitation.[6,10] Patella baja may also ensue following progressive PT shortening after surgical harvesting of the PT autograft for arthroscopic ACL reconstruction.[2,6] Moreover improper technique during total or unicondylar knee arthroplasty may lead to patella baja.[13]

The use of readily available knee radiographs and utilizing simple and reproducible morphometric parameters to establish diagnostic ISR cut-off values were the current study's strengths. However, the findings may not represent the broader Nigerian population due to the limited sample size based on the use of a single study center and retrospective study design. The sample entailed symptomatic patients hence, the data does not represent asymptomatic knees. The clinical impact of abnormal ISR values could not be ascertained from the radiographic findings of abnormal patella position.

### *Recommendations*

To increase the sample size, a multi-institutional study is recommended to ascertain the ISR thresholds obtained in this study. Future studies incorporating MRI could provide more accurate ISR measurement owing to high precision in assessing soft tissue and cartilage.

## Conclusion

This study established the normal ISR cutoff range of 0.73 and 1.4 which will assist orthopedic specialists and radiologists in accurately diagnosing abnormal patella positions regardless of patient's sex or age. Precise diagnosis will enhance better surgical outcomes through restoration of the knee's stability and biomechanics.

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## Declarations

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***Author contributions***

Conceptualization, B.S.O. and J.E.I.; Methodology, O.G.E.; Software, J.E.I.; Validation, O.F.O, P.S.I., M.T.E. and J.E.I.; Formal Analysis, O.G.E, O.J. and B.S.O.; Investigation, O.F.O., O.G.E. and J.E.I.; Resources, O.G.E. and P.S.I.; Data Curation, O.J.; Writing – Original Draft Preparation, B.S.O. and M.T.E.; Writing – Review & Editing, B.S.O., O.G.E., O.F.O. and P.S.I.; Visualization, M.T.E, P.S.I; Supervision, J.E.I. and P.S.I; Project Administration, J.E.I.

***Conflicts of interest***

The authors declare no conflict of interest.

***Data availability***

The data for this study are not publicly available to protect patient information but will be available on reasonable request from the corresponding author.

***Ethics approval***

This study due to its retrospective nature did not receive consent from subjects however ethical approval was sought from the Hospital's research and ethics committee (DELSUTH/HREC/2024/004/0742). The study was carried out in compliance with the Declaration of Helsinki.

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**ORIGINAL PAPER**

# Factors affecting prognosis in high-intermediate risk endometrial cancer in according to ESMO/ESGO/ESTRO risk classification – FIGO 2023 analysis of survival outcomes and staging dynamics compared to the FIGO 2009 system

Eren Can Guleryuz [1,2], Busra Korpe [2], Vakkas Korkmaz [3]

[1] Ankara Etlik Zubeyde Hanim Women's Health and Research Hospital, Department of Obstetrics and Gynecology, Ankara, Türkiye
[2] Ankara Etlik City Hospital, Department of Obstetrics and Gynecology, Ankara, Türkiye
[3] Ankara Etlik City Hospital, Department of Gynecologic Oncology, Ankara, Türkiye

**ABSTRACT**

**Introduction and aim.** Accurate staging is essential for determining treatment strategies and predicting outcomes in endometrial cancer (EC). The FIGO staging system was updated in 2023 to incorporate histological and molecular features. This study evaluates the impact of the FIGO 2023 system on high-intermediate risk endometrioid EC cases and compares its prognostic value with the FIGO 2009 system.

**Material and methods.** A retrospective analysis of 140 high-intermediate risk endometrial cancer cases from two tertiary hospitals was conducted. Patients were reclassified using FIGO 2023, and staging shifts were analyzed. Survival outcomes, including overall survival (OS) and progression-free survival (PFS), were assessed using Kaplan-Meier analysis and log-rank tests. Univariate and multivariate regression analyses were performed to identify prognostic factors.

**Results.** Within this high-intermediate risk group, patients were stratified into three groups: group 1 (n=79) consisted of those with LVSI (+) Stage I, group 2 (n=17) included patients with LVSI (-) Stage IB grade 3, and group 3 (n=44) comprised individuals with Stage II. Based on age, a statistically significant difference was identified between group 1 and group 3 ($p<0.05$), while no statistically significant difference in BMI was observed among the groups ($p>0.05$). Additionally, there was a statistically significant difference among the groups concerning the type of surgery performed ($p<0.05$) Although no statistically significant difference in survival outcomes was observed, a trend toward improved risk stratification in OS was noted. Positive lymphovascular space invasion emerged as a key factor influencing upstaging.

**Conclusion.** FIGO 2023 provides a refined staging approach that better aligns with clinical outcomes. Larger prospective studies incorporating molecular profiling are needed to confirm its prognostic utility.

**Keywords.** endometrial cancer, FIGO, high-intermediate risk, lymphovascular space invasion, staging system

## Introduction

Accurate staging and risk stratification are crucial for determining optimal treatment strategies and predicting patient outcomes in endometrial cancer (EC).[1,2] In this context, the staging systems developed by the International Federation of Gynecology and Obstetrics

**Corresponding author:** Busra Korpe, e-mail: busraejderoglu@yahoo.com

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(FIGO) serve as critical tools for clinicians, enabling standardized assessment and facilitating communication about disease extent and prognosis.[3]

However, the field of gynecologic oncology is constantly evolving, and as our understanding of EC improves, periodic updates to staging and risk stratification systems become necessary.[4] The growing recognition of molecular characteristics and lymphovascular space invasion (LVSI) as key prognostic indicators has demonstrated that the FIGO 2009 staging criteria are increasingly insufficient in reflecting disease biology and guiding optimal adjuvant therapy.[5-7] As a result, the FIGO 2023 staging system was introduced to incorporate these advancements and improve prognostic accuracy.[8]

The updated 2023 FIGO system integrates various histological types, tumor patterns, LVSI and molecular classifications to better reflect the improved understanding of the complex nature of several types of EC and their underlying biological behavior.[9,10] In addition to improving prognostic alignment, these changes aim to enhance clinical usability and facilitate precise risk stratification for treatment planning. Given that LVSI has been identified as a key factor in disease progression and recurrence, its inclusion in the FIGO 2023 staging criteria represents a fundamental shift in EC classification. However, the practical implications of these modifications, particularly their effect on stage migration and survival outcomes, remain unclear.

To define prognosis and estimate the risk of nodal metastasis in EC, multiple risk models have been created based on pathological information. In Europe, adjuvant treatment and surgical planning are commonly guided by the classification system established by European Society of Medical Oncology, the European Society of Gynecologic Oncology, and the European Society of Radiotherapy (ESMO, ESGO, ESTRO).[11,12]

According to this guideline, cases of EC are categorized as low, intermediate, high-intermediate, high and advanced metastatic groups. The high–intermediate group is described as: (1) stage I EC, grade 3, less than 50% myometrial invasion regardless of LVSI; (2) stage I EC, grade 1–2, positive LVSI, irrespective of myometrial invasion; or (3) stage 2 EC in the ESMO/ESGO/ESTRO risk classification.[13] Since high-intermediate risk cases include early-stage tumors with LVSI positivity, the integration of LVSI into the FIGO 2023 system is expected to significantly impact patient stratification and treatment decisions. This inclusion, along with the lack of treatment consensus, prompted our interest in evaluating changes in this risk group.

## Aim

Despite advancements, the optimal management of high-intermediate risk EC remains unclear. With LVSI now a formal staging component, assessing FIGO 2023's impact on survival and stage distribution is crucial. This study evaluates the real-world effects of these revisions, analyzing stage migration and survival outcomes to determine if FIGO 2023 improves prognostic accuracy over FIGO 2009.

## Material and methods

A retrospective cohort analysis was conducted on 1163 EC patients who underwent primary treatment at the Gynecologic Oncology Clinics of two tertiary hospitals between March 2011 and August 2023. The study design was approved by the institutional research ethics committee (Approval number: 08.06.2022-2022/78).

A total of 140 patients who met specific criteria, focusing on individuals with endometrioid-type EC classified as high-intermediate risk based on the ESMO/ESGO/ESTRO risk classification.[12] Patients categorized as low, intermediate, high, and advanced metastatic risk, those with non-endometrioid histology, individuals with irregular follow-up, and those with limited data accessibility were excluded from the study. Additionally, patients undergoing fertility-sparing treatment were excluded to maintain uniformity in treatment strategies (Fig. 1).

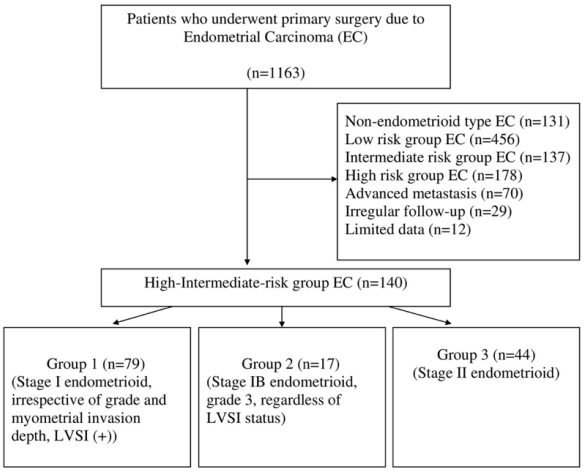


**Fig. 1.** Flow chart of the study

Cancer staging for EC was initially classified based on the 2009 FIGO staging system, and all cases were subsequently reclassified according to the 2023 FIGO system. In early stages, surgical procedures included hysterectomy, bilateral salpingo-oophorectomy (based on the age of the patient), infracolic omental biopsy and peritoneal washings tailored to specific histological subtypes. Lymph node staging, primarily was performed in the majority of cases, with some cases involving a systematic pelvic +/- paraaortic lymphadenectomy. During the study period, lymphadenectomy in our institution was guided by intraoperative frozen section, and no sentinel lymph node procedures were performed for EC cases. The decision for lymph node dissection followed the criteria established by Mariani et al.[14] Patients with a tumor diameter ≤2 cm,

myometrial invasion ≤50%, and no intraoperative evidence of macroscopic disease were classified as low risk and underwent hysterectomy without lymph node dissection. In cases that did not meet these criteria, lymph node dissection was performed. All surgeries were conducted by gynecologic oncologists.

Histopathological evaluations were performed by specialized gynecologic pathologists. LVSI was identified as the detection of tumor cells or clusters on the vessel wall through hematoxylin-eosin staining.[15] Focal LVSI was defined by the presence of a single focus around the tumor, substantial is described by a multifocal or diffuse distribution of LVSI or the detection of tumor cells within five or more lymphovascular space.16 Adjuvant treatment decisions followed ESMO/ESGO/ESTRO guidelines, including radiotherapy, chemotherapy, or combined therapies, depending on the patient's risk profile.[17]

Disease-free survival (DFS) was measured from the initiation of treatment until recurrence in patients who experienced recurrence, the date of the final follow-up for those without recurrence, or the date of death from any cause. Overall survival (OS) was calculated from the time of diagnosis to either the date of death or last hospital visit. Stage and substage-specific 5-year and 10-year DFS and OS rates were calculated and compared.

Patient follow-up records included detailed information such as age, body mass index (BMI), type of surgery (laparoscopy or laparotomy), surgery dates, LVSI status based on postoperative pathological evaluations, cancer stage, grade, myometrial invasion, risk group classification, lymph node involvement, adjuvant therapy, specifics of administered adjuvant treatments, recurrence during follow-up (if any) including location and timing, and information regarding any deaths during the follow-up period.

### *Statistical analysis*

Statistical analyses were performed using the SPSS version 26.0 software package (IBM, Armonk, NY, USA). Descriptive statistics were presented in terms of number, percentage, mean, and standard deviation, as well as median. The normal distribution of variables was assessed visually (histograms and probability plots) and analytically (Kolmogorov–Smirnov, Shapiro-Wilk tests). Numeric variables that did not show normal distribution among the three groups were analyzed by using the Kruskal-Wallis test. Chi-square analysis and Fisher's Exact test were preferred for comparing nominal data. Survival analyses were conducted using Kaplan-Meier survival analysis and the Log Rank test. Univariate analyses were performed using Linear Regression analysis, and multivariate analyses were conducted using Cox Regression analysis. In the statistical analyses of the study, comparisons with a p-value below 0.05 were considered statistically significant.

## Results

A total of 140 patients from the high-intermediate risk group of EC were included in the study. Within this high-intermediate risk group, patients were stratified into three groups: group 1 (n=79) consisted of those with LVSI (+) Stage I, group 2 (n=17) included patients with LVSI (-) Stage IB grade 3, and group 3 (n=44) comprised individuals with Stage II. All cases in Group 1 had substantial LVSI (+).

Upon comparing the groups based on age, a statistically significant difference was identified between group 1 and group 3 ($p<0.05$), while no statistically significant difference in BMI was observed among the groups ($p>0.05$). Additionally, there was a statistically significant difference among the groups concerning the type of surgery performed ($p<0.05$) (Table 1).

**Table 1.** Comparison of demographic parameters, treatment modalities, recurrence and mortality of high-intermediate risk groups*

| | Group 1 (n=79) | Group 2 (n=17) | Group 3 (n=44) | p |
|---|---|---|---|---|
| Age (y) | 60.89±8.76[a] | 60.29±10.94[a,b] | 54.61±8.50[b,c] | 0.001 |
| BMI (kg/m$^2$) | 31.72±3.57 | 32.64±6.03 | 31.41±4.88 | 0.858 |
| Type of operation n (%) | | | | |
| Hysterectomy | 7 (8.9) | 1 (5.9) | 1 (2.3) | |
| TAH+BSO+Pelvic LND+Obx | 9 (11.4) | 0 (0) | 4 (9.1) | <0.001 |
| TAH+BSO+Pelvic LND+Paraaortic LND+Obx | 63 (79.7) | 16 (94.1) | 24 (54.5) | |
| Radical Hysterectomy (Type2-Type3) + Pelvic LND+ Paraaortic LND+ Obx | 0 (0) | 0 (0) | 15 (34.1) | |
| Adjuvant treatment n(%) | | | | |
| Yes | 41 (51.9)[a] | 13 (76.5)[b] | 34 (77.3)[b,c] | 0.009 |
| No | 38 (48.1) | 4 (23.5) | 10 (22.7) | |
| Type of adjuvant treatment n(%) | | | | 0.234 |
| CT | 5 (8.8) | 2 (11.8) | 2 (5.6) | |
| Sandwich | 0 (0) | 0 (0) | 2 (5.6) | |
| Hormone treatment | 0 (0) | 1 (5.9) | 0 (0) | |
| RT after CT | 2 (3.5) | 1 (5.9) | 2 (5.6) | |
| CT after RT | 1 (1.8) | 1 (5.9) | 0 (0) | |
| Cuff brachytheraphy | 18 (31.6) | 1 (5.9) | 8 (22.2) | |
| External pelvic RT | 9 (15.8) | 4 (23.5) | 8 (22.2) | |
| EBRT | 6 (10.5) | 3 (17.6) | 12 (33.3) | |
| Recurrence | | | | |
| Yes | 16 (20.3) | 1 (5.9) | 7 (15.9) | 0.364 |
| No | 63 (79.7) | 16 (94.1) | 37 (81.4) | |
| Mortality | | | | |
| Yes | 14 (17.7) | 0 (0) | 6 (13.6) | 0.177 |
| No | 65 (82.3) | 17 (100) | 38 (86.4) | |

* different letters shows statistical significance, groups that share the same letter are not significantly different from each other, BMI – body mass index, BSO – bilateral salphingoopherectomy, CT – chemotherapy, EBRT – external beam radiation therapy, LND – lymph node dissection, Obx – omental biopsy, TAH – total abdominal hysterectomy, RT – radiotherapy

A statistically significant difference in adjuvant treatment was observed (p<0.05), specifically between group 1 and other groups (Table 1). However, there was no statistically significant difference in terms of disease recurrence and mortality (p>0.05).

**Table 2.** Stage shifts between FIGO 2009 and FIGO 2023 surgical staging systems

| FIGO 2009 | FIGO 2023 | FIGO 2009 | FIGO 2023 | FIGO 2009 | FIGO 2023 |
|---|---|---|---|---|---|
| Stage IA (n=41, 29.3%) | IA1 (n=0) | Stage IB (n=55, 39.3%) | IA1 (n=0) | Stage II (n=44, 31.4%) | IA1 (n=0) |
| | IA2 (n=0) | | IA2 (n=0) | | IA2 (n=0) |
| | IA3 (n=0) | | IA3 (n=0) | | IA3 (n=0) |
| | IB (n=0) | | IB (n=0) | | IB (n=0) |
| | IC (n=1, 0.2%) | | IC (n=0) | | IC (n=0) |
| | IIA (n=0) | | IIA (n=0) | | IIA (n=21, 47.7%) |
| | IIB (n=34, 82.9%) | | IIB (n=30, 54.5%) | | IIB (n=14, 31.8%) |
| | IIC (n=6, 14.6%) | | IIC (n=25, 45.5%) | | IIC (n=9, 20.5%) |

Table 2 presented the stage shifts between the FIGO 2009 and 2023 surgical staging systems. Among the 24 patients who experienced recurrence, after restaging according to FIGO 2023, all were upstaged into Stage IIA (n=3), IIB (n=8), and IIC (n=9). In univariate regression analysis, age, type of surgery, myometrial invasion, and the number of pelvic lymph nodes were identified as risk factors for DFS and OS in all patients (p<0.05). However, in the multivariate regression analyses, statistical significance was not observed (p>0.05).

In the survival analyses, no statistically significant difference was observed in DFS and OS among the groups in both the FIGO 2009 and 2023 surgical staging systems (p>0.05) (Fig. 2A and 2B, Fig. 3A and 3B). The p-values were determined as p=0.160 and p=0.074 for DFS and OS, respectively, according to the FIGO 2023 staging.

## Discussion

Accurate staging is fundamental for tailoring treatment strategies and predicting outcomes in EC.[1] The 2023 FIGO staging system represents a significant advancement, incorporating histological types, tumor patterns, and molecular classifications to better capture the complexities of EC.[8] It is important to assess how updated staging systems impact the classification and management of EC, with a focus on enhancing patient care and outcomes.[17] Studies examining the effects of updated guidelines on disease diagnosis and management play a pivotal role in advancing our understanding.[19]

In this study we assessed the impact of the FIGO 2023 system on 140 endometrioid EC cases in high-intermediate risk group compared to the 2009 FIGO system. Our results revealed that most of the cases progressed into a more advanced stage under the new FIGO 2023 staging system. Notably, all recurrent and deceased patients were among those upstaged, suggesting that the revised classification better aligns with actual disease progression. This highlights the potential of the FIGO 2023 system to more accurately stratify high-risk patients, potentially guiding more appropriate therapeutic decisions.

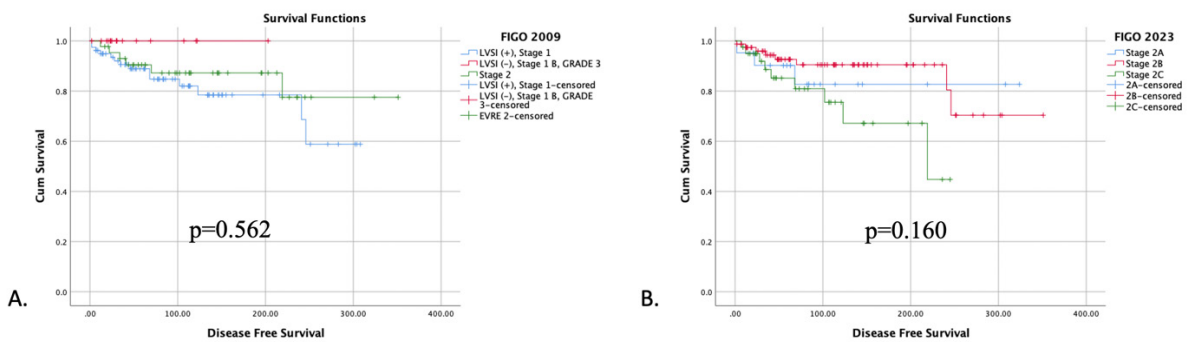


**Fig. 2.** A: Disease-free survival analysis of high intermediate risk groups according to FIGO 2009 vs. 2023, B: staging system

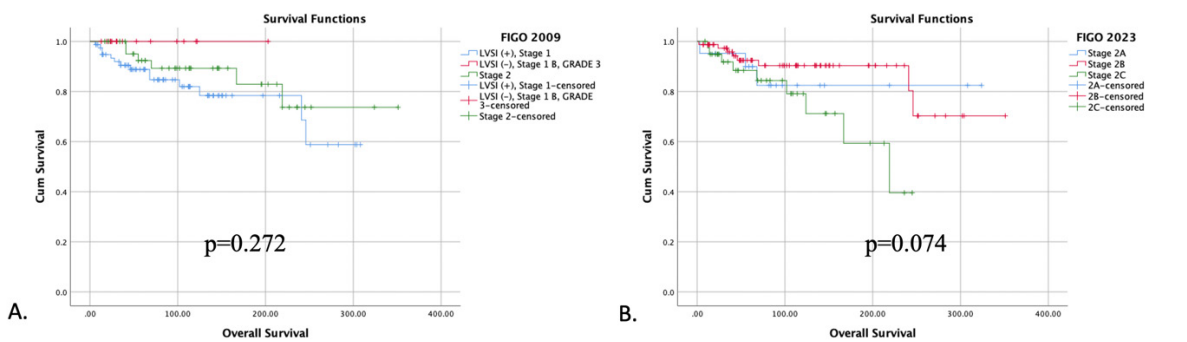


**Fig. 3.** A: Overall survival analysis of high intermediate risk groups according to FIGO 2009 vs. 2023, B: staging system

The high-intermediate risk group has consistently been associated with a significantly poorer prognosis in prior studies.[19,21] A key factor contributing to adverse outcomes is the higher prevalence of lymph node metastases, reinforcing the need for precise staging to guide management.[22-25] While our univariate analysis identified certain factors as potential risk factors for disease-free and overall survival, the multivariate analyses did not confirm statistical significance. This discrepancy suggests a need for larger, prospective studies to clarify the prognostic value of these factors.

Randomized controlled trials, such as GOG-99[26], PORTEC-1[27] have established the effectiveness of adjuvant radiation therapy in addressing intermediate and high-intermediate risk early-stage EC. Within our study, patients in group 1 received notably less adjuvant treatment. The reclassification of LVSI-positive cases to Stage IIC under the 2023 system suggests a more refined risk stratification, leading to increased eligibility for adjuvant therapy. This observation underscores the evolving role of staging systems in shaping treatment strategies and highlights the importance of periodically reassessing clinical guidelines to reflect emerging evidence.

The FIGO 2023 system remains relatively underexplored in the literature, with limited studies evaluating its clinical impact. In a recent publication by Schwameis et al.[19], it was noted that there are significant stage shifts in approximately one-quarter of patients, with prognostic implications that may influence treatment decisions. The introduction of new substages in early-stage EC has enhanced prognostic stratification, allowing for better identification of treatment-relevant subgroups. However, Schwameis et al.'s study did not specifically analyze the high-intermediate risk group, leaving a critical gap in the literature. Similarly, another recent study comparing the FIGO 2009 and 2023 systems, with and without molecular classification, demonstrated that 47% of FIGO 2009 Stage I patients were upstaged based on histopathological findings alone. Notably, in the molecular-informed FIGO 2023 system, 85% of p53-abnormal tumors were upstaged, highlighting the critical role of molecular markers in refining risk stratification. Moreover, POLE-mutated cases were frequently downstaged, suggesting that molecular data significantly influence staging accuracy and prognostic assessment.[20] However, while these findings underscore the relevance of integrating molecular markers into staging algorithms, our study was limited in this aspect, as we could not assess key molecular features such as p53 abnormalities, POLE mutations, and mismatch repair status.

In our study, although no statistically significant difference was observed between groups in the survival analyses based on this updated system, the decreasing trend in the p-value of OS and its proximity to the significance level are noteworthy. This underscores the importance of considering the FIGO 2023 staging for women monitored due to high-intermediate risk EC, urging a revision of their stages and a review of treatment plans accordingly. This observation highlights the potential significance of the evolving staging criteria in refining patient management strategies.

*Study limitations*

Despite its contributions, our study has several limitations. Its retrospective design limits control over confounding variables and prevents causal inferences. Additionally, the relatively small sample size may reduce statistical power and limit the generalizability of our findings to broader EC populations, highlighting the need for validation in larger, multicenter cohorts. The absence of molecular data further restricts our ability to assess the full impact of the FIGO 2023 system, given the growing role of molecular profiling in EC classification. Future prospective studies with extended follow-up are essential to determine whether upstaging translates into improved patient outcomes. Nonetheless, our study contributes to the literature by evaluating the new staging system in a highly homogenized cohort and providing valuable insights into its prognostic impact, an area where published data remain limited.

## Conclusion

The FIGO 2023 staging system has led to significant upstaging in high-intermediate risk endometrioid EC, with all recurrent and deceased cases being among those reclassified. The primary factor influencing upstaging was LVSI positivity, suggesting improved identification of high-risk patients. While our survival analysis did not demonstrate a statistically significant difference between FIGO 2009 and 2023 staging systems, trend toward to a lower p-value in OS analysis is noteworthy. This emphasizes the potential clinical relevance of the new staging system. In this regard, larger-scale further multicenter prospective studies, including molecular profiling, are needed.

## Declarations

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***Author contributions***

Conceptualization, V.K. and E.G.; Methodology, V.K. and B.K.; Software, B.K..; Validation, V.K., B.K. and E.G.; Formal Analysis, B.K.; Investigation, E.G.; Resources, X.X.; Data Curation, E.G.; Writing – Original Draft Preparation, B.K. and E.G.; Writing – Review & Editing, V.K.; Visualization, B.K.; Supervision, V.K.; Project Administration, E.G.

*Conflicts of interest*

The author(s) declare no competing interests.

*Data availability*

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

*Ethics approval*

The study design was approved by the instutional research ethics committee (Approval number: 08.06.2022-2022/78).

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**ORIGINAL PAPER**

# A comparative study on the utility of biomarkers – serum interleukin-13 against serum immunoglobulin E in assessing the severity of asthma

Prasanna Raju [1], Subash Sundar [1], Preethi Suresh [1], Jayaprakash Thulukanam [2], Padmanaban S. [3]

[1] Department of Paediatrics, SRM Medical College Hospital and Research Centre, SRM Institute of Science and Technology, Kattankulathur Campus, Chennai, Tamilnadu, India
[2] Department of Microbiology, SRM Medical College Hospital and Research Centre, SRM Institute of Science and Technology, Kattankulathur Campus, Chennai, Tamilnadu, India
[3] Department of Statistics, NIRT, Indian Council of Medical Research, Chennai, India

**ABSTRACT**

**Introduction and aim.** Asthma is a complex respiratory condition with fluctuating symptoms, airflow obstruction, bronchial hyperresponsiveness, and inflammation. Interleukin (IL)-13 induces various biological responses, including B-cell immunoglobulin E (IgE), eosinophil chemo-attractants, and mucus-secreting goblet cell maturation. B-cell immunoglobulin E antibodies are essential for the onset and propagation of the inflammatory cascade, triggering the allergic response. The aim was to compare the utility of biomarkers – serum IL-13 against serum IgE in assessing the severity of asthma.

**Material and methods.** A cross-sectional observational study was conducted involving 68 asthmatic children aged 6–12 years and 68 age- and sex-matched healthy controls. Asthma severity was assessed using spirometry and categorized as mild, moderate, or severe based on GINA guidelines. Serum IL-13 and IgE levels were measured using validated using enzyme-linked immunosorbent assay.

**Results.** The study confirmed elevated levels of serum IL-13 and IgE in children with asthma compared to the control group, suggesting their involvement in the development of asthma ($p<0.001$). The threshold values for identifying the existence of asthma were 1.86 pg/mL for IL-13 and 314 ng/ml for IgE. The IL-13 level could accurately classify asthmatic children as having either moderate or severe asthma, using a cut-off value of ≥2.66 pg/mL, with a statistically significant $p=0.001$. However, no such results were observed with IgE.

**Conclusion.** Bronchial asthma patients had markedly higher levels of total IgE and IL-13 compared to the healthy controls included in the study. Furthermore, it has been shown that IL-13 plays a role in discerning the extent of asthma severity.

**Keywords.** serum IgE, serum IL-13, severity of asthma

## Introduction

Asthma is a widespread and complex long-term respiratory condition marked by fluctuating and recurrent symptoms, obstruction of airflow, hyperresponsiveness of the bronchi, and underlying inflammation.[1] Asthma, according to the Global Initiative for Asthma (GINA),

**Corresponding author:** Prasanna Raju, e-mail: prasannr@srmist.edu.in

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is a diverse disorder characterized by fluctuating expiratory airflow limitation and respiratory symptoms, including dyspnea, chest tightness, and wheezing, which vary over time and in severity.[2] Asthma is commonly diagnosed in childhood, although it may develop at any stage in life.[3] Typically, 50% to 80% of asthma ailments are evident by age 5.[4]

Although it is most challenging during early childhood years, as many as 50% of children with relatively modest severity have a remission of symptoms by late adolescence; in contrast, 80% of those with more severe symptoms will continue to experience symptoms well into adulthood.[4]

Serum periostin, fractional exhaled nitric oxide (FeNO), serum immunoglobins E (IgE), and interleukin (IL)-13 are among the biomarkers of type 2 disorders that are being studied concerning asthma.

The development of mucus-secreting goblet cells, the generation of protein molecules from the extracellular matrix and myofibroblast differentiation, the eosinophil chemo-attractants, the generation of B-cell immunoglobulin E, and the increased contractility of airway cells of smooth muscle in reaction to cholinergic agonists are just a few of the numerous biological responses that IL-13 induces in relation to asthma.[5]

Allergy-induced asthma also involves a hypersensitivity reaction that is triggered by immunologic processes mediated by IgE antibodies. IgE is essential for the onset and propagation of the inflammatory cascade, which in turn triggers the allergic response.[6] IgE is considered the primary molecular target for the treatment of asthma and allergic disorders, prompting extensive research aimed at disrupting its synthesis or function within the immune system. The efficacy of anti-IgE monoclonal antibodies (mAbs) in asthma treatment substantiates this claim.[7]

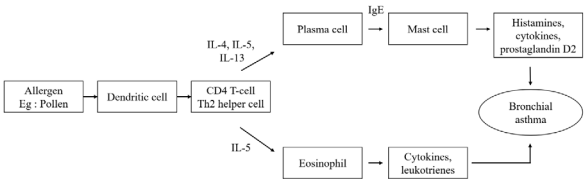


**Fig. 1.** Schematic illustration of the role of IL-13 and IgE in the pathogenesis of asthma

Biomarkers – serum periostin, fractional exhaled nitric oxide (FeNO), serum IgE, and IL-13 were analyzed in a cohort of asthmatics, and the findings demonstrated that the biomarkers were continually distributed and correlated with one another (Fig. 1).[8]

The correlation between IgE concentrations, skin tests, and pulmonary function in individuals with asthma has been demonstrated. According to clinical investigations, asthmatics exhibit an inverse correlation between IgE concentrations and $FEV_1$/FVC ratios.[9] In vitro studies indicate that IgE formation in allergic asthma patients is more reliant on IL-13 compared to non-atopic children, due to increased IL-13 levels and IgE synthesis in response to IL-13.[10]

FEV1 has established itself as the definitive tool for lung function assessment in asthma, due to its ease and speed of measurement, requiring a piece of relatively straightforward equipment. The existing asthma guidelines use $FEV_1$ in addition to daytime and nocturnal symptoms to gauge severity of asthma.[11,12]

As stated by some, children with severe chronic asthma do not experience a substantial reduction in FEV1 during asthma free periods, which is attributed to the slowly progressive nature of the disease.[13]

In light of the substantial contribution of serum IgE and IL-13 to the development of pediatric asthma, The intent of this research is to compare the efficiency of these two variables in assessing the severity of asthma in the population of pediatric asthmatics.

## Aim

To compare the utility of biomarkers – serum IL-13 against serum IgE in assessing the asthma severity.

## Material and methods

The present observational study was conducted at a tertiary hospital in Tamil Nadu after receiving ethical approval from the Institutional Ethics Committee (SRMIEC-ST0922-797). The research was initiated in September 2022 and executed over one year. Based on statistical calculations, considering effect size, a significance level of 0.05, and a study power of 80%, the analysis determined that at least a minimum of 30 participants was necessary to achieve statistical significance.[14] The trial included a cohort of 68 children, aged 6 to 12, recently diagnosed with asthma and initiated on treatment in accordance with GINA recommendations and sex and age-matched controls.[2] An informed consent form was procured from the parents.

The following categories of children were excluded from the study: children younger than the age of six, those already receiving asthma treatment, those who experienced acute exacerbations of asthma requiring systemic steroids in the preceding three months or during the study period, and those who were unwilling to give consent.

The parents provided a thorough medical history that included information about the child's age of onset of wheezing, any history of allergic rhinitis or atopic dermatitis, food allergies, prior use of inhalational corticosteroids, hospital admissions related to the wheezing, and a family history of wheezing and asthma that may have genetic implications.

Children were advised to abstain from physical activity on the morning of the procedure. Trained profes-

sionals performed spirometry, and asthma diagnosis was ascertained by bronchodilator reversibility tests (≥12%).

The patients were clothed in light clothing, with their legs uncrossed, and in an upright position when the procedure was performed. Any dentures that interfered with the process were removed. Air leakage via the nasal passages was minimized by manually blocking the nares using nose clamps. On the day of the test, the spirometer's calibration was verified. The patients were instructed to place the mouthpiece into their mouths. Following confirmation of no air leaks, the following is how the procedure was carried out:

1. The patients were instructed to take a deep breath, draw in as much air as possible, and hold their breath for less than a second at the highest capacity of their lungs.
2. Immediately after taking a deep breath, the mouthpiece was placed within the oral cavity, precisely in the space between the teeth. Tightly sealing the lips surrounding the mouthpiece was crucial to preventing any air leaks. The instructor's recommended time, or a minimum of six seconds, was used for the exhale. To measure only the forced expiratory volume, the patient was instructed to place the mouthpiece after completing step 1 and avoid breathing through the tube.
3. If any of the procedures were performed incorrectly, the technician stopped the procedure and provided the patient with a fresh description of the procedure.
4. Until two matching and good results were achieved, the process was repeated at regular intervals of one minute.
5. After the aforementioned process, the study subjects received 400 micrograms of bronchodilator (salbutamol) to evaluate reversibility.
6. After administering bronchodilators for 15 minutes, the very same procedure was carried out again. The diagnosis of asthma was established when the baseline $FEV_1$ changed by more than 12%, suggesting a positive response and reversibility.

Based on their history and pulmonary function test results, the children were categorized as having intermittent, mild persistent, moderate persistent, or severe persistent asthma, and therapy with inhalational corticosteroids was started in accordance with established guidelines.[2]

Following spirometry, blood samples for serum IL-13 and serum IgE levels were collected from the children in EDTA, endotoxin/pyrogen-free collection tubes and centrifuged at 1000 rotations/min for 10 minutes. Blood samples for the same were collected from 68 age and sex-matched controls. The generated supernatant serum (250–500 µL) was preserved at -70°C in deep freezers at the Molecular Biology Laboratory, SRM Institute of Science and Technology. The samples were subsequently examined by enzyme-linked immunosorbent assay (ELISA) upon thawing at room temperature. The assay analysis was conducted using the Human IL-13 ELISA Kit (Diaclone SAS, Besançon Cedex, France; Cat. No: 850.080.096, Batch: 1013-45T), which exhibited an intra-assay coefficient of variability of 5% and an inter-assay coefficient of 8%. Additionally, the Total IgE EIA Kit (XEMA, Moscow, Russia; Cat. No: 9398-200-18619450-2010) was utilized, with intra-assay and inter-assay coefficients of variability recorded at 5% and 10%, respectively. Absorbance measurements were recorded using the Merilyzer EIA Quant. By plotting the average absorbance of each standard against the corresponding concentrations of human IL-13 and IgE standards along the horizontal axis, a linear standard curve was generated. Using the standard curve and extrapolating optical density values against standard concentrations, the IL-13 and IgE concentrations in each sample were determined.

**Table 1.** Baseline characteristics of the subjects

| Baseline characteristics | Asthmatics | Controls | p |
|---|---|---|---|
| Age in months | | | |
| Mean (SD) | 101.4±25.2 | 102±24.9 | 0.010 |
| Gender, n (%) | | | |
| Male | 54 (79%) | 54 (79%) | 0.999 |
| Female | 14 (21%) | 14 (21%) | |
| Height (cm) | 127.8±16.9 | 129.2±16.3 | 0.768 |
| Weight (kg) | 27.8±10.9 | 16.3±9.8 | 0.338 |
| BMI (kg/m$^2$) | 16.3±3.8 | 17.2±4.2 | 0.414 |
| Family history, n (%) | | | |
| Yes | 59% | – | |
| No | 41% | | |
| History of atopic dermatitis, n (%) | 29% | – | |
| Yes | 71% | | |
| No | | | |
| History of allergic rhinitis, n (%) | | | |
| Yes | 44% | – | |
| No | 56% | | |
| Previous hospital admission, n (%) | 33% | – | |
| Yes | 67% | | |
| No | | | |
| $FEV_1$ | 74.72% | – | |
| Severity based on spirometry | | | |
| Mild asthma | 85% | – | |
| Moderate asthma | 6% | | |
| Severe asthma | 9% | | |

*Statistical analysis*

The comparison of the control versus cases group was analyzed using the students T-test. All statistical analysis was performed using SPSS version 26.0 (IBM, Armonk, NY, USA) and Medcalc version 22.030. A significant p-value was defined as one less than 0.05 for a 95% confidence interval (CI). The receiver operating characteristic (ROC) was employed to assess the ability of the

diagnostic test, specifically IL-13 in our case, to reliably differentiate between two patient conditions – mild versus moderate and severe asthma.

## Results

A total of 68 asthmatic children and 68 non-asthmatics (control group) were recruited in the study (as per inclusion and exclusion criteria). Table 1 lists the baseline characteristics of the study subjects.

It was observed that the mean concentrations of both IL-13 and IgE were higher than in controls (p<0.001). Table 2 provides details of the analysis.

**Table 2.** Comparison of IL-13 and IgE concentration in asthmatics and healthy controls

| Parameter | Asthmatics, mean±SD | Controls, mean±SD | p |
|---|---|---|---|
| IL-13 (pg/mL) | 2.12±0.82 | 1.49±0.27 | <0.001 |
| IgE (IU/mL) | 553.01±371.81 | 385.55±346.07 | <0.001 |

Given below are the ROC curves for IL-13 and IgE, which illustrate the cut-off value of IL-13 (1.86 pg/mL) and serum IgE (314 IU/mL) in evaluating the presence of asthma among cases as compared to controls (Fig. 2).

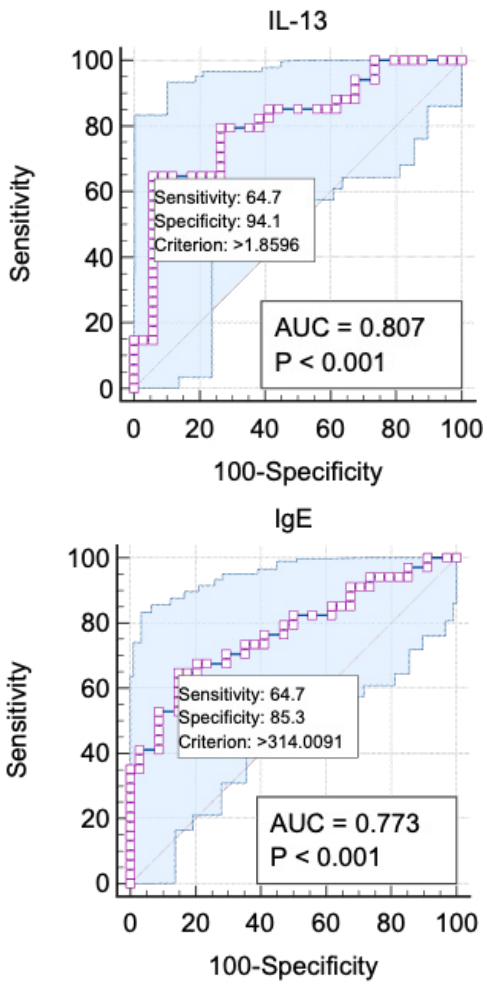


**Fig. 2.** ROC curve for IL-13 and IgE in detecting the presence of asthma in cases

The mean $FEV_1$ of those enrolled in the study was 74.72±10.18%. Serum IL-13 and total IgE levels were determined using conventional ELISA kits. The mean circulating IL-13 level in asthmatic children was 2.12±0.82 pg/mL, whilst the mean serum IgE concentration was 553.01±371.8 IU/mL (Table 3).

**Table 3.** Descriptive statistics of the study subjects

| Parameter | Mean | SD | Min–max. | Median |
|---|---|---|---|---|
| $FEV_1$ | 74.72 | 10.18 | 44–102 | 77 |
| IL-13 (pg/mL) | 2.1268 | 0.82 | 0.8–4.03 | 2.4 |
| IgE (IU/mL) | 553.01 | 371.8 | 39.10–1092.61 | 498.2 |

The study participants were categorized into mild asthmatics (85%), moderate asthmatics (6%), and severe asthmatics (9%) based on the results of the pulmonary function test.

A one-way analysis of variance demonstrated that mild asthmatics exhibited significantly lower levels of IL-13 compared to moderate asthmatics, who, in turn, had lower levels than severe asthmatics. Conversely, there was no discernible variation in IgE levels (Fig. 3).

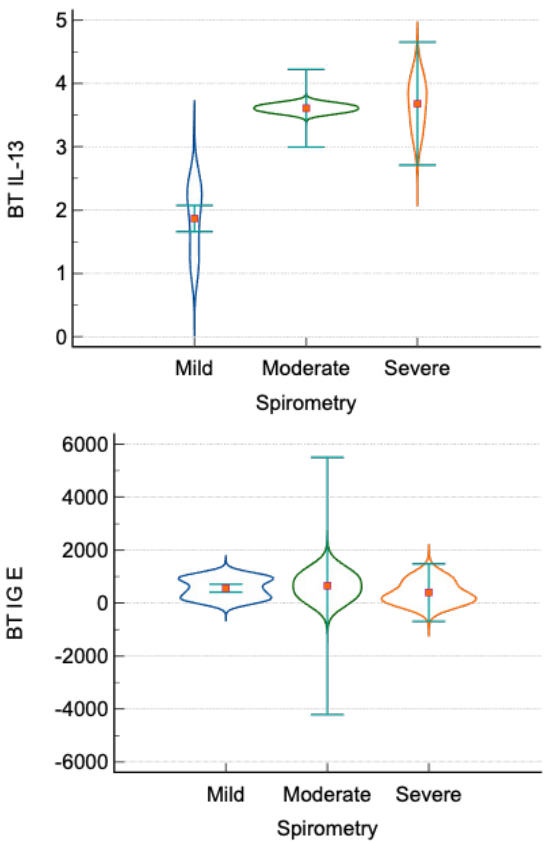


**Fig. 3.** Violin plot of IL-13 and IgE levels among mild, moderate and severe asthmatics

The results of our study indicate that IL-13 may effectively discern between individuals with mild asthma and those with moderate to severe asthma, with a sensitivity and specificity of 100%. The determined threshold

value for distinguishing between the groups was 2.6 pg/mL (p=0.001), as depicted in Figure 4.

However, a comparable degree of statistical significance was not observed concerning IgE.

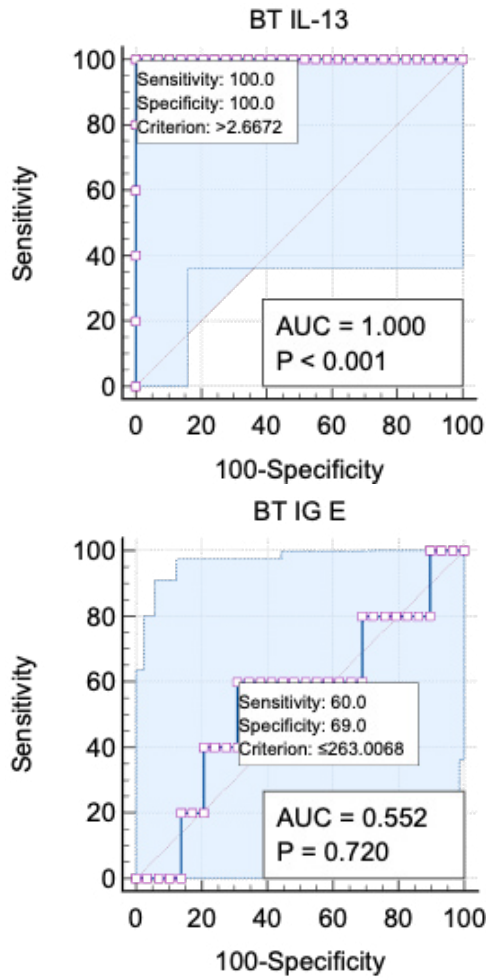


**Fig. 4.** ROC curve for IL-13 and IgE in differentiating mild, moderate and severe asthma

The closer the ROC curve is to the upper left corner of the graph, the higher the efficacy of the test because in the upper left corner, the sensitivity=1 and the false positive rate=0. The graph below illustrates the greater value of IL-13 as a marker in indicating the severity of asthma in comparison to IgE (Fig. 5).

## Discussion

The role of the Th2 cytokine interleukin IL-13 as a fundamental regulator of allergic diathesis has been convincingly demonstrated by a multitude of studies. The emerging paradigm is that, rather than eosinophils and IgE-mediated processes, which are the classic effector routes, IL-13 initiates aspects of the allergic response by acting on smooth muscle and epithelial cells.[15]

In light of these recent advances, this study explores our current comprehension of the function of IL-13 in the etiology of asthma, with a particular emphasis on determining the utility of IL-13 in distinguishing the severity of asthma and its comparison against IgE.

Our study revealed elevated concentrations of serum IL-13 and IgE in children with asthma as compared to the control group, suggesting their involvement in the development of asthma (p<0.001). The threshold values for identifying the existence of asthma were 1.86 pg/mL for IL 13 and 314 IU/mL for IgE.

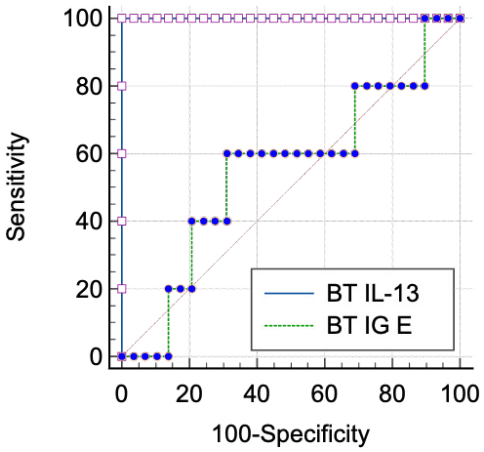


**Fig. 5.** Comparison of ROC curves between IgE and IL-13 in predicting the severity of asthma

Furthermore, it was noted that the IL-13 level could accurately classify asthmatic children as having either moderate or severe asthma, using a cut-off value of ≥2.66 pg/mL, with a statistically significant p=0.001. However, no such results were observed with IgE.

Studies have stated that patients with moderate to severe asthma exhibit elevated IL-13 levels in bronchoalveolar lavage fluid, as well as enhanced gene and protein expression of the same in bronchial tissues.[16] Previous research has shown an association between IL-13 and asthma, as well as IgE and asthma, which aligns with our current study.

Humbert M et al. utilized a semiquantitative reverse transcriptase-polymerase chain reaction approach to quantify the concentrations of IL-13 mRNA in bronchial mucosal specimens obtained from individuals with asthma, as well as control people. The biopsy specimens from the participants with asthma showed substantially higher levels of IL-13 mRNA compared to the control subjects (p≤0.02). Nevertheless, when considering the participants with asthma as a collective, no associations were found between the levels of IL-13 mRNA and measures of disease severity.[17]

Saha et al. conducted a research indicating that the prevalence of detectable IL-13 in sputum was elevated in both the moderate and severe asthma groups relative to the control group(p=0.004). Also, the quantity of IL-13+ cells within the airway smooth muscle bundle was

higher in the severe asthma group compared to the other groups ($p<0.05$).[18]

In 2022, Kursheed et al. aimed to investigate the association between serum IL-13 and IgE in bronchial asthma. The research involved 50 asthmatics aged 18 to 40 in Lahore, Pakistan. Total serum IgE and IL-13 levels were determined using enzyme-linked immunosorbent assay methods. Asthmatics exhibited increased serum IL-13 and IgE levels as opposed to controls (1574±409 pg/mL versus 390±23 ng/ml, respectively). He also concluded a positive correlation between serum IL-13 and IgE levels ($r=0.674$; $p<0.001$).[19]

In 2019, Saleh Jebur et al. conducted a study with 150 individuals with asthma and 50 healthy individuals as controls. The participants' ages ranged from 10 to 65 years. The objective of the study was to determine the levels of serum IL-13 and serum IgE in the blood of patients with allergic asthma. Before initiating inhalational corticosteroids, blood levels of total IgE and IL-13 were assessed in both the patient group and the control group. Consistent with our investigation, a statistically significant increase in serum IL-13 concentrations was noted in asthmatics as compared to controls ($p<0.001$).[20]

In a Spanish study by Davila et al., the researchers intended to assess the link between blood total IgE levels and the extent of disease in adult patients who had persistent allergic asthma. It came to light that, despite elevated serum total IgE levels in adult patients with chronic allergic asthma, a significant correlation between serum total IgE concentrations and the extent of asthma could not be established.[21]

Sandeep T sought to assess and contrast the levels of blood IgE in individuals with mild, moderate, and severe asthma, as well as in those without asthma. Elevated serum IgE levels were seen in individuals with asthma in comparison to those without the condition. Generally, the levels of asthma rose in proportion to the severity of the condition. Nevertheless, due to the substantial diversity observed within each level of asthma, no statistically significant association was found.[22]

In a study by Rathoria et al., it was observed that childhood asthmatics had elevated levels of serum IgE in comparison to individuals without asthma. Although, there was a positive correlation between the severity of asthma and the elevation of serum IgE levels. The degree of heterogeneity within each grade of asthma was substantial, making it unable to identify any statistically significant association.[23] This was a follow-up study by the same lead author.[24]

In 2020, Makieieva et al. sought to assess the clinical and prognostic implications of IL-4 and IL-13 concentrations in children with recurrent wheezing, concluding that the levels of the anti-inflammatory cytokines IL-4 and IL-13 were markedly elevated in these children, with the highest concentrations observed in those developing asthma.[25]

A study conducted by Adel Khattab et al. aimed to examine the association between the IL-13 rs20541 single nucleotide polymorphism and serum IL-13 levels concerning asthma severity in a cohort of asthmatic children. Children with asthma exhibited statistically significant elevations in IL-13 levels compared to controls (median=45 pg/mL versus 4 pg/mL; $p<0.001$). Serum IL-13 had a positive correlation with IgE levels and effectively differentiated between patients with severe asthma and those with mild to moderate asthma at a cutoff value of >83 pg/mL (sensitivity 90%, specificity 90%, positive predictive value 96.4%, and negative predictive value 75%).[26]

It is concluded that IL-13 concentrations are higher in the serum of bronchial asthma patients with allergy history and also help in distinguishing the severity of asthma.

While IgE has been extensively studied, comparative studies evaluating IL-13 alongside IgE in asthma are limited. Many emerging biological therapies (e.g., dupilumab, targeting IL-13 and IL-4 pathways) focus on type 2 inflammation, making IL-13 measurement potentially more relevant for phenotype-targeted therapies. Also, investigating IL-13 as a biomarker for inflammation in non-atopic or low-IgE asthma phenotypes could provide critical insights into the mechanisms underlying non-IgE-driven asthma. This current approach may help address current knowledge gaps, reduce the burden of asthma, and lower treatment costs in the future.

### *Study limitations*

However, conducting a comprehensive multicenter research on a broad scale with a huge sample and adding precision Recall ROC will provide evidence of the effectiveness with optimal cutoff, allowing for its application to the general population.

## Conclusion

Bronchial asthma patients had markedly higher levels of total IgE and IL-13 compared to the healthy control persons included in the study. Furthermore, it has been shown that IL-13 plays a role in discerning the extent of asthma severity. Therefore, serum IL-13 might be a beneficial focus for more research diagnostic applications, as well as for therapy objectives and monitoring.

## Declarations

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### *Author contributions*

Conceptualization, P.R. and P.S.; Methodology, P.R and P.S.; Software, P.S.; Validation, P.S.; Formal Analysis,

P.R., S.S., P.S., J.T. and P.S.; Investigation, P.R., S.S., P.S., J.T. and P.S., Resources, P.R. and S.S.; Data Curation, P.R. and S.S.; Writing – Original Draft Preparation, P.R. and P.S.; Writing – Review & Editing, P.R. and P.S.; Visualization, P.R.; Supervision, S.S.; Project Administration, P.R.; Funding Acquisition, P.R. and P.S.

***Conflicts of interest***

All authors declare that they have no conflicts of interest.

***Data availability***

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

***Ethics approval***

The current longitudinal study was conducted at the SRM Medical College Hospital and Research Center, a tertiary hospital in Tamil Nadu, with the approval of the Institutional Ethics Committee (SRMIEC-ST0922-797).

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**REVIEW PAPER**

# The pleiotropic effects of liraglutide in obesity-linked diseases

Michał Kozdrowicki [1], Konrad Kaleta [1], Aleksandra Kajtel [1], Beata Tekieli [1], Mateusz Kęska [1], Barbara Lorkowska-Zawicka [2], Beata Bujak-Giżycka [2]

[1] Student Scientific Association in the Department of Pharmacology, Jagiellonian University Medical College, Kraków, Poland
[2] Department of Pharmacology, Jagiellonian University Medical College, Kraków, Poland

**ABSTRACT**

**Introduction and aim.** Obesity, defined by a BMI ≥30 kg/m$^2$, is a global epidemic associated with increased mortality rates and an increased prevalence of chronic diseases. Such diseases include type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD), cardiovascular disease (CVD), and polycystic ovary syndrome (PCOS), besides several mental health disorders. Liraglutide, a glucagon-like peptide 1 (GLP-1) analogue, is widely recognized for its efficacy in glycemic control and weight loss and this review aims to explore the pleiotropic effects of liraglutide in obesity-related diseases.

**Material and methods.** Literature search was performed between 2022 and 2024 using the following databases: PubMed (MEDLINE) and Google Scholar. The comprehensive review of the literature focused on the action of liraglutide on NAFLD/NASH, CVD, mental disorders, and PCOS. A qualitative synthesis of the data focusing on efficacy of liraglutide in obesity-related disease outcomes was performed.

**Analysis of the literature.** Liraglutide improves metabolic outcomes by promoting weight loss, reducing appetite, and improving glycemic control. In NAFLD/NASH, liraglutide reduces intrahepatic fat, liver fibrosis, and inflammation that strongly relate to the degree of weight loss. The LEADER trial showed its cardiovascular benefits in terms of reducing all-cause mortality and major cardiovascular events in patients with T2DM, although its chronotropic effects may pose risks in patients with heart failure. In women with PCOS, liraglutide reduces hyperandrogenism, insulin resistance, and body weight, and thus has even more favorable effects compared with metformin. Liraglutide also counteracts antipsychotic-induced weight gain and improves metabolic markers in patients with severe mental disorders.

**Conclusion.** Liraglutide demonstrates significant pleiotropic effects apart from weight reduction, including improved hepatic metabolism, cardiovascular protection, and better outcomes in PCOS and mental health. While semaglutide and tirzepatide may offer enhanced efficacy, liraglutide remains a promising therapeutic option for managing obesity and its related comorbidities.

**Keywords.** cardiovascular disease, liraglutide, mental disorders, obesity, polycystic ovary syndrome

## Introduction

Obesity, is a condition characterized by excessive body weight and fat deposition in tissues and organs, defined by a body mass index (BMI)≥30 kg/m$^2$, with a BMI of 25–29.9 kg/m$^2$ classified as overweight.[1] It is affected by high incidence of chronic comorbidities, including metabolic disorders (type 2 diabetes (T2DM), hyperlipidemia, non-alcoholic fatty liver disease (NAFLD)/ non-alcoholic steatohepatitis (NASH), metabolic syndrome (MetS), cardiovascular diseases (CVD), chronic kidney

**Corresponding author:** Konrad Kaleta, e-mail: konrad.kaleta@student.uj.edu.pl

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disease (CKD), polycystic ovary syndrome (PCOS), obstructive sleep apnea (OSA) as well as it is a major risk factor for cancers.[2] Sometimes neglected, but equally significant, mental health issues such as depression and anxiety are also linked to this condition. Overweight individuals tend to overestimate the width of their own body shape, which leads to greater subjective dissatisfaction with their body appearance.[3] Over the last 30–40 years, obesity rates have surged, particularly in developed countries, exacerbated by the COVID-19 pandemic, especially in youth.[4] Projections suggest that by 2030, 38% of the global population will be overweight and 20% obese making it one of the most frequent diseases of humanity.[5] Obesity and its related comorbidities are associated with healthcare costs approx. 20% higher than compared to normal-weight individuals, amounting to $2 trillion worldwide. Obesity is currently a major public health concern affecting over 600 million adults and 100 million children globally. Diseases related to overweight and obesity result in substantial economic losses and serve as a factor reducing workforce productivity.[2,6]

Obesity is considered as a chronic metabolic disease that arises from a complex interplay of genetic, environmental, socioeconomic, behavioral, and psychological factors (Fig. 1). A key contributor is an imbalance between caloric intake and expenditure, both regulated by the hypothalamus, particularly the arcuate nucleus. Appetite is suppressed by pathways involving proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART), while neuropeptide Y (NPY) and agouti-related peptide (AgRP) stimulate hunger. Thus, NPY/AgRP neurons of arcuate nucleus stimulate feeding and inhibit satiety, while POMC/CART neurons stimulate satiety and inhibit feeding. Both groups of neurons are regulated in part by leptin. Signals from peripheral tissues include long-term energy regulation mediated by leptin and insulin and short-term satiety are regulated by gastrointestinal hormones like secretin, glucagon-like peptide 1 (GLP-1) and cholecystokinin.[7] A deficiency in leptin signaling, either via leptin deficiency or leptin resistance, leads to overfeeding and may account for some genetic and acquired forms of obesity. Equally important factor is ghrelin, secreted during fasting and promoting appetite via NPY/AgRP stimulation.[7,8]

There is large body of evidence that genetic factors play a particular role and interact with environmental factors to produce obesity. Genetic factors contribute to 40-70% of obesity cases within families and twins.[9] However, genetic and environmental factors overlap, as a healthy lifestyle can significantly reduce the effect of genetic factors. More than 300 loci have been associated with obesity, though their individual effects on BMI are small (<5%).[9,10] Additionally, familial lifestyle factors further amplify genetic risks, with a threefold increase in obesity risk in case of one obese parent and a tenfold increase if both parents are obese.[11,12] Monogenic obesity involves mutations in genes responsible for satiety and hunger regulation including POMC, MC4R, and leptin, leading to inadequate satiety signaling.[9,10,13] Polygenic obesity, in contrast, results from the interaction of multiple gene polymorphisms.[14] Epigenetic modifications, influenced by environmental factors, also play a role. Histone modifications, for example, regulate adipogenesis-related genes like PPARγ.[15] DNA methylation patterns of leptin and adiponectin correlate with LDL levels and obesity risk.[16] Russo et al. demonstrated that elevated specific miRNAs in children are strongly linked to increased BMI.[17] Intriguingly, gut microbiota dysbiosis in obesity alters the Bacteroidetes-to-Firmicutes ratio, potentially increasing pathogenic variants and affecting microbial metabolites like short-chain fatty acids (SCFA). SCFAs regulate satiety, lipogenesis, and glucose homeostasis, with obese patients showing higher fecal SCFA concentrations.[18-20]

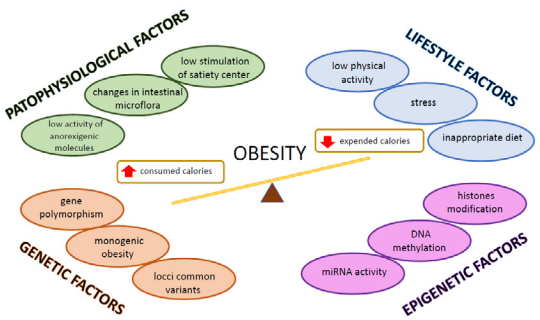


**Fig. 1.** The interplay between diverse factors leading to obesity – the interaction of multiple biological, environmental, and lifestyle influences

Better comprehension of the mechanisms driving obesity is crucial for developing pharmacological treatment strategies. Currently, lifestyle modification, which includes a balanced diet, physical activity, and behavioral therapy, remains the primary intervention, often yielding successful outcomes.[21] The choice of antiobesity pharmacotherapy depends upon medication efficacy and side effects, individual contraindications, comorbidities, and preferences, as well as insurance coverage and "out-of-pocket" costs. Available medications include lorcaserin, phentermine-topiramate and phentermine (as a single agent), orlistat, naltrexone-bupropion, tirzepatide, semaglutide, liraglutide, and setmelanotide, an MCR4 agonist reserved for a subset of POMC and leptin receptor-deficient patients.[22] Notwithstanding, patients with a BMI >30 or BMI >27 with comorbidities who do not achieve sufficient benefits from lifestyle changes or drug therapy may require surgical intervention. For individuals with severe obesity (BMI >40 or BMI >35 with

comorbidities), bariatric surgery is particularly recommended.[23] Recently, significant effectiveness in lowering body weight has been demonstrated by GLP-1 analogs. These are anti-diabetic drugs, which show additional beneficial effects in delaying gastric emptying or suppressing appetite. In 2014 U.S. Food and Drug Administration approved liraglutide to treat obesity, semaglutide in 2021, and in November 2023 tirzepatide which additionally affects the glucose-dependent insulinotropic polypeptide receptor (GIP) (Fig. 2).[24]

## Aim

The aim for conducting a review of the pleiotropic effects of liraglutide lies in the growing popularity and increasing body of research highlighting its multifaceted role in improving health outcomes beyond glycemic control. Further studies demonstrated its potential to influence other organs and systems. The current review addresses liraglutide's pleiotropic effects, the therapeutic implications, and future directions of this research area. Our findings may enhance clinical decision-making and point future research towards the optimization of liraglutide use in a wide spectrum of metabolic and cardiovascular diseases as well as other chronic diseases. The current paper will discuss the therapeutic potential of liraglutide in the treatment of mental disorders, NAFLD/NASH, PCOS, and cardiovascular diseases in obesity.

## Material and methods

Literature search was performed between 2022 and 2024 using the following databases: PubMed (MEDLINE) and Google Scholar. A literature review of articles published between 2010 and 2024 was performed. Key search terms used to identify relevant research included "Liraglutide", "Treatment of obesity", "Diseases caused by obesity", "GLP-1 receptor agonists", "Liraglutide cardiovascular risk", "Liraglutide and NAFLD", "Liraglutide and PCOS", "Liraglutide and psychopathology", "Liraglutide, clinical trial, obesity treatment", and "Liraglutide and Semaglutide in treatment of Obesity." All relevant studies were included embracing clinical trials (phase 2, 3, or 4) evaluating the effects of liraglutide on obesity and obesity-related diseases, randomized control trials, observational studies, meta-analyses and systematic reviews, animal experiments, studies that report outcomes related to obesity management (weight reduction, body mass index) and comorbid conditions such as NAFLD, cardiovascular disease, PCOS, mental disorders, researches that includes data on the mechanism of action, safety profiles, and efficacy of liraglutide. Studies that do not directly assess liraglutide as a primary intervention, studies with incomplete data or lacking rigorous methodological standards were excluded. Data was extracted independently by four reviewers, using a standardized data extraction form. A qualitative synthesis of the data conducted, focusing on efficacy of liraglutide in weight loss and obesity-related disease outcomes, comparison of liraglutide with other obesity treatments (pharmacological agents and lifestyle interventions), safety and tolerability of liraglutide, including common and rare side effects.

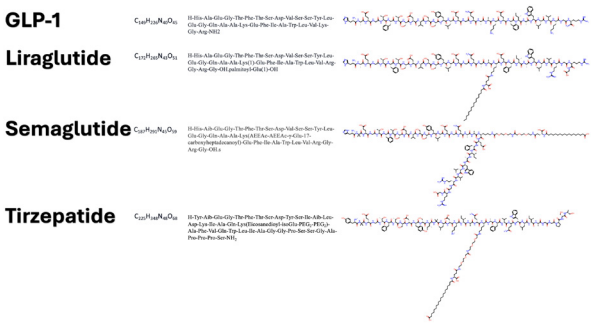


**Fig. 2.** Visual comparison of the amino acid sequences, chemical structures, and molecular formulas of GLP-1, liraglutide, semaglutide, and tirzepatide, data taken from PubChem (https://pubchem.ncbi.nlm.nih.gov, accessed November 2024), the illustrations were created using RDKit (https://www.rdkit.org) software based on SMILE formulas and the cairosvg library

### Analysis of the literature

#### *Liraglutide – mechanism of action*

Throughout the development of liraglutide, modifications such as the addition of fatty acid chains to the original GLP-1 structure were made to optimize its properties, including high receptor potency and favorable pharmacokinetics for once-daily dosing (Fig. 2). Liraglutide shares 97% homology with endogenous human GLP-1 and has a prolonged half-life of 13 hours compared to 1.5-2 minutes of endogenous GLP-1 due to resistance to inhibitors of dipeptidyl peptidase 4 (DPP-4) and neutral endopeptidases degradation, possibly due to reversible binding to albumin or direct steric hindrance. Liraglutide activates GLP-1 receptors (GLP-1R) in various locations, including the brain, where it induces satiety and reduces food intake by activating subcortical areas.[25,26] These receptors are found in the nodose ganglion of abdominal vagal afferent nerve fibers, which terminate in the nucleus tractus solitarius in the brainstem. Signals are then transmitted to the hypothalamus and forebrain regions through ascending second-order neurons. Additionally, GLP-1R are in areas of the CNS such as the parietal cortex, hypothalamus, and medulla, where they modulate desirable food signals in humans.

Activation of GLP-1R in both the PNS and CNS increases feelings of fullness and reduces food intake. This reduction is mediated by stimulation of POMC neurons and inhibition of NPY/AgRPneurons, decreasing hunger. These effects are also linked to the mesolimbic sys-

tem, where food-induced reward signals are reduced, leading to a decrease in food-seeking behavior. Liraglutide is also reported to slow 1-hour gastric emptying.[27] In a study by van Can and colleagues, it was found that 5-hour gastric emptying was equivalent for liraglutide 1.8 mg and 3 mg and liraglutide versus placebo.[27] However, reductions in 1-hour gastric emptying of 23% with liraglutide 3 mg (p=0.007) and 13% with 1.8 mg (p=0.14) versus placebo were observed. Additionally, liraglutide 3 mg improved postprandial glycemia more than liraglutide 1.8 mg, although both doses similarly increased satiety, reduced hunger, and decreased energy intake by approximately 16%.

Liraglutide at doses up to 1.8 mg is approved for the treatment of T2DM under the name Victoza.[28] It is indicated as an adjunct to diet and exercise for glycemic control in patients aged ≥10 years with T2DM and to reduce the risk of major adverse cardiovascular events in adult patients with T2DM and established CVD. The maximum recommended dose for effective glycemic control in both age groups is 1.8 mg. Another formulation, Xultophy, combines liraglutide with insulin degludec and is similarly indicated for glycemic control in adult T2DM patients, with a maximum liraglutide dose of 1.8 mg.[29]

Saxenda, a liraglutide formulation at 3 mg, is indicated for weight loss in adult and pediatric patients. Efficacy for chronic weight management at doses below 3 mg has not been established, although pediatric patients may use a reduced maintenance dose of 2.4 mg if 3 mg is not tolerated.[30] Indications for adult patients include an initial BMI of ≥30 kg/m² (obese) or ≥27 kg/m² (overweight) with at least one weight-related comorbidity (e.g., hypertension, T2DM, or dyslipidemia). For pediatric patients aged ≥12 years, indications include body weight over 60 kg and an initial BMI corresponding to 30 kg/m² for adults by international cut-offs. Studies have indicated the safety of short-term liraglutide use in pediatric patients aged 7–11 years, although the drug is not yet approved for this population.[25] Treatment should begin with 0.6 mg, with weekly dose escalation to 3 mg. If a pediatric patient does not experience a BMI reduction of at least 1% from baseline after 12 weeks on the maintenance dose, treatment should be discontinued as further benefits are unlikely. Similarly, in adult patients, treatment should be discontinued if a 4% reduction in baseline body weight has not occurred after 16 weeks.[30]

### *Side effects*

As liraglutide's indications expanded from treating only T2DM to addressing obesity in non-diabetic patients, it is increasingly important to consider adverse reactions in the growing patient population. The most relevant adverse events were collected and shown in Figure 3. Liraglutide doses up to 3 mg are associated with elevated serum lipase and amylase levels, and an increased absolute risk of acute pancreatitis, gallbladder or biliary disease, and gastrointestinal symptoms. The potential cancer-related side effects of liraglutide remain unclear, as various studies yield different outcomes.[31] However, when considering only high-quality studies, a statistically significant increase in cancer risk is observed. There are reports of elevated risks for thyroid, pancreatic, and early breast cancer, although these findings are inconclusive, with breast cancer risk specifically studied only at doses up to 3 mg.[32] Close monitoring of side effects is essential, and the balance between benefits and risks must be carefully evaluated in the context of the patient's long-term health.

The most common side effects of liraglutide are nausea and vomiting, which were the primary reasons for patient dropout in one of the pivotal clinical trials demonstrating liraglutide's effectiveness in obesity management.[33] Vomiting is relatively frequent and dose-dependent, likely due to delayed gastric emptying induced by the drug.[34-36] Other GLP-1 receptor agonists also slow gastric emptying, as this effect appears to be characteristic of the entire class of these medications.[37] This is likely due to the physiological actions of GLP-1 and other incretin hormones in slowing upper GI tract motility. However, the exact mechanism by which GLP-1 affects gastric emptying and satiation remains unclear, though it is hypothesized that CNS GLP-1 receptors may play a more significant role than peripheral GLP-1 vagal receptors in this process.[38,39]

The current liraglutide dose appears to balance efficacy with common side effects. Nonetheless, increasing the dose beyond 3 mg may not be feasible due to these adverse events, even though higher doses might further improve the treatment of obesity-related comorbidities.

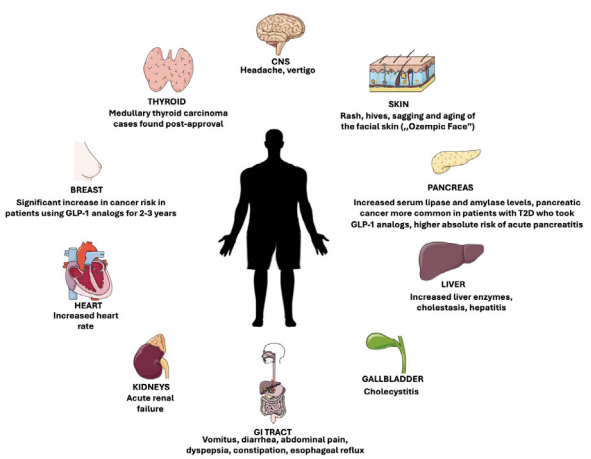


**Fig. 3.** The overview of the organ-specific adverse effects that may be associated with the use of GLP-1 analogues, this figure is created using Servier Medical Art, licensed under Creative Commons Attribution 4.0 Unported License, https://creativecommons.org/licenses/by/4.0/,

(accessed October 2024)

***Non-alcoholic fatty liver disease/non-alcoholic steatohepatitis***

Nonalcoholic fatty liver disease recently more recognized as metabolic dysfunction–associated steatotic liver disease is the most common liver disease referring to liver steatosis in patients with at least one metabolic risk factor (e.g., obesity, diabetes mellitus, dyslipidemia, hypertension). Recent studies estimate that NAFLD affects 32.4% of the global population, a prevalence that has risen sharply in recent years due to the global epidemics of obesity and type 2 diabetes. In obese patients and those with T2DM, the prevalence of NAFLD reaches 70–80%.[40] In the USA and EU, NAFLD is a leading cause of liver transplantation.[41] During NAFLD, excessive lipid accumulation in the liver (hepatic steatosis) causes hepatocyte damage, leading to liver fibrosis, cirrhosis, end-stage chronic liver disease, or hepatocellular carcinoma.[42] This lipid accumulation also triggers oxidative stress, mitochondrial dysfunction, and inflammation. Severe inflammation and necrosis of hepatocytes characterize NASH, a more severe form of NAFLD that can result in serious multisystem complications. Additionally, insulin resistance (IR) often develops during NAFLD, and patients with NAFLD have a 2–3 times greater risk of developing T2DM. This is accompanied by the increased likelihood of serious cardiovascular diseases, hypertension, dyslipidemia or CKD.[43] A vicious cycle forms, where obesity and T2DM promote the onset and severity of NAFLD/NASH, which in turn worsens the course of obesity and T2DM.

Studies have suggested several mechanisms through which GLP-1 analogues, including liraglutide, may improve the course of NAFLD/NASH, the most likely being their ability to induce weight loss. The degree of weight loss correlates most strongly with a decrease in intrahepatic fat (IHF), more so than with changes in total triglycerides, AST/ALT, or HbA1c levels ($p<0.0001$).[44,45] In patients with poorly controlled diabetes and NAFLD, no significant weight loss resulted in no reduction in IHF despite improvements in other liver parameters, whereas the greatest decrease in IHF was observed in patients with more than 5% weight loss.[45,46] Among the drugs tested for NAFLD/NASH, liraglutide and other GLP-1 analogues have shown the greatest efficacy. Liraglutide has been demonstrated as an independent factor for achieving body weight reduction of more than 5%.[47] In a study of T2DM patients with NAFLD, liraglutide treatment for 24 weeks resulted in greater weight loss (-5.60 kg) compared to metformin (-3.58 kg) or gliclazide (-0.1 kg), with a mean weight loss of 6.4% in the liraglutide group.[46]

In a study by Yan et al., patients with NAFLD and poorly controlled T2DM, previously treated unsuccessfully with metformin, experienced greater average weight loss (-3.6 kg) with the addition of liraglutide (1.8 mg daily for 26 weeks) compared to sitagliptin (-1.7 kg) or insulin glargine (-1.2 kg). Liraglutide also led to the greatest reduction in subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) in these patients.[48] Guo et al. also demonstrated that liraglutide was more effective than insulin glargine in reducing body weight (-5.1 kg vs -0.9 kg) and VAT (-47 $cm^2$ vs -16.6 $cm^2$).[49] A significant decrease in IHF (19–32%) was observed in all these studies. Additionally, Frossing et al. showed that administering liraglutide for 26 weeks to overweight women with PCOS and IR, who are at higher risk of developing NAFLD, resulted in significant reductions in body weight (5.6%), IHF (44%), and VAT (18%), reducing the prevalence of NAFLD by two-thirds ($p<0.01$).[50]

There are also weight-independent mechanisms by which liraglutide and other GLP-1 analogues may positively impact hepatic metabolism in NAFLD/NASH. Several animal studies suggest that GLP-1 plays a key role in regulating hepatic insulin sensitivity, lipogenesis/lipolysis gene expression, mitochondrial function, and endoplasmic reticulum (ER) stress in hepatocytes. Ding et al. first demonstrated that administering exendin-4, a GLP-1 receptor stimulator, to obese mice decreased the expression of lipogenesis-related genes and enzymes, such as stearoyl-CoA desaturase-1 and sterol regulatory element-binding protein 1, while increasing the expression of enzymes involved in fatty acid beta-oxidation, including PPARγ and acyl-coenzyme A oxidase 1.[47] This correlated with reductions in hepatic steatosis, serum ALT levels, HOMA-IR scores, and hepatocyte morphological changes compared to controls.[51] Similar results were found in non-obese NASH mice, where GLP-1 receptor stimulation also reduced hepatocyte inflammation.[52]

In high-fat diet mice with induced NASH, a reduction in GLP-1 receptor expression, PPARγ expression, and PPARα activity was observed. Exenatide (a GLP-1 analogue) stimulation of hepatocytes in vitro increased GLP-1 receptor expression and enhanced PPARγ expression and PPARα activity, improving insulin sensitivity and reducing lipid levels in hepatocytes.[53] Liraglutide also improved metabolic parameters in mice by inhibiting ER stress and reducing hepatocyte apoptosis.[54,55] Additionally, GLP-1 analogues have been shown to reduce lipid accumulation in hepatocytes by enhancing autophagy. In HFD mice and in vitro human hepatocytes, exendin-4 and liraglutide stimulated autophagy protein expression by activating 5'AMP-activated protein kinase (AMPK) and beclin II. Electron microscopy revealed an increased presence of autophagosomes in hepatocytes, correlating with decreased fat vacuoles. *In vivo*, liraglutide improved liver weight and serum lipid profiles.[55,56]

AMPK activation following GLP-1 receptor stimulation in mice also reduced fibroblast growth factor 21 (FGF21) expression, a key factor in liver fibrosis. In T2DM patients treated with pioglitazone, adding exen-

din significantly lowered plasma FGF21 levels and intrahepatic fat (IHF), an effect not observed without a GLP-1 analogue.[57] AMPK phosphorylation is critical for insulin signaling, explaining the reduced insulin resistance seen with GLP-1 analogue use. Exendin-4, *in vitro*, increased AMPK phosphorylation, cAMP concentrations, and phosphorylation of key insulin signaling proteins, such as PDK-1, AKT, and PKC-ƒ, in human hepatocytes.[58] These experimental findings offer promising insights into the therapeutic potential of GLP-1 analogues like liraglutide for NAFLD/NASH. However, this mechanism remains less understood in humans. Some studies do not confirm the presence of GLP-1 receptors in the human liver, suggesting indirect mechanisms behind hepatic lipid reduction.[59,60]

Despite these discrepancies, clinical trials support liraglutide's efficacy in NAFLD/NASH. In one study, administering liraglutide 1.8 mg to obese NASH patients for 48 weeks resulted in a significantly higher rate of NASH resolution (39%) compared to the control group (2%), as shown by liver biopsy histopathology. Similarly, fibrosis progression was slower in the liraglutide group (9% versus 36%). While no significant changes in serum aminotransferases were observed, modeling indicated significant differences compared to placebo, along with reductions in markers of hepatocyte damage and fibrosis. Patients in the liraglutide group also experienced greater weight loss, HbA1c improvement, and enhanced physical scores in the SF36 questionnaire.[61] Eguchi et al. reported similar results in a study of 26 poorly controlled diabetic patients with elevated ALT levels. Liraglutide therapy (0.9 mg daily for 24 weeks) led to reductions in body weight, BMI, visceral fat area, ALT, AST, GGTP, and HbA1c ($p<0.01$). Liver biopsies in 10 patients after 96 weeks of treatment showed improved NASH activity scores, although the study's small sample size was a limitation.[62] A larger study involving 128 patients with T2DM and NAFLD found that liraglutide significantly reduced AST, ALT, and HOMA-IR levels, unlike insulin glargine and placebo.[49] In contrast, Matikainen et al. found that while liraglutide 1.8 mg for 16 weeks reduced IHF (31% versus 18%) in patients with well-controlled T2DM, it did not affect hepatic de novo lipogenesis or fat oxidation. However, liraglutide did improve postprandial triglyceride levels, VLDL, chylomicrons, glycemia, and apolipoprotein C-III (apoCIII) concentrations, key regulators of postprandial lipid metabolism.[63] Some studies, however, do not confirm liraglutide's benefits on liver parameters. Tang et al. found that in T2DM patients, liraglutide for 12 weeks did not significantly reduce IHF, liver volume, or total liver fat index despite significant reductions in body weight and BMI compared to placebo and insulin.[59] Table 1 summarizes the most important studies on the role of liraglutide in the course of NAFLD/NASH.

**Table 1.** The effect of GLP-1 treatment in patients and animals on hepatic lipid's metabolism*

| Group | Intervention | Effects | Ref. |
|---|---|---|---|
| Patients with T2DM and NAFLD | Liraglutide treatment (1.8 mg per 24–26 weeks) | ↓ Body mass ↓ IHF ↓ SAT ↓VAT | 46,48, 49 |
| Obese patients with NASH | Liraglutide treatment (0.9-1.8 mg per 24–48 weeks) | ↓ Fibrosis progression ↓ NASH activity score | 61,62 |
| HFD mice with induced NASH | Liraglutide, exenatide administration | ↑ Insulin sensitivity ↓ Lipid levels in hepatocytes | 53,54, 55,56 |

* T2DM – type 2 diabetes mellitus, NAFLD – non-alcoholic fatty liver disease, NASH – non-alcoholic steatohepatitis, HFD – high-fat diet, IHF – intrahepatic fat, SAT – subcutaneous adipose tissue, VAT –visceral adipose tissue

There are notable differences among the aforementioned studies. One key distinction is the duration of liraglutide therapy. In studies where liraglutide was ineffective in reducing IHF and other liver parameters, treatment lasted only 12 weeks. Conversely, studies reporting significant improvements had much longer treatment durations, averaging 24–26 weeks. Additionally, greater reductions in IHF were observed in patients with obesity, poorly controlled diabetes, and concomitant NAFLD, suggesting that liraglutide's effectiveness may be stage-dependent and more pronounced in patients with advanced NAFLD/NASH or exacerbated comorbidities. However, the primary limitation of these studies is the small sample sizes, which constrain the ability to draw definitive conclusions. Despite this, the results offer a promising outlook for the use of liraglutide and other GLP-1 analogues in the treatment of NAFLD/NASH.[64] Figure 4 summarizes the mechanisms of action of liraglutide in NAFLD/NASH.

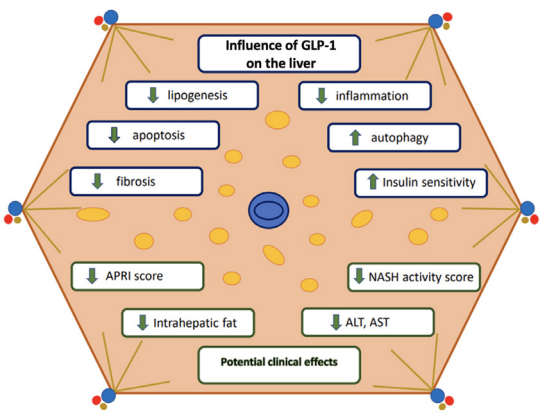


**Fig. 4.** Mechanisms and potential clinical effects of liraglutide in hepatocytes during NAFLD/NASH through various biological pathways

### *Cardiovascular disorders*

Obesity contributes to CVD and cardiovascular mortality independently of other risk factors.[65] Although

liraglutide is primarily used to lower blood glucose levels, it has been found to offer potential cardiovascular benefits.[66] T2DM is strongly associated with increased cardiovascular risk, and reducing blood glucose levels is generally thought to be beneficial. However, liraglutide also appears to have direct effects on cardiovascular health.[66] One significant way liraglutide may reduce cardiovascular risk is through weight loss, which helps mitigate obesity-related conditions such as dyslipidemia, hypertension, and T2DM, all of which are major CVD risk factors. Liraglutide has been shown to lower blood pressure, improve endothelial function, and reduce inflammation, a key driver of CVD.[67-69] Endothelial dysfunction is a direct contributor to atherogenesis and its subsequent consequences.[70]

The most well-known evidence of liraglutide's cardiovascular effects comes from the LEADER trial (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcomes Results; NCT01179048), initiated in 2010.[71] The LEADER trial demonstrated significant improvement in patient survival compared to placebo (Hazard ratio=0.78), with a lower rate of death from any cause in the liraglutide group (Hazard ratio=0.85). Although non-fatal stroke and myocardial infarction rates were lower in the liraglutide group, the results were not statistically significant.[71] Other cardiovascular effects observed in the trial included reductions in both systolic and diastolic blood pressure, an increase in heart rate by 3 beats per minute (CI, 2.5 to 3.4), and a lower incidence of composite renal and retinal microvascular events in the liraglutide group.[71]

The increase in heart rate may be explained by the presence of GLP-1R expression in all four heart chambers and the sinoatrial node.[72] Liraglutide may directly affect the cardiac conduction system, but studies in mice suggest that it exerts complex chronotropic effects, likely through inhibition of vagus nerve impulses, increasing sympathetic influence on the heart's autonomic system.[73] This is critical in liraglutide treatment because it could elevate heart workload, leading to potential complications, including death. A post-hoc analysis of the FIGHT trial revealed that liraglutide increased the risk of arrhythmias and other cardiovascular adverse events in patients with heart failure and reduced ejection fraction.[74]

Randomized controlled trials, such as the LIVE study, suggest that liraglutide significantly increases heart rate in patients with sinus rhythm, with a reported rise of 8 ±9 beats per minute compared to placebo. This effect was not observed in patients without sinus rhythm or after beta-blocker treatment.[25] While this study indicated that the heart rate increase did not elevate the risk of cardiovascular events compared to placebo, other research linked to the LIVE trial highlighted a higher risk of these events in the chronic heart failure population.[25-75] Understanding the chronotropic effects of liraglutide is crucial due to the increased risk of arrhythmias. However, it was shown that liraglutide does not cause clinically relevant increases in QTc interval, alleviating concerns about QTc prolongation.[76] Additional clinical trials have confirmed significant reductions in major adverse cardiovascular events and even all-cause mortality in patients with diabetes treated with liraglutide.[77,78]

**Table 2.** Liraglutide's effects on cardiovascular outcomes in various patient groups

| Group | Intervention | Effects | Ref. |
|---|---|---|---|
| Patients with T2DM | Liraglutide treatment (1.2-1.8 mg/day for 24-36 months) | ↓ Major cardiovascular events<br>↓ All-cause mortality | 66, 71, 77 |
| Patients with T2DM and high cardiovascular risk | Liraglutide treatment (1.8 mg/day for 36 months) | ↓ Cardiovascular death,<br>↓ Hospitalizations for heart failure | 71, 74 |
| Obese patients with CVD risk | Liraglutide treatment (1.8 mg/day for 52 weeks) | ↓ Blood pressure<br>↓ Inflammation markers<br>↑ Endothelial function | 66, 68, 69 |
| Patients with heart failure and reduced ejection fraction | Liraglutide treatment (1.8 mg/day for 48 weeks) | ↑ Heart rate<br>↑ Risk of arrhytmias<br>No significant change in QTc interval | 73, 74, 76 |

* T2DM – type 2 diabetes mellitus, CVD – cardiovascular disease

***Polycystic ovary syndrome***

PCOS is the most common endocrine disorder in women of reproductive age, affecting up to 18% of women based on the Rotterdam criteria, though prevalence estimates range from 2.2% to 26%.[79] Women with PCOS have up to a threefold higher prevalence of obesity compared to women without the syndrome.[80] Up to 70% of women diagnosed with PCOS also present with dyslipidemia, hyperinsulinemia, and IR, all of which increase the risk of developing T2DM. Additionally, these women face an elevated risk of endometrial carcinoma.[81] Obesity exacerbates PCOS symptoms, creating a vicious cycle.[82]

The pathogenesis of PCOS is complex, with no single factor fully accounting for the syndrome. Ovarian theca cells synthesize androgens in response to luteinizing hormone (LH) stimulation. Studies show that ovarian theca cells in women with PCOS are more efficient at converting androgenic precursors to testosterone compared to healthy theca cells. Moreover, women with PCOS have lower levels of progestins, which normally slow the pulse frequency of gonadotropin-releasing hormone (GnRH). The resulting acceleration in GnRH pulse frequency leads to overproduction of androgens.[83] Hyperinsulinemia, a consequence of insulin resistance, is a key driver in the pathogenesis of PCOS and hyperandrogenism. Insulin works synergistically with LH to increase androgen synthesis and inhibits the production of sex-hormone binding globulin, increasing free testos-

terone levels.[83]

Insulin resistance not only exacerbates PCOS but also raises the risk of glucose intolerance, diabetes, lipid abnormalities, and macrovascular disease.[83] Women with PCOS face a CVD risk like those with metabolic syndrome, as both syndromes share insulin resistance as a central pathogenic factor.[83]

Obesity is present in up to 70% of women with PCOS, with visceral adiposity often indicated by increased waist circumference and waist-to-hip ratio.[83] Normalizing insulin levels in these women is associated with the resolution of many metabolic abnormalities.[83] Therefore, in addition to weight loss, reducing hyperandrogenism and alleviating all PCOS symptoms are essential for improving IR.[84] Lifestyle modifications, such as physical activity and a low-carb diet, are the first-line treatments but are often reported to have limited efficacy.[85] As a result, pharmacotherapy is frequently employed to enhance weight loss and better manage clinical symptoms.[86] For weight management, metformin combined with lifestyle changes is recommended for treating PCOS. Studies show that it improves menstrual cycles, glucose levels, and adiposity in women with PCOS. Metformin also alleviates IR and improves the lipid profile, although these effects are generally mild to moderate. However, there is increasing evidence that GLP-1 receptor agonists are more effective than metformin in treating obesity in women with PCOS.[87]

GLP-1 receptor agonists stimulate endogenous insulin secretion in response to meal ingestion and inhibit glucagon secretion. Additionally, they suppress appetite, leading to changes in eating patterns, a benefit not observed with other T2DM treatments.[88] A daily dose of 3 mg liraglutide combined with lifestyle modifications has been shown to reduce body weight by 5–10%. Studies also show that liraglutide is effective for weight reduction in women with PCOS, both as monotherapy and in combination with metformin.[89]

Furthermore, higher doses of liraglutide (3 mg) have shown better outcomes compared to lower doses combined with metformin.[90]

A 32-week trial demonstrated that participants taking 3 mg liraglutide lost at least 5% of their body weight. Additionally, the free androgen index significantly decreased in the liraglutide group, while it slightly increased in the placebo group.[83] In a study by Niafar et al., BMI decreased significantly by 1.65 kg/m² after 3 months of liraglutide treatment, although waist circumference and fasting insulin levels did not change significantly. However, serum testosterone decreased, suggesting that GLP-1 receptor agonists may affect overall obesity rather than abdominal obesity.[91] In another study by Jensterle Sever et al., using a 1.8 mg liraglutide dose, 19 obese women with PCOS were recruited, and 13 completed the study. After six months, weight was reduced by 3.0±4.2 kg.[88] A lower dose of liraglutide (1.2 mg) over 12 weeks also showed a reduction in weight (3.8±0.1 kg) and significant reductions in waist circumference and visceral adipose tissue mass.[91-93]

Some studies suggest that the weight loss response to GLP-1 agonists may vary among obese patients, with those without diabetes and with a higher BMI experiencing greater weight loss than patients with diabetes and lower BMI. Genetic variability in GLP-1 receptor function, such as single nucleotide polymorphisms, may influence the efficacy of GLP-1 receptor agonists.[94] In a study by Jensterle et al., 57 women with PCOS and obesity were treated with 1.2 mg liraglutide for 12 weeks. On average, participants lost 3.96±3.24 kg, BMI decreased by 1.44±1.22 kg/m², waist circumference reduced by 3.31±4.13 cm, and VAT decreased by 7.10±18.76 cm².[94] Notably, 35% of these women showed a stronger response, losing around 5% of their weight, while others lost less (Table 3).

**Table 3.** Comparison of results of different studies concerning liraglutide in PCOS and obesity treatment

| Study | PreT weight (kg) | PostT weight (kg) | PreT BMI (kg/m²) | PostT BMI (kg/m²) | PretT WC (cm) | PostT WC (cm) | PreT WHR | PostT WHR | Dose (mg) |
|---|---|---|---|---|---|---|---|---|---|
| Elkind-Hirsch et al.[84] | 111 ±2.8 | 104.7 ±2.9 | 41.6 ±1.1 | 39.1 ±1.1 | 111 ±2.2 | 101 ±2 | 0.85 ±0.01 | 0.81 ±0.01 | 3 |
| Kahal et. al.[95] | 102.1 | 99.1 | 37.9 | 36.9 | 112 | 110.9 | – | – | 1.8 |
| Jensterle Sever et al.[93] | 108 ±15.1 | 105 ±13.8 | 39.3 ±4.2 | 37.9 ±4.0 | 124.9 ±9.9 | 121.7 ±9.6 | – | – | 1.2 |
| Jensterle et al.[94] | 102.1 | 96 | 38.7 | 35.8 | 118 | 111 | – | – | 1.2 |

* PreT – preatreatment, PostT – posttreatment, WC – waist circumference, WHR – waist hip ratio

***Treatment of obesity in patients with mental disorders***

Compared to the general population, patients with mental disorders are two to three times more likely to be overweight or obese, which is associated with increased morbidity and higher mortality due to CVD.[96] Antipsychotic medications like clozapine and olanzapine contribute to weight gain, elevated serum glucose, cholesterol, and triglycerides, primarily through mechanisms involving increased appetite and delayed satiety signaling. For individuals with severe mental illnesses, such as schizophrenia, antipsychotic treatment is often lifelong, as discontinuation increases the risk of psychotic relapse.[97] However, implementing lifestyle interventions is challenging in this population, and even short-term interventions tend to have a minimal impact on reducing BMI. Long-term efficacy of lifestyle changes is also limited, highlighting the need for additional pharmacological support. Currently, orlistat is the only licensed drug for managing obesity in these patients, but its long-term use is limited due to high discontinuation rates and limited clinical value.[96,97]

Using the GLP-1 receptor agonists was, however,

limited in this group of patients, due to concerns highlighted by Icelandic medicines agency following the reports of suicidal thoughts and self-injury.[98] The review of available data made by European Medicines Agency's (EMA) safety committee, the Pharmacovigilance Risk Assessment Committee published on 12th of April 2024, has concluded that the gathered evidence does not support a casual association between the GLP-1 receptor agonists, including liraglutide, and suicidal and self-injurious actions or thoughts.[99] This review incorporates the recent study made by Wang et al. published in Nature Medicine on 5th of January 2024 on "Association of semaglutide with risk of suicidal ideation in a real-world cohort" which found the risk of incident and recurrent suicidal ideation to be lower in comparison to the group of non-GLP1R agonist anti-obesity medications. Those results were consistent across sex, age and ethnicity stratification and replicated in both the group of overweight or obese patients and ones with T2DM.[100]

Thus, the GLP-1 receptor agonists still offer a promising alternative to achieve clinically significant weight loss in this population, as will be discussed in the following paragraphs.

In a qualitative sub-study by Barnard-Kelly et al., interviews with patients who had undergone liraglutide treatment at a 3 mg dose reported improved quality of life and minimal side effects.[96] The randomized clinical trial by Larsen et al. demonstrated significant weight loss with liraglutide (1.8 mg) compared to placebo after 16 weeks, with a mean weight loss difference of -5.3 kg (95% CI, -7.0 to -3.7 kg).[97] This trial included 103 overweight or obese patients with prediabetes and schizophrenia spectrum disorders treated with clozapine or olanzapine. Compared to placebo, liraglutide significantly reduced waist circumference (-4.1 cm; 95% CI, -6.0 to -2.3 cm), BMI (-1.8; 95% CI, -2.4 to -1.3), systolic blood pressure (-4.9 mmHg; 95% CI, -9.5 to -0.3 mmHg), total cholesterol (-19.3 mg/dL; 95% CI, -30.9 to -7.7 mg/dL), and LDL cholesterol (-15.4 mg/dL; 95% CI, -23.2 to -7.7 mg/dL). Liraglutide also reduced visceral fat and total body fat, as evaluated by DXA scans.[97] In a study conducted by Whicher et al., which was a pilot-randomized, double-blind, placebo-controlled trial, 47 participants were randomized to intervention with liraglutide 3 mg and placebo.[101] Patients were overweight or obese with at least one weight-related consequence such as dysglycaemia (prediabetes or T2DM), hypertension, dyslipidemia, or OSA. Eligible participants were aged from 18 to 75 years and had diagnoses of schizophrenia, schizoaffective disorder, or first-episode psychosis and had been prescribed antipsychotic medication for at least 1 month. 79% of randomized patients completed the trial. Intention-to-treat analysis was performed on 15 intervention participants and 19 control participants. Participants in the liraglutide group lost a mean of 5.7±7.9 kg (4.5%; 95% CI -8.3% to -0.8%) after six months compared with no significant (0.3±5.7 kg [0.0%; 95% CI -2.5% to 3.1%]) weight change in the placebo group (treatment difference -6.0 kg, p=0.015). BMI, waist circumference, and HbA1c were reduced in the intervention group. Furthermore, 53% of those who completed the trial on the trial medication in the liraglutide treatment group lost 5% or more of their body weight in comparison to 10% of the placebo participants (p=007).[101] In the systematic review of licensed weight-loss medications in treating antipsychotic-induced weight gain and obesity in schizophrenia and psychosis carried out by Lee et al. authors reviewed three RCTs (two liraglutide, one naltrexone-bupropion), one unpublished open-label trial (naltrexone-bupropion), and seven observational studies (five liraglutide, one semaglutide, one multiple WLMs).[102] In regard to liraglutide, a meta-analysis was conducted for RCTs previously mentioned, carried out by Larsen et al., and by Whicher et al., giving in a total of 131 participants. Findings were statistically significant ($p<0.05$) for improvement in weight, BMI, waist circumference, glycated in (HbA1c), total cholesterol, and LDL favoring the liraglutide intervention over placebo. However, the difference in systolic blood pressure was not found to be statistically significant. These findings were supported by the observational studies reviewed by the authors, which found liraglutide intervention beneficial and significant.[102]

There is currently limited data available on this subject, highlighting the need for additional studies. Table 4 summarizes the key findings of the studies discussed above while indicating the type of the study. Authors believe that, priority should be given to conducting more randomized clinical trials, as only two on liraglutide treatment have been published thus far. Furthermore, despite the current stance of the EMA, reports of suicidal thoughts or self-injury suggest the necessity for further research into the potential risks of these outcomes in patients treated particularly with liraglutide.

**Table 4.** Summary of key findings from studies on liraglutide's effects in patients with psychotic disorders, overweight or obese

| Group | Intervention | Key outcomes | Ref. |
|---|---|---|---|
| Overweight/obese patients with schizophrenia, schizoaffective disorders or first episode psychosis | Liraglutide 3 mg vs placebo for 6 months | Improved quality of life, minimal side effects, weight loss reductions in BMI, waist circumference, HbA1c | 96, 101 |
| Overweight/obese patients with prediabetes and schizophrenia spectrum disorders on clozapine or olanzapine | Liraglutide 1.8 mg vs placebo for 16 weeks | Significant weight loss, reduced waist circumference and BMI, systolic blood pressure, total cholesterol, LDL, visceral fat, and total body fat | 97 |
| Review of participants with a diagnosis of a psychotic disorder from RCTs, and observational studies | Liraglutide, semaglutide, naltrexone-bupropion | Significant improvements in weight, BMI, waist circumference, HbA1c, total cholesterol, LDL, no significant effect on systolic blood pressure | 102 |

***Future directions***

Obesity-related diseases are currently one of the main denominations of medicine. The action of liraglutide and other GLP-1 analogues described above have a positive effect on the course of diseases. In NAFLD/NASH, liraglutide has a protective effect on hepatocytes, reduces inflammation, fibrosis, apoptosis and lipid deposition. In patients with NAFLD and T2DM used for at least 24 weeks, it led to a significant reduction in IHF, liver parameters AST and ALT. It resulted in a significant weight loss (more than 5%) which is the most important indicator of a slower progression of NAFLD. Liraglutide has a significant impact on the cardiovascular system. The use of liraglutide in obese patients was associated with limitation in the cardiovascular mortality rate in clinical trials, though it could be dangerous in patients with developed chronic heart failure most presumably due to increase heart rate. Treatment with liraglutide can be beneficial for patients with severe mental disorders, as it shows effectiveness in reducing the mass gain on the psychiatric drug course, and improvement in the level of factors connected to CVD, without the necessity of using other interventions. There has been a growing interest in GLP-1 agonists as a potential treatment for women suffering from PCOS and obesity. As stated above, studies have shown that use of liraglutide helps with weight loss, as well as it improves hyperandrogenism, insulin resistance and hyperinsulinemia.

An important aspect is a brief comparison of the action and efficacy of liraglutide with semaglutide (GLP-1 analogue) and tirzepatide (GLP-1 and GIP agonist). They are registered for once-weekly subcutaneous administration, which is much more convenient than daily subcutaneous injections of liraglutide. In addition, recent meta-analyses indicate greater efficacy of semaglutide and tirzepatide in reducing body weight. The mean weight loss with tirzepatide 15 mg/week was on average 5.1% greater than with semaglutide 2.4 mg/week and 13% greater than with liraglutide 3 mg/day.[103] The latest randomized control trials from the last 2–3 years confirm that both tirzepatide and semaglutide effectively control glycemia, show beneficial effects on the cardiovascular system, reduced arterial hypertension, lower the level of total cholesterol, reduce the degree of fatty liver disease and fibrosis in the course of NAFLD/NASH.[104,105,106] Semaglutide and tirzepatide have a similar safety profile, the main side effects include gastrointestinal symptoms such as nausea and diarrhea, similar to liraglutide. Further studies are needed to determine which drug is the best for overweight patients with specific obesity-related diseases.

## Conclusion

The findings of our study imply the possibility of developing the new indications for GLP-1 analog intervention, particularly for states associated with obesity. That could offer improvement in quality and length of the patients' life.

The obtain results may be applied to create combination therapies for treating certain conditions.

For instance, in the treatment of PCOS, standard pharmacotherapy involves the use of hormonal contraception, metformin, antiandrogenic agents, and infertility treatment. The use of GLP-1 analogs could enhance the principal effects of such drugs. Other applications of liraglutide embrace mental health disorders, such as depression, in which obesity might be both an etiological factor and a consequence. GLP-1 analogs are also likely to reduce the noxious metabolic effects of antipsychotic treatment, though the interaction of liraglutide with such treatments is not yet fully understood.

Future studies, especially about the efficacy of liraglutide in these conditions, should be directed toward meta-analyses or network meta-analyses.

Intriguingly, use of GLP-1 analogs with other antidiabetic drugs, such as SGLT2 inhibitors or DPP-4 inhibitors, represents a promising direction in the pharmacotherapy of obesity. Dual or triple antiobese therapy utilizing these agents could enhance the synergistic effects of treatment ensuring significant results.

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***Author contributions***

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***Conflicts of interest***

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

***Data availability***

Not applicable.

***Ethics approval***

The study does not involve any human or animal subjects.

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**REVIEW PAPER**

# The role of ABCA12 in neurodegenerative diseases – a review of molecular mechanisms and potential therapeutic implications

Daksh Kumar [1], Falguni Goel [1], Sachchida Nand Rai [2]

[1] Department of Pharmaceutical Technology, Meerut Institute of Engineering and Technology (MIET), Meerut, India
[2] Center of Experimental Medicine and Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

**ABSTRACT**

**Introduction and aim.** ABCA12 a member of the ATP-binding cassette transporter superfamily, is known to be involved in lipid transport and in the formation of the skin barrier. However, recent evidence also suggests its implication in the pathophysiology of neurodegenerative diseases. This review focuses on the molecular mechanisms that could link ABCA12 to neurodegenerative processes and its potential as a therapeutic target.

**Material and methods.** A literature review search was conducted between 200 and 2024 via the databases, which included PubMed, Scopus, and Web of Science. There, pertinent studies with relevance to ABCA12 involvement in neurodegenerative diseases were searched. This study reviewed pertinent articles on the expression patterns of ABCA12 and its molecular interactions, as well as its contribution to cellular processes, such as lipid homeostasis, inflammation, and neuronal integrity. The analysis further included studies on ABCA12 mutations and their associations with neurodegenerative pathologies such as Alzheimer's disease, Parkinson's disease, and Huntington's disease.

**Analysis of literature.** The results from the analysis showed that ABCA12 dysfunction led to disturbances in lipid metabolism, accompanied by increased oxidative stress, neuroinflammation, and compromised integrity of the neuronal membrane. The results imply that mutations or dysregulation of ABCA12 exaggerates amyloid-beta aggregation in Alzheimer's disease and dopaminergic neuron loss in Parkinson's disease. Finally, pathways of ABCA12 functionally interact with other core neurodegenerative mechanisms, which include autophagy dysregulation and mitochondrial dysfunction. Preliminary preclinical data indicate that altering ABCA12 expression or function diminishes neuroinflammation and restores cellular homeostasis.

**Conclusion.** ABCA12 plays an important role in maintaining neuronal health and its dysfunction contributes to neurodegenerative processes. Targeting pathways related to ABCA12 seems promising to mitigate disease progression in neurodegenerative diseases. More research is still required to elucidate its precise molecular mechanisms and identify specific interventions.

**Keywords.** ABCA12, central nervous system cholesterol homeostasis, lipid metabolism, neurodegenerative diseases

## The list of abbreviations:

**ABCA12** – ATP binding cassette transporter A12, **CNS** – central nervous system, **AD** – Alzheimer's disease, **PD** – Parkinson's disease, **HD** – Huntington's disease, **CRISPR** – clustered regularly interspaced short palindromic repeats, **NFTs** – neurofibrillary tangles, **Aβ** – amyloid beta, **αSyn** – alpha synuclein

**Corresponding author:** Falguni Goel, e-mail: falguni.goel@miet.ac.in or falgunigoel2908@gmail.com

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## Introduction

ABCA12 has been considered a critical member of the family of transporters of the ATP-binding cassette (ABC), for which members have primarily been known for lipid translocation across cellular membranes, especially in skin keratinocytes.[1] Its role has been quite well characterized with respect to the function of the skin barrier, in which the major role of ABCA12 is the transport of glucosylceramides, prime constituents of the lipid bilayer as lamellar granules in the process of keratinization. Main defects in this process, attributed to mutations in the ABCA12 gene, are the major cause for a severe skin disorder known as Harlequin ichthyosis, wherein there is defective lipid transport leading to defective barrier function. This is characterized by thickened skin, severe desquamation, and a compromised epidermal barrier that often leads to life-threatening dehydration and infections. Although such a gene has been very important in maintaining health of the skin, it appears current evidence indicates that ABCA12 functions outside of skin homeostasis and lipid metabolism is central to most other tissues, particularly the, central nervous system (CNS).[2] Recent studies have shown the expression of ABCA12 in the CNS suggesting involvement in lipid metabolism within the brain.[3] Lipid homeostasis is crucial to neuronal integrity, synaptic function, and membrane fluidity, and its disruption is increasingly implicated in neurodegenerative disease pathology.[4] For example, aberrant lipid metabolism has been linked to protein aggregation, oxidative stress, and mitochondrial dysfunction in diseases like Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD).[5] ABCA12 has been known for ages to be associated with lipid trafficking. As such, dysfunction in it is thought to lead to increased imbalance in lipids that may cause neurodegeneration.[3]

Dysregulation of lipids can be neurotoxic and could influence the properties of neuron membranes, impairing membrane fluidity and composition and thus affecting synaptic plasticity and neurotransmission.[6] Moreover, lipids such as sphingolipids and ceramides play a significant role in cell signaling pathways, inflammation, and programmed cell death. Any form of dysregulation in such lipid species may worsen neurodegenerative processes.[7] For instance, AD-amyloid-β plaques and tau protein tangles, which are similar in this case, are hallmarks of the disease; both of these are modulated by lipid environments.[8] PD includes α-synuclein aggregation, which is sensitive to lipid interactions. HD also includes disturbances in lipid metabolism especially cholesterol and phospholipids that have been implicated in neuronal dysfunction and death.

These links of lipid dysregulation to neurodegeneration make ABCA12, in particular through its function in lipid trafficking, a highly promising candidate to pursue studies on neurodegenerative diseases. Dysfunction in ABCA12 could potentially lead to lipid imbalance in neurons, providing a new mechanism by which these diseases might develop and progress. Being particularly relevant because glucosylceramides are crucial to cell membrane structural integrity, its further potential role in the transport of glucosylceramide makes ABCA12 particularly relevant.[9] Disruptions in this transport pathway potentially affect the stability of neuronal membranes leading to a chain reaction and neurodegeneration.[10]

Besides mechanistic roles, ABCA12 is a promising potential therapeutic target for neurodegenerative diseases. Modulation of ABCA12 activity or correction of its dysfunction could restore lipid homeostasis, thus providing an avenue for attenuating the pathological processes that might drive neurodegeneration.[11] Targeted therapies directed at enhancing the function of ABCA12 or compensating for its loss might have much broader implications than aiding to alleviate the dermatological condition called Harlequin ichthyosis because they might influence all neurological disorders.[12] Further, ABCA12, in terms of being a therapeutic target, will lead to treatments that are aimed at gaining back balance in lipid level-a feature not yet implicated in neurodegenerative disease research.

ABCA12 has been studied for quite a long time due to its function regarding lipid transport in skin. However, because of its involvement with neurodegeneration, this might open very new fields of doing further research. The increasing strength of the evidence for expression within the CNS and critical function in lipid trafficking indicate that ABCA12 may play a very significant role in the causation of diseases like AD, PD, and HD.[13] Knowledge of the molecular mechanisms by which ABCA12 contributes to lipid dysregulation in the brain could open the gates to innovative therapeutic strategies for these devastating conditions.

## Aim

This review focuses on the molecular mechanisms that could link ABCA12 to neurodegenerative processes and on its potential as a therapeutic target.

## Material and methods

A thorough literature search was conducted via the databases, which included PubMed, Scopus, and Web of Science. There, pertinent studies with relevance to ABCA12 involvement in neurodegenerative diseases were searched. The articles highlighted were between 2000 and 2024. This study reviewed pertinent articles on the expression patterns of ABCA12 and its molecular interactions, as well as its contribution to cellular processes, such as lipid homeostasis, inflammation, and neuronal integrity. The analysis further included studies on ABCA12 mutations and their associations with neu-

rodegenerative pathologies such as Alzheimer's disease, Parkinson's disease, and Huntington's disease.

## Analysis of the literature

### *Molecular structure and protein function*

The ABCA12 gene is one of the largest transmembrane proteins encoded at chromosome 2q34 and belongs to the ABC transporter family.[14] Like the entire family of the ABCs, ABCA12 is composed of two nucleotide-binding domains (NBDs) and two transmembrane domains. These NBDs use the energy from the hydrolysis of ATP to transport lipids out of the plasma membrane. This opens up the possibility that ABCA12 drives glucosylceramides and other lipids into the lamellar bodies of skin cells, a necessary aspect of forming the epidermal barrier.[15]

### *Transport of lipids and cholesterol metabolism role of ABCA12*

The central role of ABCA12 in lipid metabolism is in the transport of ceramides and glucosylceramides, both key components of the lipid bilayer that play a prominent role in maintaining membrane integrity within the neurons in the CNS while modulating synaptic transmission.[16] Another possible function of ABCA12 is in cholesterol homeostasis, since cholesterol is itself integral to the lipid bilayer and lipid rafts – the microdomains implicated in protein trafficking and signal transduction.

### *Potential roles in neuronal lipid regulation*

Although the exact function of ABCA12 in the CNS is yet to be fully elucidated, it is clear that lipid transporters, such as ABCA12 can impact neuronal health. ABCA12 may regulate the balance of cholesterol and ceramides within the cell membranes of neurons, a balance critical for synaptic function, myelination, and neuronal survival.[3] CNS ABCA12 dysregulation could lead to lipid imbalances contributing to neurodegeneration.[17]

### *Factors regulating ABCA12 expression*

The expression of ABCA12, a critical lipid transporter implicated in epidermal barrier function, is finely controlled by a variety of factors, including transcription factors, microRNAs, epigenetic mechanisms, lipid levels, oxidative stress, inflammation, hormonal regulation, pharmacological agents, and keratinocyte differentiation signals.[18] Understanding these regulatory mechanisms can provide insight into the molecular basis of skin homeostasis and disorders associated with ABCA12 dysfunction.[19]

#### *Transcription factors*

Specific transcription factors influence ABCA12 expression by binding to its promoter or enhancer regions. For instance, peroxisome proliferator-activated receptors (PPARs), which regulate genes involved in lipid metabolism, might directly or indirectly affect ABCA12. PPARγ, highly expressed in keratinocytes, could enhance ABCA12 expression to facilitate lipid transport necessary for forming the skin barrier. Dysregulation of such transcription factors can lead to abnormal ABCA12 levels and associated skin conditions.[1] Several transcription factors regulate ABCA12 expression:

<u>PPARs</u>

PPARs, especially PPAR-α and PPAR-γ, are nuclear receptors. They regulate lipid metabolism and skin barrier integrity, positively regulating ABCA12 transcription. This enhances lipid transport necessary for epidermal differentiation.[20] PPAR-γ and PPAR-β/δ activation was found to highly induce ABCA12 mRNA in cultured human keratinocytes in a dose- and time-dependent manner. The upregulation of ABCA12 mRNA is followed by an increase in the protein levels, thus implying a biologically significant effect. In contrast, ABCA12 expression is not significantly changed when PPAR-α or retinoic acid receptor, retinoid X receptor, or vitamin D receptors are activated.[21]

<u>Sterol regulatory element-binding protein 1</u>

Sterol regulatory element-binding protein 1 (SREBP-1) is a primary regulator of lipid biosynthesis. Direct evidence is scarce, but it can be noted that SREBP-1 is linked to the regulation of ABCA12 through its influence on expression. Related ABC transporters, such as ABCA1, have been studied extensively, providing information on mechanisms of regulation. For example, the close relative of SREBP-1 is SREBP-2.[22] This has been demonstrated to activate ABCA1 by enhancing the synthesis of oxysterol ligands that activate liver X receptors for the promotion of transcription of ABCA1. With structural and functional similarities existing between ABCA1 and ABCA12, SREBP-1 may have an indirect influence on ABCA12 through similar lipid metabolism pathways involving LXR activation.[23]

<u>Specificity protein 1 and 3 (SP1 and SP3)</u>

SP1 and SP3 directly bind to the GC-rich promoter regions of the ABCA12 gene. They respond to stress signals, differentiation, and metabolites, thereby modulating ABCA12 expression tightly. SP1 has activator function, while SP3 can act as activator or repressor under certain cellular conditions, creating a dynamic control. Thus, it can control the gene transcription at several cellular signals.

#### *MicroRNAs*

MicroRNAs, abbreviated as miRNAs, are short, non-coding RNA that modulate gene expression

post-transcriptionally.[24] Certain specific miRNAs that are targeted to the ABCA12 mRNA reduce its level of protein, thus inhibiting lipid transport and altering skin barrier formation. Important miRNAs involved in regulating ABCA12 are as follows:

miR-21

MicroRNA-21 (miR-21) is a well-characterized miRNA involved in various biological processes, including development, cancer, cardiovascular diseases, and inflammation.[25] It regulates gene expression by binding to target mRNAs, leading to their degradation or translational inhibition. As an inflammation and cell proliferation-related miRNA, it may downregulate ABCA12 expression, associating it with the dysfunctional lipid barrier in inflammatory disease.

miR-29b

MicroRNA-29b (miR-29b) is part of the miR-29 family, which includes miR-29b. This family is known to regulate various genes involved in processes such as fibrosis, apoptosis, and lipid metabolism.[26] Notably, miR-29b has been shown to target DNA methylation-related enzymes, influencing epigenetic modifications. This miRNA is related to the differentiation of skin and can target pathways associated with ABCA12, thus modulating its expression during keratinocyte maturation.[27]

*Epigenetic mechanisms*

Epigenetic regulation by DNA methylation and histone modifications can also significantly affect ABCA12 expression.[28] DNA methylation involves the addition of methyl groups to cytosine bases in the promoter region of CpG islands. Generally, DNA methylation suppresses gene expression, while hypermethylation of its promoter could silence transcription. For this reason, in certain disorders affecting the skin, one could anticipate this to be due to silencing of its transcription.[29] For example:

DNA methylation

DNA methylation is an epigenetic modification that involves the addition of methyl groups to cytosine residues in the DNA. The role of DNA methylation is important in the regulation of gene expression.[30] Hypermethylation of promoter regions results in gene silencing, but hypomethylation might lead to gene activation. Hypermethylation of the ABCA12 promoter region would result in gene silencing, thereby contributing to the defects of the epidermal barrier.[1]

Histone modifications

Histone modifications, including methylation and acetylation, play a significant role in the regulation of gene expression through changes in chromatin structure and accessibility.[31] These epigenetic changes can either promote or inhibit the transcription of specific genes. In the case of ABCA12, a gene involved in lipid transport in keratinocytes and critical for skin barrier function, direct studies linking histone modifications to its expression are scarce. Histone acetylation and methylation patterns influence chromatin accessibility and transcription of ABCA12, especially in the presence of environmental stressors.

***Lipid levels***

ABAC12 is a critical lipid transporter in keratinocytes responsible for maintaining the skin lipid barrier. Although the amount of studies directly related to systemic lipid levels with expression is limited, the knowledge on the general relationship of lipid metabolism with ABCA12 function does provide insightful knowledge.[32] ABCA12 activity is coupled with lipid metabolism Particular lipids, such as:

Ceramides

Ceramides are critical lipid components in the epidermis; they are involved in an important function of regulating ABCA12, a membrane transporter critical for lipid export in keratinocytes. The scientific evidence suggests that ceramides up-regulate the expression of ABCA12 through the signaling pathway of PPARδ. These are needed for the formation of the lipid barrier, ceramide levels can act as response to regulate ABCA12 expression.[33]

Sphingolipids

The family of diverse lipids comprises ceramides, sphingomyelins, and sphingosine-1-phosphate, all very important in regulating ABCA12 expression as a necessary transporter for lipid transport across keratinocytes.[34] However, studies suggest that certain ceramides activate this transporter through the PPARδ-mediated signaling pathway. These lipids are crucial for skin homeostasis and may regulate ABCA12 activity through signaling pathways that affect keratinocyte differentiation.[1]

*Oxidative stress*

Oxidative stress regulates ABCA12 expression through reactive oxygen species (ROS) mediated signaling pathways. ROS can alter the activity of transcription factors or induce epigenetic changes, resulting in dysregulated ABCA12 expression and impaired skin barrier function.[35]

*Inflammation*

Chronic inflammation in the skin, as in psoriasis or eczema, can affect the expression of ABCA12. The pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, may interfere with normal lipid transport either by directly repressing the transcription

of ABCA12 or by changing the differentiation process of keratinocytes.[36] The interference leads to impaired function of the skin barrier and worsens the skin condition. Inflammatory cytokines can regulate ABCA12 expression:

TNF-α
TNF-α is a proinflammatory cytokine that significantly regulates lipid transporters, which include the ABC transporter family.[37] The expression of ABCA1 by TNF-α is mediated by the NF-κB pathway. The upregulation leads to increased cholesterol efflux during the phagocytosis of apoptotic cells, linking inflammatory signals to lipid efflux pathways. Prolonged exposure to TNF-α decreases ABCA12 expression, which affects lipid transport.[38]

IL-1β and IL-6
The cytokines change the expression pattern of ABCA12 through mechanisms dependent on altering keratinocyte proliferation and differentiation, thereby promoting inflammation and disrupting the skin barrier.[39]

*Hormonal regulation*
This vitamin A metabolite increases ABCA12 transcription by binding to nuclear retinoic acid receptors, which activate keratinocyte differentiation and lipid metabolism.[1] Retinoic acid generally activates genes responsible for keratinocyte differentiation and lipid transport; therefore, it is likely to promote ABCA12 expression. Corticosteroids, although anti-inflammatory, tend to suppress the expression of some skin barrier genes, which may affect ABCA12 levels.

*Keratinocyte differentiation signals*
The differentiation of keratinocytes, a critical process for the formation of the epidermal barrier, is tightly coupled with ABCA12 regulation. Differentiation signals, including calcium gradients and activation of epidermal growth factor receptor pathways, enhance ABCA12 expression to support lipid transport and skin barrier maturation.[27]

***ABCA12 and neurodegenerative diseases***
ABCA12 plays a crucial role in neurodegenerative diseases like AD, PD and HD (Fig. 1).

*Lipid homeostasis in neurodegeneration*
There is a crucial requirement for lipid homeostasis in neuronal function, and it has been observed that alterations in lipid metabolism can directly contribute to neurodegenerative diseases.[40] Cholesterol and ceramides are used by the CNS to maintain cell membrane integrity, synaptic function, and intracellular signaling. ABCA12 plays a critical role in lipid transport; mutations or dysfunction in ABCA12 may cause imbalances in lipids, which in turn contribute to neurodegenerative disease processes, such as protein aggregation, oxidative stress, and mitochondrial dysfunction.[41] ABCA12 in AD is characterized by the accumulation of amyloid plaques and neurofibrillary tangles (NFTs), and increasing evidence has come to indicate that lipid metabolism is a key component in these pathological processes.[3]

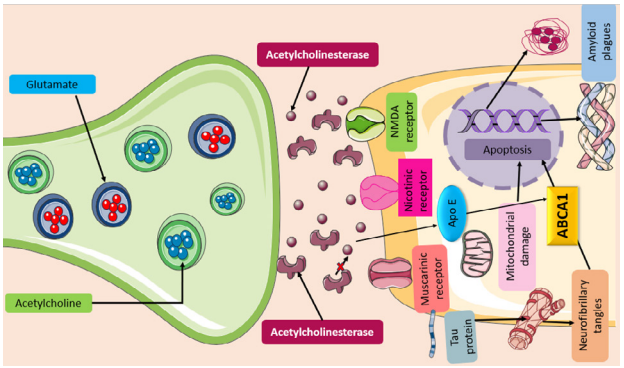


**Fig. 1**. Molecular pathway linking ABCA12 dysfunction via amyloid plaque formation in AD, α-synuclein aggregation in PD, and huntingtin protein aggregation in HD

Cholesterol transport and amyloid plaque formation
An essential role of cholesterol plays in Aβ plaques is the formation of the Alzheimer's disease. Dysregulation of lipid transport will probably disturb cholesterol homeostasis, and these disturbances might augment the Aβ plaque formation.[42] This interaction between cholesterol and amyloid-β peptide production takes place through the processing of APP by beta-secretase.[43] The impaired cholesterol transport could enhance amyloidogenic APP processing, thereby promoting plaque deposition.[3]

ABCA12 and tau pathology
In addition to amyloid plaques, tau protein hyperphosphorylation and aggregation into NFTs are the hallmark of Alzheimer's pathology. Lipid metabolism is considered to impact tau phosphorylation, while ABCA12, by modulating membrane lipid composition through lipid transport, indirectly acts on tau pathology. It has been suggested that levels of ceramides regulated by ABCA12 could be influential in tau aggregation and neurodegenerative processes.[44]

***ABCA12 in PD***
PD is typified by the aggregation of α-synuclein (αSyn) in Lewy bodies and the degeneration of dopaminergic neurons in the substantia nigra.[45] Emerging evidence ties lipid metabolism to αSyn aggregation and the development of PD.

*Lipid dysregulation and αsynuclein aggregation*
αSyn synuclein is a lipid-binding protein whose interaction with neuronal membranes may be enhanced by

lipid imbalance.[46] The role of ABCA12 in lipid homeostasis may play an important role in countering the misfolding and aggregation of αSyn.[47] ABCA12 disruption can impair lipid transport and alter membrane composition, thus hastening αSyn aggregation that may cause neuronal toxicity and PD.[48]

*Mitochondrial dysfunction and ABCA12*
Mitochondrial dysfunction is characterized by PD, and recent evidence has implicated lipid transporters such as ABCA12 in maintaining mitochondrial integrity.[49] As lipids make up a significant part of the molecules present in mitochondria, lipid transport is of significant importance for the preservation of mitochondrial membrane structure and function.[50] Lipid metabolism disruptions associated with ABCA12 may therefore lead to compromised mitochondrial function and enhanced oxidative stress and neuronal death, potentially making them contributory factors in PD pathogenesis.[51]

***ABCA12 in HD***
HD is one of the classic genetic neurodegenerative diseases, characterized by aggregation of the mutant huntingtin protein and progressive neuronal loss.[52]

*Lipid metabolism and protein aggregation*
A more recent emphasis has been placed on lipid metabolism itself, in that it could substantially contribute to the aggregation of the mutant huntingtin protein-a central aspect of HD pathology. The lipid imbalance due to ABCA12 dysfunction may accelerate huntingtin aggregation. Of the sphingolipids, ceramides are highly associated with cellular stress responses and may modify aggregation of misfolded proteins in HD.[53]

*Neuronal lipid imbalance and cell death mechanisms*
Synaptic function is primarily supported by membrane lipids, the homeostasis of which is essential for the survival of neurons in HD. ABCA12-mediated lipid transport can modulate the neural membrane composition.[54] Disrupted function of this protein may trigger apoptosis via the lipid-dependent pathways of cell death. Further, disrupted ceramide and cholesterol transport could facilitate increased neuronal susceptibility to stress and more rapid neurodegeneration in HD.[55]

***Molecular mechanisms implicating ABCA12 in neurodegenerative processes***
*ABCA12, lipid rafts, and membrane dynamics in neurons*
Lipid rafts are highly enriched plasma membrane microdomains mainly composed of cholesterol and sphingolipids; these microdomains play an important role in neuronal signal transduction and protein trafficking.[56]

ABCA12 regulates these lipids; aberrant ABCA12 function may disrupt the lipid raft composition and impair synaptic signaling, which seems to contribute to neurodegenerative processes.[2] Changes in lipid raft integrity have been associated with the pathology of AD and PD, where disrupted cascades of signaling promote protein aggregation and neuronal loss.[57]

*Oxidative stress and neuroinflammation*
Oxidative stress and chronic neuroinflammation are major characteristics of neurodegenerative diseases, and ABCA12 could influence such processes through its function in lipid transport.[58] Ceramides, whose levels the ABCA12 modulates, have been demonstrated to be implicated in neurons as oxidative and inflammatory mediators.[41] High accumulation of ceramides attributed to defective ABCA12 functionality may enhance neuroinflammatory responses and promote damage and degeneration of neurons.[2]

*Lipid transport disruption and mitochondrial dysfunction*
ABCA12 is essential in the maintenance of lipid balance in cellular membranes, including mitochondrial. Maintenance of high ATP production and the avoidance of ROS formation in mitochondrial membranes are contingent upon correct lipid composition.[59] Dysregulation of ABCA12 can cause mitochondrial lipid imbalance leading to mitochondrial dysfunction and oxidative stress-apoptosis in neurons, which form some of the fundamental features of diseases such as Alzheimer's and Parkinson's.[17]

*Neuronal apoptosis and ABCA12 dysregulation*
Neuronal apoptosis in neurodegenerative diseases often appears to be associated with disorders in lipid metabolism. ABCA12 plays a role in the protection of the cell from lipid-induced apoptosis through regulation of ceramide. Ceramides are pro-apoptotic lipids, and abnormalities in its levels due to dysfunction of ABCA12 can activate the apoptotic pathway leading to subsequent neuronal death.[60] Being able to comprehend how this dysfunction may associate with apoptotic signaling pathways in neurons might thus open fresh avenues for the investigation of neurodegenerative mechanisms.[61]

*Mechanisms of endoplasmic reticulum stress and protein misfolding in ABCA12-induced neurodegeneration*
ABCA12-induced neurodegeneration could be related to complicated mechanisms involving endoplasmic reticulum (ER) stress and protein misfolding. The ER has great importance in the synthesis, folding, and lipid metabolism of proteins.[62] Pathological consequences may occur in neural tissues when the ABCA12 is dysfunctional and disrupts these processes. Genetic mutations in ABCA12 can result in improperly folded proteins that fail to achieve functional conformation, accumulating in the ER. Misfolded ABCA12 may aggregate, over-

whelming the ER's protein quality control systems and leading to proteostasis imbalance.[38] Accumulated misfolded proteins activate the unfolded protein response (UPR), a protective mechanism aimed at restoring ER homeostasis. Without being resolved, chronic UPR signalling becomes inappropriate and leads to neuronal apoptosis while intensifying neurodegenerative processes.

*UPR major pathways*

Protein kinase RNA-like ER kinase (PERK)

The PERK pathway represents one of the major limbs of the UPR, active during ER stress.[63] PERK has a protective function to reduce the load of unfolded or misfolded proteins in the ER, however, its prolonged or otherwise dysregulated activation can lead to pathological outcomes, which are particularly pronounced in neurons.[64] When ER stress is present, PERK phosphorylates eIF2α, leading to an inhibition of global protein translation. This event avoids the accumulation of misfolded proteins in the ER and allows the cell to channel its resources towards quality control of proteins. Meanwhile, PERK-induced phosphorylation of eIF2α selectively increases the translation of ATF4, which is a transcription factor that activates genes involved in redox balance, autophagy, and amino acid metabolism.[65] But chronic activation of PERK leads to a disruption of cellular homeostasis. The availability of crucial proteins for neuronal survival and maintenance is reduced by persistent translation inhibition. In addition, extended ATF4 expression leads to the activation of CHOP (C/EBP Homologous Protein) and drives apoptotic pathways. Overactivation of PERK has been associated with synaptic dysfunction, impaired memory, and neurodegenerative diseases such as Alzheimer's and Parkinson's.[66] PERK activity also leads to interference with axonal transport and mitochondrial function that worsens neuronal vulnerability. Targeting the PERK pathway therapeutically is complex. While PERK inhibitors can mitigate neurodegeneration, they risk increasing ER stress by allowing uncontrolled protein synthesis. Balancing PERK activity remains a critical challenge in developing treatments for ER stress-related neurological diseases.[67]

Inositol-requiring enzyme 1

Inositol-requiring enzyme 1 (IRE1) is another critical sensor of ER stress and the most conserved UPR pathway. It has dual functionality: an endoribonuclease activity that processes mRNA and a kinase activity that regulates downstream signalling.[68] Upon ER stress, IRE1 autophosphorylates, thus activating its endoribonuclease domain. It splices the X-box binding protein 1 mRNA into a spliced variant known as XBP1s, a potent transcription factor that induces the genes associated with ER-associated degradation (ERAD), folding, and lipid metabolism for the efficient removal of proteins and restoration of ER homeostasis.[69] However, sustained IRE1 activity may be shifted from adaptive to maladaptive responses. Uncontrolled endoribonuclease activity causes degradation of specific mRNAs and microRNAs, disturbing cellular homeostasis. Prolonged activation also leads to inflammation due to the production of pro-inflammatory cytokines and c-Jun N-terminal kinase activation.[70] In neurons, IRE1 activity for extended periods contributes to oxidative stress and apoptosis, causing neurodegeneration. Moreover, excessive IRE1 activity enhances inflammatory cascades and, hence, it may also be related to chronic neuroinflammation, a feature of diseases such as amyotrophic lateral sclerosis and multiple sclerosis.[71]

Activating transcription factor 6

Activating transcription factor 6 (ATF6) is a transcription factor that is activated upon ER stress to improve the cell's adaptive capacity. Normally, ATF6 is a precursor residing in the membrane of the ER as an inactive form.[72] Upon the onset of ER stress, ATF6 translocates to the Golgi apparatus, where it is cleaved by site-1 and site-2 proteases. This cleavage releases its cytosolic domain, which enters the nucleus and activates genes related to protein folding, ERAD, and chaperone production.[73] ATF6 promotes the transcription of molecular chaperones like BiP/GRP78 and GRP94 to assist in the folding of proteins and in the mitigation of ER stress. The other effectors upregulate components of ERAD to ensure removal of terminally misfolded proteins. In neurons, this is crucial to maintain proteostasis and prevent the accumulation of aberrant proteins. These mechanisms, however, are compromised through the dysregulation of ATF6.[74] Reduced activity of ATF6 impairs cellular responses to ER stress and promotes protein aggregation and neurodegeneration. On the other hand, prolonged ATF6 activation leads to an imbalanced proteostasis that exhausts cellular resources and exacerbates neuronal dysfunction. In AD, where misfolded proteins overwhelm the ER, inadequate ATF6-mediated responses correlate with synaptic loss and cognitive decline.[75]

*Lipid dysregulation and membrane instability*

The ER is the key site for lipid synthesis and homeostasis. Dysfunctions in ABCA12 interrupt lipid transport, which can result in altered lipid composition and instability of ER membranes. Lipid dysregulation contributes to ER stress through impairment of membrane fluidity and the function of membrane-bound proteins. ABCA12 plays an important role in transporting ceramides and glucosylceramides into the cell, critical to the maintenance of membrane integrity. Deficiency of

ABCA12 impairs lipid delivery to the ER and thus affects membrane composition. The lipid imbalance disrupts ER membranes, causing stress, which makes the membranes more vulnerable to it, thus impairing the ER's ability to support protein folding and trafficking.[76] Changes in lipid composition amplify ER stress by changing the environment required for protein folding. Lipid imbalances can affect the function of resident ER chaperones and enzymes, which leads to protein misfolding and aggregation. This forms a vicious cycle, as lipid dysregulation aggravates ER stress, which in turn increases the burden of misfolded proteins.[77]

*Neuroinflammatory amplification*

ER stress in neurons provokes neuroinflammation, which increases the loss of neurons and enhances pathology. This reaction involves cytokine release and the activation of microglia, which are immune cells present in the brain.[78] In cytokine release ER stress-induced neuronal damage results in the release of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. These cytokines attract immune cells to the site of injury, thus increasing inflammation. Acute inflammation is protective; however, chronic cytokine release interferes with neuronal homeostasis, enhancing synaptic dysfunction and cell death. Sustained inflammation results in the activation of microglia, which then produces more pro-inflammatory mediators and ROS.[79] Overactivated microglia are part of secondary neuronal damage that creates a feedback loop in the continuation of inflammation and neuronal injury. This is the mechanism in neurodegenerative diseases such as Alzheimer's and Parkinson's, where chronic neuroinflammation accelerates the progression of the disease.[80] Targeting neuroinflammatory amplification includes inhibition of cytokine signaling or microglial modulation. Anti-inflammatory drugs and microglial inhibitors are being researched as potential therapeutic agents in neurodegenerative disorders.

*Apoptotic pathways*

Prolonged ER stress activates apoptotic pathways to eliminate highly damaged cells. CHOP is a transcription factor induced by sustained UPR signaling, mainly through the PERK-eIF2α-ATF4 pathway. CHOP promotes apoptosis by disrupting mitochondrial function and upregulating pro-apoptotic genes such as Bim and Bax. It also suppresses anti-apoptotic factors, thus shifting the balance toward cell death. In neurons, activation of CHOP is particularly damaging because neuronal loss leads to irreversible functional deficits.[66] CHOP's role in neurodegeneration has been well documented in ALS and Huntington's disease. Chronic ER stress activates CHOP in these conditions, leading to neuronal apoptosis and contributing to disease progression.

***ABCA12-ceramide pathogenesis in neurodegeneration***

Impairment in ABCA12 function leads to the accumulation of ceramide inside cells. Increased ceramide levels activate pro-inflammatory pathways, such as the NF-κB pathway, causing chronic neuroinflammation, characteristic of neurodegenerative diseases like Alzheimer's and Parkinson's.[2] Ceramide accumulation can induce oxidative stress by activating several pro-apoptotic signaling pathways. This oxidative damage is particularly harmful in neurons, which are highly vulnerable to oxidative injury.[81] Excess ceramide can also activate caspase-dependent apoptosis pathways, contributing to the loss of neurons in neurodegenerative conditions. In AD, the levels of ceramide in the brain have been reported to be high, especially in the hippocampus.[7] The imbalance in ceramide synthesis and its clearance has been related to the formation of amyloid plaques and tau tangles, which are typical features of the disease. The involvement of ABCA12 in ceramide transport may imply that disruption of this protein function will exacerbate these processes leading to neuronal loss. In PD, ceramide accumulation has been linked to dopaminergic neuron death.[41] ABCA12 dysfunction could contribute to the degeneration of these neurons, as the impaired transport of ceramide disrupts cellular functions, including synaptic vesicle trafficking and neurotransmitter release. HD has been associated with altered lipid metabolism, such as increased ceramide levels.[53] Considering the role of ABCA12 in maintaining lipid balance, its dysfunction may promote the progression of HD through enhancing neuroinflammation and cell death.

Ceramides at higher levels can disrupt the normal functioning of the cytoskeleton, which is critical for maintaining the structure and function of neurons.[82] It leads to neuronal atrophy and dysfunction. Accumulation of ceramides impairs autophagy, which is the mechanism by which cells eliminate damaged organelles and proteins.[83] In neurons, dysfunctional autophagy leads to the accumulation of toxic aggregates, such as tau and α-synuclein, associated with various neurodegenerative diseases. ABCA12 dysfunction may also affect the lipid rafts, which are membrane microdomains that play a role in cell signaling. Disruption of these lipid rafts due to the impaired ceramide transport may interfere with synaptic signaling and contribute to neuronal dysfunction.[84] Understanding the ABCA12-ceramide pathway opens avenues for potential therapeutic strategies targeting ceramide metabolism or ABCA12 activity. Modulation of ceramide metabolism or enhancement of ceramide clearance by drugs might alleviate neurotoxic effects due to its accumulation.[85] Small molecules or gene therapy approaches for enhancement of the function of ABCA12 could restore lipid transport, thus reducing pathologi-

cal effects associated with ceramide accumulation. Inhibition of inflammation pathways activated due to the accumulation of ceramide also offers a potential therapeutic avenue for neurodegenerative diseases caused by dysfunction of ABCA12.[86]

***Therapeutic opportunities in the targeting of ABCA12 in neurodegenerative diseases***

*Manipulation of lipid transport pathways in neurodegenerative disease*

However, since the ABCA12 gene participates in lipid metabolism, manipulation of lipid transport pathways might represent a novel therapeutic approach to the treatment of neurodegenerative diseases, either by normalizing lipid homeostasis through targeting the ABCA12, thus alleviating protein aggregation and oxidative stress, or through indirect compensation by an alternative lipid transport pathway in the case of ABCA12 dysfunction.[87,88]

*Gene therapy and ABCA12*

Gene therapy is a developing field that ensures cure for genetic dysfunctions such as those related to ABCA12.[2] In principle, gene therapy should deliver functional copies of the ABCA12 gene to affected neurons, which can correct lipid transport defects and cease the progression of neurodegenerative diseases.[89] Early-phase clinical trials of gene therapies targeting other lipid transport disorders form the basis for future ABCA12-targeted therapies.

*Drug development: lipid modulators and neuroprotective agents*

Restoration of lipid homeostasis in the CNS might have pharmacological interventions that could benefit neurological patients afflicted by neurodegenerative diseases.[90] Compounds that modify cholesterol and ceramide can rescue neuronal cells from lipid stress. Small molecules developed to enhance or correct imbalances through the functioning of ABCA12 may be useful candidates for neuroprotective agents, potentially retarding the progression of diseases such as Alzheimer's, Parkinson's, and Huntington's.

***Challenges and future directions***

Although targeting ABCA12 opens new avenues for therapy, several hurdles still need to be overcome. The CNS is a challenging environment with tightly regulated lipid metabolism; producing a change in activity of ABCA12 within this system without affecting critical pathways will require careful modulation.[87] Other research will be needed as well, to clarify ABCA12's role in the brain and its interface with other lipid transporters or pathways involved in neurodegeneration.[3]

*Elucidating the function of ABCA12 in CNS lipid homeostasis*

While the involvement of ABCA12 in CNS lipid homeostasis is yet to be fully determined, there are important points it controls in lipids in terms of neuronal composition and its implications in neurodegenerative processes.[44] Such points need to be studied further. Animal models in which the brain would lack or malfunction due to the absence of the ABCA12 gene may help better understand the functioning of this gene in neurodegeneration.[44]

*Prospect of CRISPR-based gene therapy in ABCA12-associated therapies*

CRISPR is also the revolutionary tool to correct genetic mutations. If the correct mutations in the ABCA12 gene are accurately edited by using CRISPR, it may prevent the further progress of neurodegenerative diseases that are due to ABCA12related disruption. So, there should be further investigations into the feasibility of this approach for treating the ABCA12 dysfunction within the CNS.[2]

*Bridging the gap between dermatology and neurology: research on ABCA12*

ABCA12 has had significant studies with dermatological disorders, but there remains an unexploited area of its function in the brain. Interconnection between studies involving ABCA12 in the skin and the CNS may be the light at the end of the tunnel in understanding its functions in lipid transport and disease on a larger scale. Hence, cross-interdisciplinary research will help further our knowledge of the role of ABCA12 in neurodegeneration.

**Challenges**

***Lack of comprehensive models***

The current animal and cell-based models fail to replicate the neural-specific effects of ABCA12 dysregulation. Such inadequacy makes it difficult to fully understand its role in the nervous system. Most models are focused on established roles in the skin, such as lipid transport in the epidermis, but ignore its unique functions in neurons.[91] The absence of models that specifically recapitulate ABCA12's neural functions makes it difficult to evaluate its contributions to processes like synaptic signaling, neuronal survival, or degeneration.

*Lipid metabolism complexity*

The lipid environment within the brain is unique because it's dominated by sphingolipids, gangliosides, and other complex lipids.[91] It becomes particularly challenging to study the interaction between ABCA12 with these lipids in the brain as they are very dynamic and region-specific. The diversity and complexity of lipid

profiles in the brain make it difficult to identify specific pathways affected by ABCA12.[60] The interaction of ceramides with other lipids involved in neural processes such as myelination and synaptic plasticity remains poorly understood.

*Functional overlap with other transporters*
ABCA12 is functionally similar to other members of the ABC transporter family.[60] Such transporters often share overlapping functions in lipid transport, making it challenging to distinguish ABCA12-specific functions. For instance, other transporters such as ABCA1 and ABCG1 are crucial in the metabolism of neural lipids and neurodegenerative diseases.[40] Elucidating the specific role of ABCA12 in these processes is a challenge.

*Limited biomarkers*
To date, there are no definitive biomarkers for diagnosing ABCA12 brain dysfunction.[60] This further reduces the ability to identify neural disorders at an early stage and monitor them for proper management.[92] Since specific biomarkers are lacking, monitoring the progression of neurodegenerative diseases is hard, and so is understanding the treatment efficacy or potential therapeutic value of targeting ABCA12.

***Future outlooks***
*Creation of high-order models*
More advanced models, such as neural-specific knockouts or overexpression systems, will be used to explore the role of ABCA12 in neurodegeneration.[93] Technologies such as organoids and brain-on-chip technologies can recreate the neural environment, giving deeper insights into ABCA12 function in the context of the nervous system. These models can be designed to replicate conditions such as Alzheimer's disease, Parkinson's disease, or other neurodegenerative disorders to explore ABCA12-linked mechanisms.[44]

*Lipid pathway elucidation*
Future research should focus on mapping out the specific roles of ABCA12 in sphingolipid and ganglioside metabolism within neurons.[94] Techniques such as mass spectrometry-based lipidomics may be used to identify specific lipid substrates and pathways affected by ABCA12 dysfunction. This may help establish direct links between ABCA12, lipid dysregulation, and specific neurodegenerative disorders.[95]

*Therapeutic interventions*
Small molecules or drugs, like retinoid-based therapies, can be developed to restore or modulate ABCA12 function in the nervous system. Improved systems of CRISPR and viral vectors might present with therapeutic options for correcting mutations of ABCA12 or up-regulating its expression in neural tissues. Drugs that diminish ceramide accumulation or oppose its harmful action might supplement strategies involving the modulation of ABCA12.

*Multi-omics strategies*
The combined genomics, lipidomics, and proteomics approaches will elucidate the functions of ABCA12 in promoting healthy neural environments. The patient-specific pattern of ABCA12 dysfunction that can be derived from multi-omics datasets opens up opportunities for precision medicine approaches. Secondary pathways and compensatory mechanisms, potentially targeted therapeutically, also are found through multi-omics approaches.[96]

*Interdisciplinary research*
There is a potential for researchers in dermatology, where the function of ABCA12 is better understood, and neurology, where it remains an area of exploration, to collaborate on shared mechanisms and therapeutic targets.[97] Insight from ABCA12-associated skin diseases (like harlequin ichthyosis) may have implications for its neural functions and vice versa, with a potential for better management of both dermatological and neurological disorders. Technologies and approaches in dermatology, like lipid imaging and targeted lipidomics, can be applied in neurology to better analyze ABCA12 in the brain.[34]

## Conclusion

As the number of reports linking ABCA12 to lipid deregulation in neurodegenerative diseases is on the rise, this transporter is emerging as a potential therapeutic target for intervention. Activation of inhibition of ABCA12 might normalize the lipid balance against oxidative stress toward neurons and reduce protein aggregation. Future models of neurodegenerative diseases must contain lipid transport mechanisms with a role for ABCA12 so that the contribution of lipid dysregulation in disease progression can be understood better. Such integrative approaches could lead to much more effective. There is hope in personalized medicine for future treatment of neurodegenerative diseases. These distinct lipid transport dysfunctions, including mutations in ABCA12, can serve to create specific targeted therapies that address the molecular deficits underlying neurodegeneration. This research on ABCA12 shall not stop anytime soon since research in this line can greatly push forward improving the outcomes of patients affected with neurodegenerative disorders. This review now provides the ABCA12 in neurodegenerative diseases with a systematic discussion of its molecular mechanisms and therapeutic potential. Further, more detailed research will help widen the un-

derstanding of how functional dysfunction of ABCA12 propagates its effects during neurodegeneration and, more importantly, will help open up avenues for novel therapeutic strategies.

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***Data availability***
Data will be made available as per the policy of Journal.

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Not applicable.

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**REVIEW PAPER**

# The role of vitamin D and its supplementation in sarcoidosis – current status

Anna Bieda [1], Monika Kondracka [2], Michał Bielecki [3], Agata Zapałowska [4], Kamil Kondracki [5], Milena Szczepańska [6], Wojciech Kozłowski [7]

[1] Masovian Bródnowski Hospital, Warsaw, Poland
[2] Prague Hospital of Lord's Transfiguration, Warsaw, Poland
[3] The Independent Group of Public Ambulatory Care Institutions Warsaw-Mokotów, Warsaw, Poland
[4] John Paul II Independent Public Specialist Western Hospital, Grodzisk Mazowiecki, Poland
[5] Medical Center named after the Battle of Warsaw 1920 in Radzymin Independent Public Health Care Facility Complex, Radzymin, Poland
[6] Independent Clinical Hospital named after Prof. Witold Orlowski in Warsaw, Warsaw, Poland
[7] Marynin Medical Center, Marynin, Poland

**ABSTRACT**

**Introduction and aim.** Sarcoidosis is a chronic autoimmune-related inflammatory disease characterized by non-caseating granuloma formation. The macrophages accumulating in granulomas express increased 1alpha hydroxylase activity. Increased extrarenal 1,25-dihydroxyvitamin D synthesis can lead to hypercalcemia and its complications. However, 25-hydroxyvitamin D deficiency and insufficiency are virtually universal among sarcoidosis patients. The aim of this study was to explain the complex role of vitamin D and to its metabolites and discuss the possible benefits and risks associated with the administration of exogenous vitamin D to patients with sarcoidosis.

**Material and methods.** PubMed and Scopus databases were searched for reviews about The role of vitamin D and its supplementation in sarcoidosis. The authors have analyzed a total of 107 full-text articles published between January 2000 and November 2024, with additional articles identified by bibliography analysis.

**Analysis of literature.** The potential benefits of vitamin D supplementation in sarcoidosis are promising in terms of reducing the inflammatory response, counteracting disease progression, reducing bone fracture risk, and minimizing the pharmacotherapy needed for disease control. However, the risk of hypercalcemia should not be neglected.

**Conclusion.** Despite the increased risk of hypercalcemia, vitamin D supplementation in patients with sarcoidosis should be considered. Each patient's benefits-to-risks ratio of vitamin D supplementation should be assessed individually and the intervention should be closely monitored both before and during implementation.

**Keywords.** bone mineral density, disease course, hypercalcemia, sarcoidosis, supplementation, vitamin D

## Introduction

Sarcoidosis is a chronic autoimmune-related inflammatory disease characterized by non-caseating granuloma formation. The lungs and lymphatic system are the sites most frequently affected. Other organs such as the skin, heart, eyes, liver, and spleen may also be involved.[1]

**Corresponding author:** Anna Bieda, e-mail: annabieda23@gmail.com

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The symptoms of sarcoidosis are nonspecific and include persistent cough, dyspnea, fatigue, weight loss, fever, night sweats, and erythema nodosum. More characteristic clinical manifestations may be observed depending on the affected area.[2,3] The definitive etiology of sarcoidosis remains unknown; however, various hypotheses have been proposed, including disruption of innate immunity or the role of mycobacteria.[4]

Currently, the development and phenotype of sarcoidosis in individual patients are widely dependent on the interplay between genetic, environmental and non-environmental factors.[2]

The prevalence of sarcoidosis varies between one and 160 per 100,000 depending on the geographical region and ethnicity.[5] The incidence among Blacks is three times higher than among Whites. Sarcoidosis usually occurs between 20 and 60 years of age.[6] Additionally, women are more commonly affected than men.[5] Other populations at increased risk of developing sarcoidosis are individuals of black ancestry or relatives with sarcoidosis.[7]

The challenges of sarcoidosis treatment include the variety of manifestations and the heterogeneity of the disease's course.[8] A wide range of medicines, including glucocorticosteroids (GCS), methotrexate, azathioprine and monoclonal antibodies, such as infliximab or adalimumab, are used.[9] Despite evolving pharmacotherapy options, GCS remain the mainstay of therapy in sarcoidosis patients.[10] https://pubmed.ncbi.nlm.nih.gov/35838355/ The optimization of treatment efficacy and disease activity, as well as counteracting the side effects of chosen pharmacologic interventions remain a challenge.

Calcitriol is one of the key hormones in calcium and phosphorus metabolism. Its interaction with PTH, calcitonin, and fibroblast growth factor 23 allows one to maintain adequate levels of calcium and phosphorus in the body, protecting from risks associated with both their abundance and scarcity.[11] Additionally, the impact on the immune system is complex.

Due to the most common side effect of GCS, osteoporosis, paired with a complicated role of vitamin D and its metabolites in sarcoidosis, together with the anti-inflammatory properties of calcitriol, the role of vitamin D supplementation in sarcoidosis is particularly intriguing.

However, a consensus on vitamin D supplementation in patients with sarcoidosis has not yet been reached. Due to risks about the concerns of hypercalcemia risks and the potential benefits of vitamin D supplementation in sarcoidosis patients, understanding the role of vitamin D in the pathogenesis of the disease and its metabolites' mutual impact and influence of its metabolites on the course of the disease is crucial. Previous research has addressed these connections; however, due to the rapid evolution of data on vitamin D's role and impact on sarcoidosis, we believe a timely re-evaluation is necessary. Although previous reviews discuss several aspects addressed in our review, by including recent studies, our article provides an up-to-date analysis of the current knowledge. Furthermore, in our article we include aspects of sarcoidosis and vitamin D supplementation in this population not recently juxtaposed in a single article, including disease pathophysiology, genetic predispositions, bone health, disease course, hypercalcemia, and its potential consequences in patients with sarcoidosis, offering to bridge gaps left by previous reviews. We propose a clinical approach based on the currently available data, underline knowledge gaps, and suggest areas for future research.

## Aim

In this review, our aim is to explain the complexities of vitamin D's role in sarcoidosis pathogenesis, the complex relationship between its metabolites, and discuss possible benefits and risks associated with exogenous vitamin D administration in sarcoidosis patients. We propose a clinical approach to vitamin D supplementation among sarcoidosis patients.

## Material and methods

The authors reviewed the literature by searching PubMed and Scopus databases. The search entries included various combinations of the following phrases: sarcoidosis, vitamin D, cholecalciferol, supplementation, bone mineral density, coarse, protracted treatment. The selected articles were published between January 2000 and November 2024 by titles and abstracts, excluding conference presentation abstracts, posters, and studies not consistent with and exceeding our review's purpose. Additional articles were extracted from the bibliographies of the screened articles. In the final analysis the authors included a total of 107 full-text articles: reviews, meta-analyses, randomized-controlled trials, cross-sectional studies, research articles, retrospective cohort studies, register-based studies, cohort studies, case-control studies, and a few case reports. The studies were focused on the pathophysiology of sarcoidosis, altered vitamin D metabolism, risk of hypercalcemia, bone health, disease course, and current knowledge on vitamin D supplementation in patients with sarcoidosis.

## Analysis of literature

### *Sarcoidosis – pathophysiology*

Several factors have been consistently associated with the development of sarcoidosis. At the genetic level, the Annexin A11 (ANXA11) gene – involved in the regulation of apoptosis – and its variations have been reported to impact the susceptibility to sarcoidosis.[12,13] The T allele of the rs1049550 ANXA11 gene was found to be

protective against the development of sarcoidosis, reducing the susceptibility to sarcoidosis by 30-40% depending on the studied population.[13,14] Conversely, the A allele of rs2789679 was more frequent among sarcoidosis patients compared to controls (p=0.00004, odds ratio (OR) 1.42, 95%, confidence interval (CI) 1.17 to 1.73), similarly to the T allele of rs2819941 (p=0.0006, OR 1.41, 95%; CI 1.16 to 1.71).[13]

Although a direct link is still being discussed, mycobacterial infections are considered a factor in the development of sarcoidosis. The connecting agent is cathelicidin, an antimicrobial peptide directed against mycobacteria.[15]

In their review, Gupta et al. indicate that the prevalence of sarcoidosis is greater in populations with a higher tuberculosis (TB) incidence, especially when a decline in the prevalence of TB occurs. This is consistent with molecular and immunological studies that suggest mycobacterial antigens (*Mycobacteria tuberculosis* in particular) as inciting agents of sarcoidosis.[16]

Sarcoidosis is a mainly granulomatous disease. The formation of non-caseating granulomas in sarcoidosis is related to overstimulation of the inflammatory system, often in the absence of a clear infectious agent. The exaggerated immune response is related to CD4+ T lymphocyte activation, their accumulation at the affected site, macrophage aggregation and subsequent formation of non-caseating granulomas. The overproduction of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin (IL) 6, interferon gamma (IFN-γ) contribute to tissue inflammation. B lymphocytes also accumulate in granulomatous tissue, with their altered maturation and function further contribute to the disease progression.[15,17] Their role in the pathogenesis is related to accompanying hypergammaglobulinemia, antigen presentation, and formation of immune complexes.[18,19]

***Vitamin D – physiological metabolism***

Physiologically, 7-dehydrocholesterol in the skin absorbs UVB radiation and undergoes isomerization to become vitamin D (cholecalciferol). However, vitamin D is considered inactive until hydroxylation. It is first hydroxylated in the liver to form a partially active 25-hydroxyvitamin D (25OHD). The full metabolic potential is reached after the second hydroxylation, most of which occurs in the kidneys when 1,25-dihydroxyvitamin D (calcitriol, 1,25-(OH)2D) is formed. Although dietary sources of cholecalciferol, such as fish, egg yolks, and some dairy products, continue to play a role, sunlight exposure remains the main source of cholecalciferol.[20] The kidneys play a crucial role in the formation of calcitriol by catalyzing the formation of last step in the active hormones and they are also primarily responsible for the catabolism of calcitriol. The renal CYP24A1 is key in the breakdown of calcitriol, helping maintain its adequate levels.[21] This negative feedback loop, which consists of decreased calcitriol synthesis with increased catabolism, is tightly regulated by calcium and phosphorus levels, parathormone (PTH) and is also product dependent: Increased calcitriol levels inhibit PTH release and decrease renal expression of 1alpha hydroxylase, decreasing the risk of hypercalcemia.[22] The 1,25(OH)2D synthesized at granulomatous sites also stimulates macrophage expression of 24-hydroxylase. It facilitates the conversion of calcitriol to less active metabolite and also thought to limit 25OHD availability to enzymes, thus increasing the 25OHD:1,25(OH)2D ratio.[23]

***The role of vitamin D in immunity***

Calcitriol modulates both the innate and adaptive immune responses.[24] It stimulates the expression of antimicrobial proteins, especially cathelicidin. This may help combat mycobacteria, which are considered inciting agents of the development of sarcoidosis.[25] Cathelicidin promotes autophagy in human monocytes and macrophages killing mycobacteria directly. It induces an inflammatory response by activating scavenger receptors, activating pro-inflammatory intracellular pathways, and enhancing cytokines and chemokines, including IL-6 and IL-10, monocyte chemoattractant protein (MCP) 1 and 3 in human peripheral blood mononuclear cells.[26,27] In severe sarcoidosis, cathelicidin levels are reduced compared to healthy individuals. Cathelicidin deficiency can impede the resolution of lung inflammation in patients with severe sarcoidosis, as the regulation of immune cells and pro-inflammatory cytokines is then impaired.[28]

In addition to cathelicidin stimulation, calcitriol promotes autophagy and thus destruction of intracellular pathogens. It reduces intracellular replication of phagocyte-free bacteria. Moreover, local synthesis of calcitriol, mediated by macrophages, promotes anti-inflammatory reactions by modulating T lymphocyte response. It suppresses the effector Th1 and Th17 lymphocytes while promoting the regulatory lymphocyte response of T, leading to a reduction in the inflammatory reaction. Studies show that calcitriol modulates cytokine expression in a concentration-dependent manner, generally promoting anti-inflammatory cytokine production and reducing anti-inflammatory cytokine stimulations, particularly IL-1, IL-2, IL-12, and IFN-γ. Research shows that calcitriol may also reduce prostaglandin production and TNF-α expression and secretion, further modulating the immune response.[24,29]

Calcitriol also affects B-lymphocyte-dependent immune processes. It reduces B lymphocyte memory and autoantibody production, crucial in the pathogenesis of sarcoidosis.[30-32] Despite conflicting evidence, it is suggested that the hormone affects B lymphocyte dif-

ferentiation, aggregation, activation threshold, and immunoglobin production.[33–38]

***Altered vitamin D metabolism in sarcoidosis***

The aggregation of macrophages is particularly important in cholecalciferol metabolism among sarcoidosis patients. Activated macrophages exhibit 1alpha hydroxylase expression. Their accumulation in sarcoid granulomas leads to increased enzyme levels, resulting in an increased 25OHD to 1,25(OH) 2D. Although renal calcitriol production is tightly regulated by the negative feedback loop, there is no product-controlled inhibition in immune cells. The production of 1alpha hydroxylase by immune cells is mainly controlled by proinflammatory cytokines produced in granulomas. Calcitriol production is promoted by production at site IL-1 and IL-2, TNF-α with IFN-γ shown to directly increase 1 alpha hydroxylase expression in macrophages, further increasing calcitriol production in sarcoid granulomas.[22,39]

***Hypercalcemia risk in sarcoidosis***

The increased extrarenal 1,25(OH)2D synthesis that occurs in sarcoid granulomas may lead to hypercalcemia and its complications. Hypercalcemia occurs in around 11% of patients with sarcoidosis - it may be symptomatic or asymptomatic; unmasked by exogenous vitamin D administration, or, with its complications, present as the first clinical characteristic of sarcoidosis.[40–52] Hypercalcemia, especially when chronic, can lead to various complications such as digestive tract dysfunction, coma, life-threatening arrhythmias, or kidney damage.[53] The increased levels of serum calcium may affect the electrical conductivity resulting in abnormal heart rhythms. Electrocardiographic manifestations include PR and QRS interval prolongation, T wave flattening, or the presence of a Q-wave.[54,55] The abnormal calcium metabolism is also linked to hypercalciuria. It occurs in approximately 50% of patients with sarcoidosis and may lead to nephrolithiasis, noted in 10% to 14% of sarcoidosis patients.[56–59] In up to 3.6%, kidney stones can be the first clinical presentation of sarcoidosis.[60] In addition to nephrolithiasis, nephrocalcinosis leading to kidney injury may occur. This complication is seen in up to 50% of patients with renal involvement.[61] Calcium level monitoring and evaluation of vitamin D metabolites assessment in correlation with clinical image are of key importance in patients with sarcoidosis.[48,49,62]

***Sarcoidosis and vitamin D status***

According to Burke et al. 25OHD deficiency and insufficiency are virtually universal among sarcoidosis patients, with 97% of sarcoidosis patients in the studied group with a 25OHD concentration below 28 ng/mL. Despite the 25OHD deficiency, 71% of these patients had 1,25(OH)2D levels equal to or greater than the median clinical value in the reference population (33.5 pg/mL).[22] The paradox presented by the authors sheds light on the importance of studies on the safety of vitamin D supplementation among patients with sarcoidosis, with a focus on possible risks of vitamin D toxicity. However, while the review, published in 2010, highlights challenges in adequate management of vitamin D metabolites levels, it does not include recent findings on complicated calcium homeostasis in sarcoidosis patients. Furthermore, while the authors advise caution, they do not include clinical trials on vitamin D supplementation – its safety and efficacy – among patients with sarcoidosis. The review lacks a patient-centered focus and focuses on biochemical findings without addressing outcomes such as the management of concentration-dependent symptoms of vitamin D metabolites.

***Sarcoidosis and bone health***

Studies show that the risk of bone deformities and bone fractures among sarcoidosis patients is significantly greater compared to studies on their incidence among healthy populations. In their cross-sectional study, Heijckmann et al. reported a prevalence of vertebral deformities among patients with sarcoidosis despite unchanged bone mineral density (BMD), 32% ($p < 0.05$) in the follow-up study, and 26% of patients developing either new or progressive deformities. Despite the unchanged BMD, the described deformities bore marks of osteoporotic fractures. Furthermore, the described pre-follow-up deformities occurred in a population with a mean age of 43 years, with half of the affected populations receiving GCS therapy.[63,64] These results suggest that patients with sarcoidosis may be at increased risk of vertebral deformities regardless of their BMD. This underlies the importance of early assessment and the introduction of appropriate interventions in this group of patients, as well as the need to establish more appropriate indicators of fracture risk among sarcoidosis patients. However, the lack of a control group, the small sample size (66 patients), and the short follow-up (4 years) limit the study's generalizability. Furthermore, potentially confounding factors have not been extensively assessed.

A similar study by Saidenberg-Kermanac'h et al. on 142 patients with histologically confirmed sarcoidosis delivered similar results, with 23.5% of patients with a history of fractures despite the mean age of 51 years and normal mean BMD. The risk factors associated with the increased risk of fracture included GCS treatment, low dietary calcium, as well as serum 25OHD levels that did not meet the 10–20 ng/mL interval.[65] This suggests that standard supplementation guidelines for the general population may not be adequate for patients with sarcoidosis and randomized-controlled trials in this population are necessary. Again, the lack of a control group,

not enough extensive analysis of potentially confounding factors, cross-sectional study design, and lack of implementation of advanced imaging techniques limit the ability to draw general conclusions from this study.

Boures et al. conducted a retrospective cohort study in a large population of 5 722 sarcoidosis patients matched with 28 704 controls, concluding that patients with sarcoidosis are at increased risk of clinical vertebral fractures (adjusted relative risk (RR) 1.77; 95%; CI 1.06–2.96) but not any fractures (adjusted RR 0.87; 95% CI 0.77–0.99); and the use of GCS use among sarcoidosis patients causes an increased risk of any fractures (adjusted RR 1.50; 95% CI 1.20–1.89).[66] While recent GCS use is taken into consideration, no details on the total dose or duration of therapy have been included. Furthermore, lack of BMD measurements or attribution of potential confounding factors prevents fully informed conclusions on the causes of fractures. Further prospective research on the relationship between fracture prevalence and vitamin D metabolites' levels is necessary for establishing vitamin D's role in bone health in sarcoidosis patients.

In a 2018 meta-analysis by Yong et al., no increased risk of fracture or loss of bone mineral in sarcoidosis has not been identified.[67] The authors did not include the previously described studies in the analysis and mostly focused mainly in patients treated with the available studies on premenopausal and not with GCS. Other studies have also found no correlation between sarcoidosis and decreased BMD regardless of baseline vitamin D status and oral GCS use, and one study suggesting possible decreases in BMD in female patients. 1,25(OH)2D has been suggested to the increased bone turnover as a cause of increased fracture risk among sarcoidosis patients.[63,65,68] A single center cross-sectional study on 262 patients, matched with healthy controls, found that in sarcoidosis patients, the increased fragility fracture risk correlates with the severity of lung disease severity and potential disease activity.[69] The BMD T scores of the sarcoidosis patients were significantly lower than those of healthy controls in the lumbar spine and total hip ($p<0.01$ and $p<0.05$, respectively). Furthermore, fragility fractures were more prevalent among patients with sarcoidosis, reaching 30.6% compared to 12.3% in the control group. The link between bone health and lung function of sarcoidosis patients showed decreases in forced vital capacity, lungs diffusion capacity of the lungs for carbon monoxide, and forced expiratory volume in the first second among sarcoidosis patients with low BMD and multiple vertebral fractures.[69] Again, the study lacks details on GCS dosage, proper assessment of confounders. Together with the cross-sectional design of the study, the limitations suggest that more longitudinal research is needed. A summary of the studies analyzed, with their main focus and key findings, is presented in Table 1.

**Table 1**. Sarcoidosis and bone health summary

| Study | Focus | Key findings |
|---|---|---|
| Bolland et al. [63] | Bone turnover and hip BMD | Bone turnover normal |
| Heijckmann et al. [64] | Vertebral deformities and BMD change over 4 years | Progressive vertebral deformities occurred despite stable BMD |
| Saidenberg-Kermanac'h et al. [65] | Bone fragility and calcium metabolism disorders | Increased fracture risk correlated with calcium metabolism disorders |
| Bours et al. [66] | Risk of vertebral and non-vertebral fractures | Increased fracture risk in sarcoidosis patients compared to controls |
| Yong et al. [67] | Bone mineral loss and fracture | Lower BMD and increased risk of fracture in sarcoidosis patients compared to the general population |
| Bolland et al. [68] | Changes in BMD over 2 years | Hip BMD normal and stable over 2 years |
| Cameli et al. [69] | Evaluation of BMD and fracture risk | Lower BMD and higher fracture risk identified in specific sarcoidosis populations |

### *Vitamin D status and course of sarcoidosis*

Studies show that despite an increase in serum 1,25(OH)2D sarcoidosis patients often suffer from 25OHD deficiency.[22,70] Furthermore, an association between decreased serum 25OHD to the 1,25 (OH) 2D ratio and disease activity, severity, chronicity, and protracted treatment has been noted.[65,71–74] Higher serum 25OHD is also associated with lower disease activity implicating that described dysregulation may affect prognosis.[65,75]

Kiani et al. and Mihailovic-Vucinic et al. describe a statistically significant correlation between vitamin D deficiency and sarcoidosis chronicity.[74,76] According to a cross-sectional study on 80 sarcoidosis patients by Kiani et al., the 0–1 respiratory involvement in sarcoidosis is not correlated with vitamin D deficiency (Mann-Whitney test; $p=0.243$). However, a possible progression of pulmonary sarcoidosis to stage 2-4 lung involvement by 25OHD deficiency has been described (Pearson Chi-Square=4.266; degrees of freedom (df)=1; $p=0.039$). 25OHD deficiency has also been suggested to play a role in the development of acute and active sarcoidosis. However, the study by Kiani et al. involves only serum 25OHD measurements but not 1,25(OH)2D3 levels. Evaluation of the levels of both metabolites would provide more comprehensive understanding of the impact of vitamin D on disease course. Additionally, the lack of a control group, the evaluation of disease activity based on 24-hour urinary calcium levels – which is not a definitive marker of sarcoidosis – are important limitations of the study.[76]

Mihailovic-Vucinic et al. have also noted an inverse correlation between serum 25OHD levels and disease activity markers. They found a statistically significant correlation between disease chronicity and low serum 25 (OH) D levels (Chi-Squar e=6.044; df=2; $p=0.014$).

Furthermore, the majority of patients with high calcium levels in the 24h urine sample had absolute 25(OH)D deficiencies, despite a statistically significant correlation between calcium urine levels (24 hour urine) and serum vitamin D in the studied group (ChiSquare=6.759; df=2; p=0.034).[74] Their study underscores the complex role of vitamin D in sarcoidosis pathogenesis, immune function and dysfunction, as well as disease activity, measured by serum angiotensin-converting enzyme levels. Despite promising results, the cross-sectional study design, limited sample size and sample diversity, lack of control group, and conclusions drawn based on 25OHD3 levels not juxtaposed to the level (not measured) limit the generalizability and its potential role in guidelines development.[74]

Similar results were obtained in a retrospective study by Kamphius et al., where the authors concluded that vitamin D scarcity may affect sarcoidosis activity. Although a negative correlation ($p < 0.001$) between 25OHD levels and disease activity – measured by somatostatin receptor scintigraphy - no additional markers of either disease activity or inflammation were implemented, limiting the comprehensibility of this aspect of the study. Furthermore, serum calcium levels were not significantly correlated with 25OHD or 1,25(OH)2D levels ($p = 0.52$ and $p = 0.07$, respectively).[75] However, this study did not investigate only vitamin D supplementation but calcium and vitamin D supplementation, and the study lacked a standardized supplementation protocol. The retrospective study design and limited follow-up data are additional study limitations.[75]

Kavathia et al. have conducted a complementary study linking increased 1,25(OH)2D levels with prolonged systemic treatment in patients with sarcoidosis. The authors demonstrated that serum 1,25(OH)2D levels are positively correlated with the severity and prolonged treatment of sarcoidosis. Patients in the highest quartile of 1,25(OH)2D levels had significantly increased odds (OR: 1.82; with 95% CI: 1.11–2.99) of chronic phenotype. In particular, 71% of patients with $1,25(OH)_2D$ levels greater than 51 pg / ml had chronic treatment status (SCAC Class 6 requiring repeated regimens of systemic immunosuppressive therapy or >1 year of therapy). However, no significant differences were observed in serum 1,25(OH)2D3 levels by chest radiograph stage or Sarcoidosis Severity Score were noted. Despite clear cutoff levels and specific and targeted focus of the study, its cross-sectional design, small sample size (59 patients), limited diversity of the studied population, and lack of evaluation of 25OHD levels limit the study and suggest that more research is necessary to fully assess the role of vitamin D in the treatment of patients with chronic disease.[73] A summary of the analyzed studies, with their main focus and key findings, is presented in Table 2.

**Table 2**. Vitamin D status and sarcoidosis course summary

| Study | Focus | Key findings |
|---|---|---|
| Burke et al. [22] | Calcium and vitamin D in sarcoidosis | The high 1,25(OH)2D associated with hypercalcemia and granuloma activity suggested cautious monitoring of vitamin D metabolites |
| Heijckmann et al. [64] | BMD, calcium, and vitamin D in sarcoidosis | The dysregulation of calcium and vitamin D metabolism affects bone health and potentially disease activity |
| Saidenberg-Kermanac'h et al. [65] | Bone fragility and calcium-vitamin D metabolism | Elevated 1,25(OH)2D levels observed in a subset of patients despite low to normal 25OHD levels, patients with high 1,25(OH)2D levels exhibited markers of increased disease activity |
| Filipovic et al. [71] | Vitamin D deficiency and sarcoidosis activity | Low 25OHD is associated with increased disease activity and worse outcomes |
| Scullion et al. [72] | Role of vitamin D in pulmonary inflammation | Vitamin D deficiency linked to increased inflammation highlighted potential role of Vitamin D in modulating activity of lung sarcoidosis |

### *Vitamin D supplementation and risk of hypercalcemia in sarcoidosis patients*

In their study discussed previously, Kamphius et al. determined that sarcoidosis patients without calcium and vitamin D supplementation were at increased risk of developing hypercalcemia than those supplemented; however, this finding was described as not statistically significant. Simultaneous GCS use and vitamin D supplementation correlated with a lower risk of hypercalcemia than sole vitamin D supplementation ($p<0.001$). Although the calcium concentration being higher than healthy controls, calcium levels were not correlated with vitamin D status.[75] Gwadera et al. found no significant correlation between calcium or vitamin D levels and disease activity, despite some inflammatory markers are correlating with increased calcium level. The design of the cross-sectional study, the small sample size of 58 patients, lack of a 1,25(OH)2D assessment significantly limit the generalizability of the study.[77] These results may indicate that vitamin D supplementation with concurrent GCS therapy may not only decrease the risk of hypercalcemia, but both interventions can act jointly to limit inflammation and disease progression, further reducing the risk of hypercalcemia. However, more detailed large-scale longitudinal studies are necessary to elucidate the relationship between calcium, vitamin D, and sarcoidosis.

In their randomized controlled trial, Bolland et al. introduced vitamin D supplementation in a group of patients with sarcoidosis with normal calcium levels and serum 25OHD levels below 20 ng/L. Compared to the control group, vitamin D did not exhibit significant dif-

ferences in serum calcium levels, but showed increases in their 25OHD serum levels. The intervention included supplementation of 50 000 IU of cholecalciferol per week for 4 weeks and 50 000 IU per month in the following 11 months. The authors noted one case of asymptomatic hypercalcemia and one case of asymptomatic hypercalciuria. Despite an appropriate study design, it only included 27 patients with sarcoidosis, limiting its statistical power.[78]

Again, a positive correlation between vitamin D supplementation and serum 25OHD levels but not with serum calcium levels has not been confirmed in the cross-sectional study previously discussed in 142 sarcoidosis patients by Saidenberg-Kermanac et al.[65]

In their study, Capolongo et al. concluded that hypercalciuria and nephrolithiasis associated with sarcoidosis are not related to endogenous calcitriol synthesis. They have also noted a surprising decline in serum 1,25(OH)2D levels after vitamin D replacement. Despite a relatively small sample size, the study included a total of 86 Caucasian and African American sarcoidosis patients, providing a comparative perspective. The results provide valuable information on the role of vitamin D in sarcoidosis, however a relatively short-follow up time, the observational design of the study and the lack of adequate analysis of the correlation with disease activity limit the study's potential as the basis for safe and effective supplementation strategies.[79] Table 3 presents a summary of the studies analyzed, with their main focus and key findings.

**Table 3**. Vitamin D supplementation and hypercalcemia risk in sarcoidosis patients summary

| Study | Focus | Key findings |
|---|---|---|
| Saidenberg-Kermanac'h et al. [65] | Calcium metabolism disorders in sarcoidosis | Vitamin D dysregulation associated with hypercalcemia in a significant subset of patients |
| Kamphuis et al. [75] | Safety of vitamin D and calcium supplementation | Supplementation often led to hypercalcemia and increased 1,25(OH2)D levels detected in susceptible individuals |
| Gwadera et al. [77] | Vitamin D, calcium and phosphate status | Hypercalcemia associated with high 1,25(OH)2D levels, even in mild supplementation cases, an individual approach to supplementation needed |
| Bolland et al. [78] | Vitamin D supplementation randomized controlled trial | Hypercalcemia developed in 11% of supplemented patients |
| Capolongo et al. [79] | Vitamin D and mineral metabolism in sarcoidosis | Hypercalcemia is more frequent in individuals with elevated baseline 1,25(OH)2D |

***Vitamin D supplementation – current guidelines***

Despite increasing knowledge on the pathogenesis of sarcoidosis and the mechanism by which the immune system, vitamin D and calcium metabolisms are dysregulated, there are still no clear guidelines on vitamin D supplementation in sarcoidosis. The available literature underlines the importance of an individual approach to each patient, with an emphasis on appropriate pre-testing. Laboratory tests should precede the decision on vitamin D supplementation in a particular patient. Patients with sarcoidosis who receive vitamin D supplementation should undergo regular assessments not only 25OHD serum levels but also of 1,25(OH)2D serum levels. Serum calcium, urine calcium concentration, and renal function should also be monitored. In each case, supplementation should be adjusted to change sources of cholecalciferol, calcitriol, and calcium, such as diet and sun exposure, individually. Such an approach is advised to minimize the risk of hypercalcemia and its complications.[80]

There is no consensus on the appropriate nor completely safe dose of vitamin D that can be universally recommended to patients. However, starting with low doses (eg 400 800 IU per day), with close monitoring of laboratory and clinical parameters, seems reasonable. High-dose vitamin D supplementation is not recommended as it may cause a hypercalcemic crisis in patients with sarcoidosis.[22,62,68,75,78]

The increased sensitivity to vitamin D and its metabolites among patients with sarcoidosis supports some authors' suggestion that the desirable ranges of 25OHD and 1,25(OH)2D may be lower than those proposed in the general population[65], however, more research on advised reference range is necessary. In their recent review, Gianella et al. extensively discuss the role of vitamin D in sarcoidosis. The authors underscore that despite the potential beneficial effects of vitamin D supplementation on innate immunity in sarcoidosis patients, the possible risks of the intervention have limited the number of prospective clinical trials that address the intervention's efficacy and safety of the intervention.[81]

## Conclusion

The number of potential benefits of vitamin D supplementation in sarcoidosis is highly promising in terms of reducing the inflammatory response, counteracting disease progression, reducing bone fracture risk, and minimizing pharmacotherapy needed for disease control. Despite the increased risk of hypercalcemia in patients with sarcoidosis, vitamin D is not indisputably responsible. The potential risks of hypercalcemia associated with vitamin D supplementation in this group should not outweigh the potential benefits of supplementation, as well as the risk of vitamin D hypovitaminosis. Due to the lack of universal guidelines, each patient's benefits to risks ratio of vitamin D supplementation should be assessed individually and the intervention should be closely monitored both before and during implementation.

## Declarations

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The authors declare no competing interests.

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Not applicable.

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**REVIEW PAPER**

# Implications of labor analgesia on labor outcomes – a systematic review

Manpreet Kaur Tehalia , Shubhra Agarwal , Astha Lalwani

Department of Obstetrics and Gynaecology, Teerthanker Mahaveer Medical College and Research Centre, Moradabad, Uttar Pradesh, India

**ABSTRACT**

**Introduction and aim.** Labor analgesia is a key component in ensuring maternal comfort during childbirth and impacts several maternal and neonatal outcomes. The selection of pharmacological and nonpharmacological analgesic methods significantly affects labor progression, delivery methods, neonatal health, and maternal satisfaction. This systematic review sought to assess the implications of labor analgesia on these outcomes by synthesizing evidence from various study designs.

**Material and methods.** Searches on the following electronic databases comprehensively: PubMed, Scopus, Web of Science, Cochrane Library, Embase, and CINAHL; Using Boolean operators and MeSH terms, six studies were included. These comprised randomized controlled trials, cohort studies, and observational studies that assessed maternal and newborn outcomes in the presence of labor analgesia. Data on types of analgesia, onset times, maternal hemodynamic outcomes, labor durations, delivery modes, neonatal Apgar scores, adverse events and maternal satisfaction were extracted. The exclusion criteria were studies that did not meet the inclusion criteria, such as reviews, editorials, and non-human studies.

**Analysis of the literature.** The analysis involved a wide range of studies employing analgesia methods such as epidural, combined spinal-epidural (CSE), programmed intermittent epidural bolus (PIEB), and non-pharmacological interventions. Ropivacaine (0.1–0.2%) with fentanyl (7.5–25 µg/mL) was the most commonly used combination. The onset times ranged from immediate to 200 minutes for prolonged durations of PIEB. Labor durations were variable. Some techniques, such as peripheral nerve blocks, reduced second stage labor by 33.8 minutes, whereas epidural analgesia prolonged labor duration in some cohorts. The modes of delivery outcomes were characterized by relatively minimal variations in cesarean rates between techniques, while operative vaginal deliveries were more likely with routine epidurals. Neonatal outcomes were otherwise favorable with normal Apgar scores, although some studies reported lower 1 minute Apgar scores with epidurals. Adverse events, such as motor blockade and postdural puncture headaches, were usually technique-dependent and minimal. Maternal satisfaction was high in all methods, with ultrasound-guided CSE, PIEB, and nonpharmacological methods receiving particularly positive feedback.

**Conclusion.** Labor analgesia showed overall safety and efficacy but varied impacts on labor duration, mode of delivery, and neonatal outcomes with the technique used. Although most of them had high maternal satisfaction and stable maternal hemodynamics, some increased operative deliveries or adverse newborn outcomes. These results underscore the importance of tailoring analgesic strategies to individual clinical needs to optimize maternal and neonatal outcomes.

**Keywords.** epidural analgesia, labor analgesia, maternal outcomes, newborn outcomes, patient satisfaction

**Corresponding author:** Astha Lalwani, e-mail: asthalalwani7@gmail.com

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## Introduction

Labor analgesia is today a part of routine obstetric practice to provide relief from pain in labor without risking the safety and welfare of the mother and fetus.[1] Pain in labor results from a multifactorial interaction of physiological processes involving uterine contractions, cervical dilation, and fetal transit through the birth canal, which activate visceral and somatic afferent nerves.[2] Unrelieved, this pain triggers a severe physiological stress response, expressed as increased catecholamine release, increased cardiac output, and hyperventilation, which can adversely affect maternal hemodynamics and fetal oxygenation.[3] Successful control of labor pain, thus, not only provides maternal comfort, but also optimizes maternal and neonatal outcomes.[4]

Among the many pharmacologic alternatives, epidural analgesia is well established as the gold standard for labor pain relief because of its effectiveness and the potential to preserve maternal awareness and activity during delivery.[5,6] The procedure consists of the injection of local anesthetics, usually combined with opioids, into the epidural space, providing focal and prolonged pain relief. Despite its benefits, epidural analgesia is controversial with respect to its possible correlation with adverse outcomes of labor, including prolonged second-stage labor, higher instrumental delivery, and maternal complications, such as hypotension and urinary retention.[7] Other pharmacologic approaches, such as parenteral opioids and nitrous oxide, are employed as alternatives or adjuncts to epidural analgesia, but tend to be less effective and accompanied by side effects such as nausea, sedation, and drowsiness.[8]

Currently, non-pharmacological methods such as transcutaneous electrical nerve stimulation (TENS), hydrotherapy, acupressure, and hypnosis have also become popular, especially in areas where limited medical intervention is desired or pharmacologic options are not available.[9] Although these modalities have demonstrated some effectiveness in alleviating pain and maternal satisfaction, their effect on labor outcomes such as labor duration and mode of delivery is less consistent owing to inconclusive evidence.[10] Additionally, cultural acceptability, access to healthcare, and provider skill also determine the use of pharmacologic versus non-pharmacologic analgesia between and within systems.[11]

In addition to pain relief, labor analgesia is also central to maternal and neonatal health outcomes. Epidural analgesia has been blamed for prolonged second-stage labor and rising rates of assisted vaginal delivery rates, with controversies about its larger implications.[12] Evidence for its link to cesarean delivery remains conflicting, some research attributing increased cesarean rates of cesareans to confounders in the form of maternal obesity, advanced age, and pre-existing pregnancy complications and not the analgesic method itself.[13] In the same vein, neonatal outcomes such as Apgar scores, umbilical cord pH, and the requirement for resuscitation have been the subject of investigation concerning labor analgesia, with evidence yielding conflicting conclusions.[12,13]

Considering the pivotal position of labor analgesia in obstetric practice and its multi-pronged effect on maternal and neonatal outcomes, it is imperative to have a systematic assessment of its effects.

## Aim

The purpose of this review is to integrate the available data on the effect of various analgesic methods on key maternal and neonatal outcomes, such as labor progress, delivery method, maternal satisfaction, and neonatal health indicators.

## Material and methods

### *Inclusion and exclusion criteria*

The inclusion and exclusion criteria for this review were selected with great concern for the selection of relevant and quality studies. Only studies have been considered that have compared the effects of labor analgesia, pharmacological or non-pharmacological on maternal and neonatal outcomes. Only studies with adequate data on labor outcome – duration of labor, mode of delivery, level of maternal satisfaction, neonatal Apgar scores, or any adverse events reported were considered. For this review, RCTs, cohort studies, and observational studies conducted in human subjects who had received analgesia for labor were considered for this review. Studies were excluded if they were reviews, case reports, editorials, or conference abstracts, and if they lacked sufficient outcome data or focused on analgesia in non-labor settings. Also, studies involving nonhuman subjects or those not published in peer-reviewed journals were excluded.

### *PECOS protocol and PRISMA construction*

The PECOS protocol was constructed to align with the systematic approach mandated by the PRISMA 2020 reporting guidelines.[14] The population (P) to be exposed was pregnant women when in labor and delivery. Exposure (E) was any type of analgesia for labor, pharmacological or non-pharmacological. The comparator (C) had no analgesia or alternatives forms of analgesia. Outcomes (O) encompassed maternal outcomes such as time elapsed by labor, delivery method as perceived by the mother, and others. Neonatal results including Apgar scores and adverse events that follow. The eligible study design (S) included RCT, cohorts, and case series / case control studies.

### *Protocol for database search*

Six electronic databases were searched to find relevant studies for this review, PubMed, Scopus, Web of Sci-

ence, Cochrane Library, Embase, and CINAHL. The concept was further refined by having the first set of terms of all the databases using Boolean operators and MeSH terms. Some of the concepts that had been used were "labor analgesia", "pain management in childbirth", "maternal outcomes", "neonatal outcomes," and "epidural analgesia" with Boolean terms including AND, OR, and NOT to narrow the searches. Further narrowing was done using other MeSH terms such as "Analgesia, Obstetrical", "Labor, Obstetric," and "Maternal Health". Filters applied for language and type of study, such as RCTs, cohort, and observational studies.

### *Data extraction protocol and data items*

Data were extracted using a structured protocol to ensure consistency and completeness in data extraction. A standardized data extraction form was developed that included identifiers (author, year, journal), study design, population characteristics (age, parity), details of exposure (type of analgesia, administration protocol), details of the comparator, and reported results (labor duration, mode of delivery, maternal satisfaction, neonatal Apgar scores, adverse events). The data were independently extracted by two reviewers, and any discrepancies were resolved by consensus or arbitration by a third reviewer. This process ensured that all relevant data items were captured for subsequent analysis.

### *Bias assessment protocol*

The risk of bias was assessed using the ROBINS-I tool[15] for nonrandomized studies and Cochrane RoB 2.0 tool for RCTs.[16] For ROBINS-I, confounding domains, selection bias, intervention classification and outcome reporting were considered in the evaluation. For RoB 2.0 domains that comprise the randomization process, the deviation of patients from the intended interventions, as well as selective reporting, were studied. In all of these areas, risk was considered low, moderate or high risk, and general bias was applied to all of them.

### *Protocol for sensitivity analyses*

Sensitivity analyzes were conducted to test the robustness of the review findings. The analyzes included exclusion of studies at high risk of bias, excluding study design-for example-and checking for the effects of key variables such as maternal age and parity. Subgroup analyzes were performed by type of analgesia used, for instance, pharmacological versus non-pharmacological, to explore the heterogeneity in outcomes.

## Results

The process of selecting the studies was in accordance with the PRISMA guidelines, which included the stages of identification, selection, eligibility, and inclusion stages (Fig. 1). In the initial step, 451 records were identified from databases such as PubMed (81), Embase (91), Scopus (93), Web of Science (53), Cochrane Library (57), and CINAHL (76). After eliminating 58 duplicate records, the search was performed for 393 unique records. No records were excluded in the screening stage and a total of 393 reports were recovered for assessment. Of these 355 reports, 38 were not recovered. Of the remaining 355 reports, a total of 343 were excluded because they did not meet the PECOS criteria (86), were off topic (74), literature reviews (59), these articles (66), or editorials (58). In addition, 121 records identified through other methods (websites, organizations, citation search) were screened, and 28 were not retrieved. Among the 93 evaluated reports, 52 were excluded as in vitro studies and 41 as animal studies. Finally, 12 new studies [17-28] were included in the review.

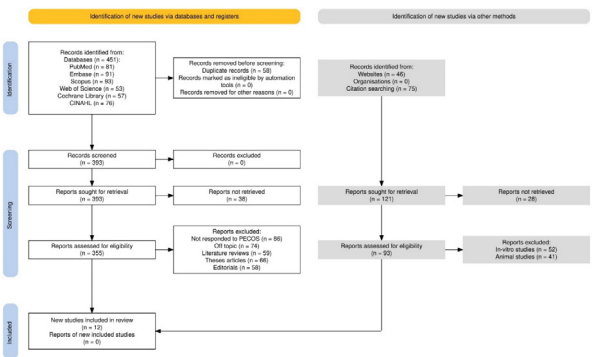


**Fig. 1.** Representation of the study selection process for this review

### *Baseline variables assessed*

The included studies spanned various years, from 2014 to 2024, highlighting a diverse temporal range (Table 1).[21,23,27] From a geographical point of view, they represented studies performed within different countries: South Korea, USA, Australia, Brazil, Poland, Taiwan, Malaysia, Netherlands and China.[17-28] The study designs were quite diversified; most of the studies were RCTs focusing on methodological rigor.[17,19,20,23,24,26,28] Other designs included retrospective observational studies, retrospective cohorts, controlled before and after cohorts, prospective sampling, and a non-inferiority trial.[18,21,22,25,27] The sample sizes varied greatly, from as few as 71 participants to 16,852, reflecting a wide scope of investigation.[22,28] The mean age of participants also differed between studies, ranging from 21.5 years to the oldest mean age at 34.25 years, which implies that there was a wide demographic range of pregnant women.[17,20] Mean age was not reported in one study.[27] Follow-up periods ranged from immediate postpartum periods to longer durations as six weeks postpartum, or until certain milestones: neonatal outcomes, or discharge from the maternity unit.[17-21,23,25]

**Table 1.** Demographic characteristics of the included studies

| Study ID | Year | Country | Study design | Sample size | Mean age (in years) | Follow-up Period |
|---|---|---|---|---|---|---|
| Bae et al [17] | 2023 | South Korea | Randomized controlled trial | 84 | 34.25 | Immediate postpartum |
| Bullingham et al [18] | 2018 | Australia | Controlled before-and-after cohort | 397 | 28.1 | Immediate postpartum |
| Cahill et al [19] | 2018 | USA | Randomized controlled trial | 2414 | 26.5 | 6 weeks postpartum |
| Gallo et al [20] | 2018 | Brazil | Randomized controlled trial | 80 | 21.5 | Discharge from maternity unit |
| Hincz et al [21] | 2014 | Poland | Retrospective observational | 5593 | 30.2 | Neonatal outcomes |
| Hung et al [22] | 2015 | Taiwan | Retrospective cohort | 16852 | 29.5 | Perinatal period |
| Kim et al [23] | 2024 | South Korea | Randomized controlled trial | 85 | 33 | Immediate postpartum |
| Sharawi et al [24] | 2023 | USA | Randomized controlled trial | 140 | 30.1 | Postoperative day 1 |
| Sra et al [25] | 2016 | Malaysia | Prospective sampling | 110 | 28.7 | Immediate postpartum |
| Tan et al [26] | 2022 | USA | Double-blind randomized controlled trial | 132 | 29.5 | Delivery and postpartum day 1 |
| Wassen et al [27] | 2014 | Netherlands | Randomized non-inferiority trial | 488 | Not reported | Not specified |
| Xu et al [28] | 2020 | China | Randomized controlled trial | 71 | 27.5 | Until delivery |

***Sample type and type of analgesia***

The included studies included various types of samples (Table 2), such as comparing ultrasound-guided techniques with palpation-guided techniques, epidural analgesia against controls or dural puncture against standard epidural techniques.[17,21,22,24,26] The types of analgesia were also vastly different, including combined spinal-epidural (CSE), neuraxial analgesia, dural puncture epidural, and programmed intermittent epidural bolus (PIEB).[17,19,23-26] Other comparator was non-pharmacological interventions that include massage, exercise and showers.[20] Such heterogeneity occurs as these strategies and their application of analgesia labor is diversified.

***Dose and regimen***

The dose and regimen varied according to the type of analgesia. Ropivacaine 0.1–0.2% was frequently used with fentanyl 7.5–25 μg/mL, whereas other studies administered boluses of chloroprocaine 15–20 ml.[22-26] Other studies utilized standard protocols without detailing the dosages.[17,18] This variability highlights institutional differences in the practices and preferences of labor analgesia.

***Time to onset of analgesia***

The onset of analgesia varied from immediate starts to more delayed onsets as 30–60 min after catheter insertion or 422 seconds after dural puncture epidurals.[18,22,24,28] PIEB had more protracted onset times, with the technique taking 200 minutes to begin.[23] CSE had quick onset.[25] The discrepancies between the onset times require the choice of technique be made based on the urgency of the clinical procedure and the needs of each individual patient.

***Labor stage at onset***

The onset of analgesia occurred at different stages of labor. Most interventions started in active labor, with thresholds for cervical dilation ranging from 2 cm to 4–7 cm.[20,27] Dural puncture epidurals were started during presurgical anesthesia, while others targeted the second stage of labour.[19,24,28] The heterogeneity is a reflection of the flexibility of analgesia strategies across the labor stages.

***Maternal hemodynamic outcomes***

Most studies demonstrated stable maternal hemodynamics without significant hypotension.[17,22,23,26] However, with routine epidural analgesia, increased hypotension (9.5% difference).[27] PIEB produced significantly reduced motor blockade compared to continuous infusion techniques.[18] Therefore, most of the analgesic methods show a high safety profile with minimal occurrence of hemodynamic complications.

***Length of labor***

The effects of analgesia on the duration of labor varied. Some techniques, such as peripheral nerve block (PNB), reduced the duration by 33.8 minutes, while nonpharmacological interventions led to an 18-minute reduction.[20,28] Conversely, epidural analgesia prolonged both the first and second stages of labor in some cohorts.[21,22] PIEB was associated with shorter second stages compared to continuous epidural infusion.[18]

***Mode of delivery***

The mode of delivery results showed little variation in the rate of cesareans in most techniques.[17,26] There were increased operative vaginal deliveries among routine epidural groups and nulliparous groups who received epidural analgesia.[22,27] Nonpharmacological interventions greatly reduced operative deliveries.[20] Therefore, these results indicate that although analgesia tends to promote vaginal delivery, certain methods would increase assisted delivery rates.

***Neonatal outcomes***

The newborn results were generally positive in all studies, with normal Apgar scores reported in most cases.[17,20,23,24,26,28] However, epidural analgesia was associated with lower 1-minute Apgar scores and increased risk of low cord pH in some cohorts.[21,22] Therefore, the results

**Table 2.** Outcomes related to labour analgesia as observed in all included trials

| Study ID | Sample type assessed | Type of analgesia | Dose and regimen | Onset time of analgesia | Labour stage at initiation | Maternal hemodynamic outcomes | Duration of labour | Mode of delivery | Neonatal outcomes | Adverse events | Patient satisfaction |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Bae et al [17] | Ultrasound-guided vs Palpation-guided CSE | Combined Spinal-Epidural (CSE) | Not specified; standard CSE protocols | 134.5 seconds in ultrasound group | Active labour (dilation ≥3 cm) | Stable; no significant differences between groups | No significant difference in total duration | No significant difference in cesarean rates | No significant differences; normal Apgar scores | None significant; fewer dural punctures in ultrasound group | Higher in the ultrasound group (median 10/10) |
| Bullingham et al [18] | CEI vs PIEB + PCEA | Epidural analgesia | CEI: Ropivacaine 0.2% + Fentanyl; PIEB + PCEA: Ropivacaine 0.1% + Fentanyl | 30-60 minutes post-catheter insertion | First stage | Stable with reduced motor block (21.8% vs 1.0%) | Second stage in PIEB + PCEA group (69.4 min vs 89.1 min) | No significant difference | No significant difference reported | Lower motor block with PIEB + PCEA | No significant difference between groups |
| Cahill et al [19] | Immediate vs Delayed Pushing | Neuraxial analgesia | Standard neuraxial protocols | Not reported | Second stage | Stable | Shorter in the immediate push group (102.4 min vs 134.2 min) | Similar spontaneous vaginal delivery rates (85.9% vs 86.5%) | Similar composite rates of neonatal morbidity rates (7.3% vs 8.9%) | Lower chorioamnionitis and postpartum hemorrhage in immediate group | High satisfaction reported |
| Gallo et al. [20] | Nonpharmacological Interventions vs Standard Care | Non-pharmacological techniques (massage, exercise, shower) | Not applicable (non-pharmacological) | Not applicable | 4 to 7 cm dilation (stage-specific interventions) | Not measured | 18 minutes faster in the experimental group (95% CI 5-30) | Reduced operative delivery in experimental group | Improved neonatal outcomes in experimental group | None reported | Higher satisfaction in experimental group |
| Hincz et al [21] | EA vs Control | Epidural Analgesia | Protocol not specified | Not reported | First stage (≥37 weeks) | Increased labor augmentation (EA group) | Prolonged first stage with EA | Higher forceps delivery rate with EA | Lower 1-min Apgar score with EA | Increased risk of low cord pH with EA | Not assessed |
| Hung et al [22] | Nulliparous vs Multiparous with EA | Epidural Analgesia | Ropivacaine 1mg/mL + Fentanyl 7.5 µg/mL | Immediate | First stage | Stable, no significant hypotension | Prolonged first and second stages (EA group) | Increased operative vaginal delivery in nulliparous | Higher rate of 1-min Apgar <7 in EA group | None significant beyond study-defined outcomes | Not explicitly reported |
| Kim et al [23] | PIEB vs Continuous vs Manual | Programmed Intermittent Epidural Bolus | Ropivacaine 0.2% + fentanyl 20 µg | PIEB: 200 min | Active labor (2-5 cm dilation) | Stable | Prolonged interval to breakthrough pain | Mixed (vaginal and cesarean) | Normal Apgar scores | None significant | High |
| Sharawi et al[24] | DPE vs Standard Epidural | Dural-puncture epidural | Chloroprocaine 15-20 mL | 422 seconds | Pre-surgical anesthesia | Stable | Not applicable (cesarean) | Cesarean delivery | Normal Apgar scores | Minimal (e.g., PDPH) | High |
| Sra et al [25] | CSE vs Non-CSE | Combined Spinal Epidural (CSE) | Ropivacaine 0.2% + Fentanyl 25 µg | Rapid onset with CSE | Active labour (3-4 cm dilation) | Stable, no reported hypotension | No significant differences | Similar between groups | Similar Apgar scores between groups | Pruritus is most common in CSE group | High satisfaction with CSE |
| Tan et al [26] | Dural Puncture Epidural vs Standard Epidural | Dural Puncture Epidural | 0.1% Ropivacaine + 2 µg/mL Fentanyl | Within 30 minutes | 2 to 7 cm dilation | Stable with no significant hypotension | No difference in second-stage duration | No significant differences | No significant differences; normal Apgar scores | No significant adverse events reported | High satisfaction in both groups |
| Wassen et al[27] | Routine EA vs Analgesia on Request | Epidural analgesia | Routine EA: Continuous infusion of Ropivacaine/ Bupivacaine + Sufentanil | Immediate upon administration | Active labor (cervical dilation ≥2 cm) | Increased hypotension (difference of 9.5%) | No significant difference reported | Higher operative deliveries in the routine EA group (34.8% vs 26.7%) | No significant differences in Apgar scores or NICU admissions | Higher motor blockade with routine EA (6.8% difference) | Not explicitly reported |
| Xu et al [28] | PNB vs Control | Epidural + PNB | 0.25% ropivacaine 10 mL per side | Immediate | Second stage | Stable | Reduced by 33.8 min | Vaginal delivery | Normal Apgar scores | None significant | High |

show that, in general, the newborn is safe from analgesia methods but sometimes has adverse effects.

### *Adverse events*

The frequency of significant adverse events is small. Common issues involved include pruritus with CSE, postpartum dural puncture headaches with dural puncture epidurals, and motor blockage with routine epidurals.[24,25,27] Motor block was substantially lower in PIEB than it was for continuous infusion.[18] Thus, adverse events generally occur infrequently and seem to depend on technique.

### *Patient satisfaction*

Patient satisfaction was consistently high in all studies, with ultrasound-guided CSE and PIEB receiving particularly positive responses.[17,18,25] Non-pharmacological interventions also led to greater satisfaction than standard care.[20] This reflects the effectiveness of these techniques in meeting maternal expectations during labor.

### *Bias levels observed*

The bias assessment across the included studies was analyzed using domain-specific evaluations for each study type. For RCTs, bias levels across seven domains (D1-D7) were determined to show heterogeneity in methodological rigor (Fig. 2). Bae et al. and Xu et al. were assessed as having a moderate overall risk due to moderate concerns in specific domains such as D3 and D6, respectively.[17,28] Cahill et al. Gallo et al., and Kim et al. are in general low risk.[19,20,23] Sharawi et al. and Tan et al. had low overall risk but presented moderate risks in specific domains.[24,26] Wassen et al. had moderate overall risk mainly due to serious concerns in D4.[27]

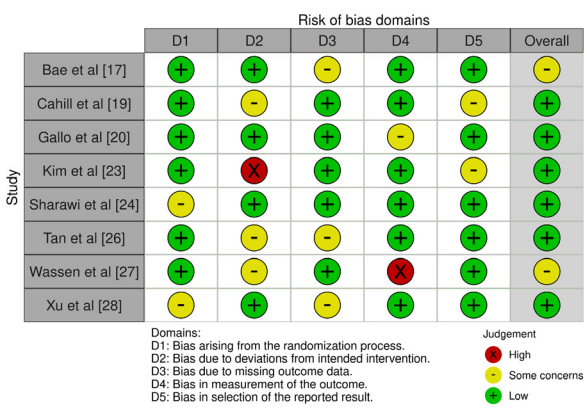


**Fig. 2.** Bias levels assessed across the RCTs included in the review

In cohort studies, the bias assessments were equally mixed (Fig. 3). Bullingham et al. were rated with moderate overall risk mainly due to moderate concerns in D2 and D6.[18] Hincz et al. and Hung et al. were rated with low general risk with consistent low ratings in most of the domains except with moderate concerns in D1 and D5 for Hincz.[21,22] Sra et al. scored with low general risk even though there were serious concerns on D2 with moderate concerns on several of the domains.[25]

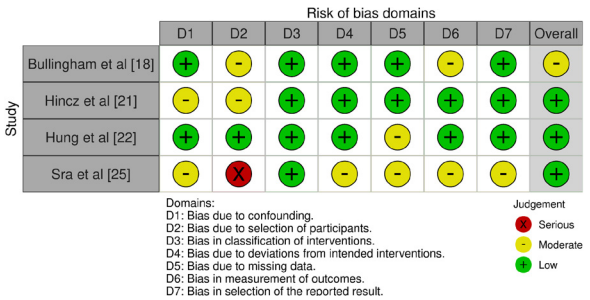


**Fig. 3.** Bias levels assessed across the cohort studies included in the review

### *Sensitivity analyses observations*

A sensitivity analysis was performed to evaluate the robustness of the findings in all the studies included in the review. Study designs, sample sizes, analgesia techniques, and results were all taken into account to draw conclusions from the analysis. RCTs, such as those of Bae et al. Cahill et al., Kim et al., and Sharawi et al., comprised the bulk of the database, ensuring methodological stringency, while observational and cohort studies, such as those by Hincz et al. and Hung et al. offered additional context of real-world variability.[17,19,21-24] The trial by Hung et al. with 16,852 participants, had more weight in results concerning epidural analgesia but also reported longer duration of labor and higher operative deliveries.[22] Conversely, smaller trials such as Bae et al. with 84 participants and Gallo et al. with 80 participants, were successful in reporting benefits from ultrasound-guided CSE and nonpharmacological techniques with a higher level of maternal satisfaction and decreased complications during the procedure.[17,20] Although the size of the subjects was small, consistency of their findings across multiple parameters assured their reliability.

The nature of analgesia profoundly affected both maternal and neonatal outcomes. The epidurals differed significantly in hemodynamic stability and labor progression among the continuous infusion, PIEB, and dural puncture epidurals. Bullingham et al. and Kim et al. found consistent reductions in motor block and second-stage duration with PIEB by Bullingham et al. and Kim et al., whereas routine epidural techniques, as described by Wassen et al., were associated with increased operative delivery rates and maternal hypotension.[18,23,27] Gallo et al. and Xu et al., respectively, in which consistent benefits were derived in reducing operative delivery rates and enhancing patient satisfaction, thus agreeing with pharmacological methods in the context of neonatal safety.[20,28] The analysis has also considered varying follow-up periods that range from immediate postpartum in Bae et al. and Kim et al. to

six weeks in Cahill et al.[17,19,23] The long follow-up period in studies such as those of Cahill et al. enabled maternal morbidity to be assessed, thereby showing fewer complications in the postpartum period with immediate pushing during neuraxial analgesia.[19] Studies with

## Discussion

### *Impact of labor pain on maternal and neonatal outcomes*

The pain experienced during labor can exert multiple deleterious impacts on the mother. For example, pain can trigger the physiological stress response, disrupt uterine contractions, extends the time taken to complete labor, and contributes towards mental health disorders such as post-traumatic stress disorder or depression postdelivery.[1-4] Labor pain also has been reported to play a very significant role in the overall increasing rates of Caesarean delivery rates around the world.[28-29] The effects of uncontrolled labor pain could be transmitted to neonates and can result in complications such as neonatal hypoxia, metabolic acidosis, cognitive and emotional developmental problems, and even death in extreme situations.[30-33]

Our review offers a new and extensive synthesis of labor analgesia methods, with a focus on their effect on maternal and neonatal outcomes. It is the first to compare various methods, including pharmacological methods such as CSE, PIEB, and dural puncture epidurals, with non-pharmacological methods such as massage and hydrotherapy.[17,18,20,23,25] In contrast to earlier research, this review discusses variations in dosage regimens, including ropivacaine-fentanyl combinations and chloroprocaine boluses, and institutional and practitioner-specific preferences.[22-25] It also discusses differences in time to onset and stage of labor at initiation, offering key insights into the flexibility of analgesia strategies to clinical urgency and patient needs.[20,24,27]

The findings also highlight the new advantages of newer methods such as PIEB, which reduces motor block and hypotension compared to continuous epidural infusions.[18,27] The research also measures the extent to which non-pharmacological methods decrease operative delivery rates and improve maternal satisfaction, providing significant alternatives to scenarios where little medical intervention is desirable.[20] Also highlighted is neonatal safety, where most methods depict favorable results, while some procedures such as routine epidurals are questioned due to their suspected association with reduced Apgar scores and cord pH alteration.[21,22]

### *Role and implications of epidural labor analgesia (ELA)*

ELA is the most widely used method to treat labor pain.[30] It effectively blocks the nociceptive signaling pathways and reduces maternal stress response.[15,16] Emerging evidence suggests that ELA can play a positive role in reducing the risk of postpartum depression and could probably reduce the risk of long-term depressive disorders.[31-35] The impact of ELA on neonatal outcomes, lactation, and long-term neurodevelopmental pathways is also being explored.[32-36] Findings in such areas are not conclusive enough and warrant further research to establish clarity on the long-term implications associated with this widely used analgesic approach.

### *Findings from included studies in this review*

The findings of the studies included in this review showed similarities and differences in the implications of labor analgesia on the outcomes of both the mother and neonate. The main reason for the variability was the variation in the methods, regimens, and populations used for the studies. Several studies, including Bae et al., Xu et al., and Sra et al. demonstrated that ultrasound-guided CSE and peripheral nerve blocks improved maternal outcomes, reducing complications from procedure and length of second-stage labour.[17,25,28] This was consistent with what Gallo et al. showed regarding operative deliveries and neonatal results that were lower compared to the group receiving pharmacological interventions.[20] However, Hincz et al. and Hung et al. pointed out that epidural analgesia was protective against cesarean delivery, but had the disadvantages of prolonged labor durations and increased operative vaginal deliveries, demonstrating that different results were found based on the type of analgesia.[21,22]

### *Maternal hemodynamic stability*

Regarding hemodynamic stability, most reports indicated that there was no statistically significant hypotension from studies such as Sharawi et al. Tan et al., and Kim et al.[23,24,26] However, Wassen et al.[27] reported increased maternal hypotension with routine epidural techniques, which means that there is some divergence in safety profiles.[27] PIEB, evaluated by Bullingham et al.,[18] had significant decreases in motor blockade from start to termination compared to a continuous infusion, as previously found and discussed in improved safety and efficacy such as in Sra et al.[25] Results of neonates were relatively good across most studies and were frequently given normal Apgar scores similar to studies such as Cahill et al., Sharawi et al., and Xu et al.[19,24,28] However, there was a trend for higher risks of adverse neonatal outcomes, including lower 1-minute Apgar scores and increased risk of low cord pH, in some cohorts receiving epidural analgesia, as reported by Hincz et al. and Hung et al.[21,22] These differences underscore the importance of careful technique selection with appropriate consideration to maternal and neonatal safety considerations.

### *Adverse events and patient satisfaction*

Adverse events were generally minimal but technique-dependent. PIEB and non-pharmacological

methods had lower incidences of complications, as highlighted by Bullingham et al. and Gallo et al.[18,20] Conversely, dural puncture epidurals (Sharawi et al.[24]) and routine epidurals (Wassen et al.[27]) were associated with specific issues such as headaches after puncture and motor blockade, underscoring the importance of method-specific evaluations. Patient satisfaction was uniformly high for all techniques, most favorable responses to ultrasound-guided CSE by Bae et al., PIEB by Kim et al., and non-pharmacological interventions by Gallo et al.[17,20,23] Thus, this uniform satisfaction may suggest that most of the techniques do meet the expectations of their patients, although clinical results might vary.

***Comparison with other reviews***

The findings of the studies presented in this review show a number of tangential similarities, as well as differences with the other reviews conducted in this same regard.[37-42] Both our review and the studies by Halliday et al., Callahan et al., and Lu et al. recognized epidural analgesia (EA) as the most effective and widely used method for pain relief during labor.[37,40,42] The conclusions regarding the relationship of EA with the duration of first and second stages of labor were concurred in with the results reported by Callahan et al. and Lu et al., where a similar prolongation was observed in our results.[40,42] Also, the negative finding on increased rates of cesarean delivery after EA agreed with the conclusion reported by Callahan et al.[42] As reported by Liu et al., the decreased post-labor maternal depressive symptoms correlated well with our review's observations that techniques such as PIEB and non-pharmacological interventions have high maternal satisfaction levels.[4] Furthermore, the observation of transient maternal hypotension with EA, which was

Both our review and that of Guasch et al. noted advantages of CSE techniques: faster onset of analgesia with minimal effect on neonatal Apgar scores, ensuring their efficacy over standard EA.[39] Although our review identified an association of specific epidural techniques with an increased incidence of operative vaginal delivery, Callahan et al. noted that improvements such as low-concentration local anesthetics and PIEB have reduced this risk; more recent studies have failed to demonstrate a significant difference between EA and nonepidural analgesia.[42] This may represent a change in practice regarding the use of anesthesia. Unlike our results, where neonatal outcomes such as 1-minute Apgar scores and cord pH were sometimes affected, Callahan et al. and Liu et al. repeatedly reported no neonatal adverse effects and better acid-base status was found in neonates whose mothers received EA.[41,42]

Lu et al. and Liu et al. highlighted the elevated risk of intrapartum maternal fever with EA that was identified less often in our summary.[40,41] This is likely due to differences in the study population, the definition used for fever, or the type of analgesia applied. While Halliday et al. have pointed out significant heterogeneity in epidural technique research and a lack of standardized outcome reporting, our review has focused on synthesizing findings across diverse study designs and methods. This divergence points to a methodological limitation in both bodies of research.[37]

***Limitations of the review***

Although systematic, has a number of limitations that must be acknowledged. First, the included studies were highly heterogeneous with respect to sample populations, analgesia methods, doses, and outcomes, which could reduce generalizability of the results. For example, variation in institutional practice and regional taste dictated the selection and delivery of analgesia, resulting in heterogeneity of the results. Second, the heterogeneity in the timing of onset of analgesia, from the initiation of labor to the second stage of labor, complicates the comparison across studies. Third, some studies did not report high levels of detail on dosing regimens or information, which can affect the interpretation of efficacy and safety profiles of particular methods. An additional limitation was the reliance on various study designs, e.g., RCTs and observational studies. Although this allows for more richness in the analysis, it generates potential for bias because observational studies are more vulnerable to confounding. Furthermore, some of the included RCTs had small numbers, which could decrease statistical power to identify significant differences or rare adverse effects. Although sensitivity analyzes were performed to try to minimize these issues, the strength of evidence from larger trials could overbalance valuable findings from smaller trials. Brief follow-up intervals in most studies also constrain the ability to evaluate long-term maternal and neonatal outcomes, e.g. postpartum complications or neurodevelopmental impact. The absence of consistent outcome measures, especially for patient satisfaction and maternal hemodynamics, is also a problem in synthesis and comparing across studies. Furthermore, although bias evaluation was undertaken, moderate risks of bias in certain domains, especially in observational studies, remind us to be cautious when interpreting the results.

***Recommendations for future research and implications***

The focus of future research should be placed on multicenter trials with standardized protocols for reducing variability in methods and dosages of analgesia. Further studies would be required to explore the long-term outcomes of mother and newborn resulting from differing analgesia techniques, especially in the population or clinical settings. Integration of patient-reported outcomes, including satisfaction and quality of life, would be beneficial in offering a more holistic understanding of the effectiveness of labor analgesia. Other studies on cost-effectiveness

may be useful to inform the use of resources in clinical practice. Lastly, filling gaps in the evidence for non-pharmacological interventions could be useful in the exploration of alternative approaches to managing labor pain.

## Conclusion

This systematic review was shown to have generally effectiveness and safety, with a high level of maternal satisfaction and favorable neonatal outcomes for labor analgesia. However, the effects of labor duration, mode of delivery and maternal or neonatal adverse events were quite varied depending on the type of analgesia used. Although non-pharmacological techniques and PIEB had benefit in reducing operative deliveries and diminishing adverse events, routine epidural techniques were associated with an increased incidence of maternal hypotension and prolonged labor in some cases. Therefore, it calls for the tailoring of analgesic approaches to specific clinical needs and maternal preferences.

## Declarations

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***Author contributions***

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

***Conflicts of interest***

The authors have no conflicts of interest to declare.

***Data availability:***

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

***Ethics approval***

Not applicable.

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**REVIEW PAPER**

# Endogenous and exogenous factors influencing anti-Müllerian hormone levels in women of reproductive age

Olga Jankowska [1], Wojciech Kraśnik [1], Jacek Kurzeja [1], Katarzyna Piotrowicz [2], Hubert Piotrowicz [2], Agnieszka Bajkacz [3], Anna Rogala [4], Joanna Osmólska [4]

[1] J. Strus City Multispecialty Hospital, Poznań, Poland
[2] University Clinical Hospital in Poznan, Poznań, Poland
[3] University Hospital in Wroclaw, Wrocław, Poland
[4] Poznan University of Medical Sciences, Poznań, Poland

**ABSTRACT**

**Introduction and aim.** Anti-Müllerian hormone (AMH) is a key marker of ovarian reserve (OR), which declines with advancing reproductive age. Certain pathological conditions can reduce OR or lower AMH levels independently of age, potentially impairing fertility. This study aims to review the scientific literature on factors influencing AMH levels and the causes of diminished OR, including lifestyle, diet, supplementation, pathogenic factors, environmental influences, and genetic predispositions.

**Material and methods.** This review was conducted using electronic databases, including PubMed and Google Scholar. A comprehensive search was carried out across these databases, covering the period from 2007 to 2024. The inclusion criteria encompass studies on AMH and factors influencing ovarian reserve, that present either quantitative or qualitative data.

**Analysis of the literature.** The most important factor determining the level of AMH is age. In addition, factors that may influence hormone levels include genetic background, autoimmune diseases, polycystic ovary syndrome (PCOS), environmental toxins, diet, supplementation, oral contraception, physical activity, and smoking.

**Conclusion.** The causes of reduced OR and abnormal AMH levels remain unclear in many cases. Recommendations for the prevention of pathologically reduced OR include lifestyle modifications, a diet rich in antioxidants, avoiding toxins, refraining from smoking, appropriate supplementation, genetic testing, and regular blood tests.

**Keywords.** anti-Müllerian hormone, fertility, ovarian reserve, reproduction

## Introduction

### *Anti-Müllerian hormone*

Anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting substance, is produced by the gonads-Sertoli cells of the testes and granulosa cells of the ovaries.[1-3] Its name derives from its role during fetal development, where it induces the regression of Müllerian ducts in male fetuses.[2,3] Anti-Müllerian hormone is a glycoprotein belonging to the transforming growth factor β superfamily.[2,3] It is an important regulator of specific stages of folliculogenesis.[2] During reproductive maturity, AMH is produced by granulosa cells of primary, preantral, and small antral follicles. The highest concentration of AMH is observed in small antral follicles, while its expression is absent in atretic follicles.[2,3] Anti-Müllerian hormone production in female fetuses is detected in the granulosa cells of follicles starting around the 23rd week of fetal development.[4] The hor-

**Corresponding author:** Olga Jankowska, e-mail: jankowskaolga.jo@gmail.com

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mone level gradually rises until roughly the age of 25, after which it generally decreases with age due to the diminishing follicle pool until menopause, when it becomes undetectable.[1-4] The annual decline in AMH levels in healthy women aged 20 to 50 years is estimated to be approximately 5.7%.[1] However, AMH concentrations and the rate of decline are highly individual.[1] Research suggests that the rate of AMH decline could help predict the onset of menopause, regardless of baseline AMH levels or age.[5] The prediction of menopause timing could be improved by multiple AMH measurements. This approach may help identify women at risk of early menopause.[5] However, some studies suggest that using AMH measurements for this purpose is imprecise and, therefore, not recommended.[6] Most studies indicate that AMH levels remain relatively stable throughout the menstrual cycle and do not significantly vary between cycles.[2,3] Therefore, AMH measurements are recommended regardless of the menstrual cycle phase. Although some literature reports fluctuations, these are not considered clinically significant enough to justify AMH testing during specific menstrual phases.[3,4] Notably, there are pathological conditions in which AMH levels do not correlate with ovarian reserve (OR).[7] AMH concentrations may be falsely elevated or reduced due to follicular arrest at specific stages rather than the total pool of primordial follicles.[7] Clinically, AMH is useful in the diagnosis of polycystic ovary syndrome (PCOS) and primary ovarian insufficiency (POI). It is a key tool in assisted reproductive technologies (ART), aiding in the selection of the most appropriate procedure and predicting ovarian response to stimulation.[2,3,4,8]

### *Ovarian reserve*

Ovarian reserve refers to the reproductive potential of the ovaries, defined as the number of primordial ovarian follicles capable of maturing into viable oocytes.[9,10] It is genetically determined and declines with age. As the ovaries age, the follicular pool diminishes, oocyte quality decreases, and chromosomal abnormalities of the oocytes and miscarriage rates increase.[3,9] Clinicians consider several ovarian reserve tests, including follicle-stimulating hormone (FSH), estradiol (E2) and inhibin B levels on the third day of the menstrual cycle, AMH levels, antral follicle count (AFC) via ultrasound during the early follicular phase (days 2–5 of the cycle).[9] Among all the tests, serum AMH and AFC are the most reliable indicators of the ovarian pool.[11] Diminished ovarian reserve (DOR) refers to a reduced quantity and quality of oocytes in the ovaries. It affects nearly 10% of women seeking infertility treatment.[8] When DOR occurs at a young age, it often involves a reduced follicle count while maintaining normal oocyte quality. It remains unclear whether the reduced follicle pool results from an initially lower count or accelerated depletion due to excessive atresia. Anti-Müllerian hormone levels can help identify patients with DOR, even when menstrual cycles are regular and FSH levels are not yet elevated.[3] Signs of DOR include shorter menstrual cycles and lower estrogen levels, which may cause symptoms such as hot flashes, vaginal dryness, and night sweats.[10] However, many women with DOR are asymptomatic.[3] Diminished ovarian reserve increases the risk of early reproductive decline, infertility, poor ovarian response to stimulation, suboptimal ART outcomes, and recurrent miscarriage.[8,10] A severe form of DOR is premature ovarian insufficiency/failure (POI/POF), defined as ovarian failure before the age of 40.[10] According to the European Society of Human Reproduction and Embryology (ESHRE), the diagnostic criteria for POI include women under 40, menstrual disturbances lasting at least four months, and elevated FSH levels measured twice, at least one month apart.[2,10,12,13] Distinguishing between DOR and POI is crucial, as women with POI face additional health risks, requiring specialized care. Reduced estrogen levels increase the risk of osteoporosis, coronary artery disease, and psychological disorders such as anxiety and depression.[10,12]

The exact etiology of ovarian dysfunction often remains unknown, with idiopathic causes accounting for 50–90% of cases.[8,10,12] Proposed causes of DOR include genetic, autoimmune, iatrogenic, environmental factors, pharmacological and surgical treatments, infections, and lifestyle-related factors such as stress and nutrition (Fig. 1).[2,8,9,10,12,14] Modifiable factors, including lifestyle, diet, physical activity, and supplementation, are of growing interest. Identifying these modifiable factors to enhance OR and AMH levels is highly desirable and has been the subject of recent observational studies.[8] Raising awareness among women about the factors influencing fertility may facilitate timely diagnosis and help preserve reproductive potential. Currently, therapeutic alternatives for DOR include ovarian stimulation with gonadotropins, in vitro fertilization, and cryopreservation of oocytes, embryos or ovarian tissue[15] In certain cases, OR may be supported by medical treatments, such as ovarian platelet-rich plasma therapy.[16] Although this remains an experimental approach, it is a promising new treatment option.[16] For patients with the poorest prognoses for successful pregnancy, egg donation is often the only viable option, though it may be unacceptable to many patients. Therefore, the search for new therapeutic solutions is highly desirable.[16]

## Aim

The aim of this study is to review the scientific literature on factors influencing AMH levels and the causes of DOR, including lifestyle, diet, supplementation, pathogenic factors, environmental influences, and genetic predispositions. This study seeks to raise aware-

ness among women about the factors affecting ovarian reserve, with a particular focus on high-risk groups at an increased risk of premature fertility decline. It also emphasizes the importance of lifestyle modifications, preventive measures, and the necessity of regular testing to monitor reproductive health.

**Material and methods**

This review was conducted using electronic databases, including PubMed and Google Scholar. Articles were selected based on their relevance to the topic. A comprehensive search was carried out across these databases, covering the period from 2007 to 2024. A thorough analysis of the literature was conducted, with a focus on the most recent articles published within the last five years. However, older studies were also considered if they presented significant findings. The articles were identified using a combination of keywords (in both Polish and English): ovarian reserve, fertility, anti-Müllerian hormone, AMH levels, premature ovarian failure/insufficiency, diet, supplementation, reproduction, environment. The inclusion criteria for this study encompass studies on AMH and factors influencing ovarian reserve, published in English or Polish, that present either quantitative or qualitative data. The exclusion criteria include irrelevant articles, studies with weak methodologies, and research published more than 17 years ago.

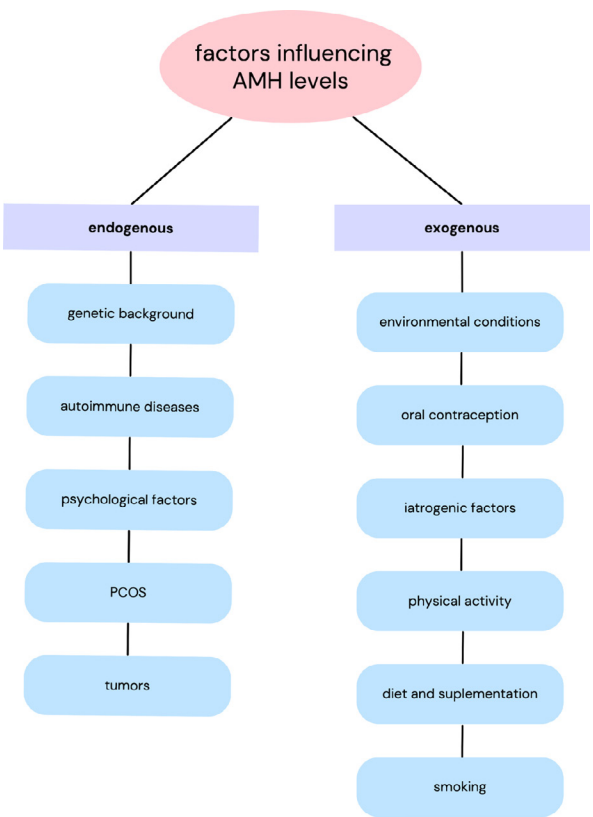


**Fig. 1.** Endogenous and exogenous factors influencing AMH levels

## Analysis of the literature

***Endogenous factors influencing AMH levels and OR***

*Genetic mutations and chromosomal aberrations*

Multiple genetic mutations in both sex chromosomes and autosomes have been linked to POI.[10,15] X chromosome-related defects, including structural abnormalities or aneuploidy, account for the majority of genetic cases of POI.[10,15] The most common associated conditions include: Turner syndrome, fragile X syndrome, and trisomy X.[2,10,15] Women with Turner syndrome may experience follicular loss either prenatally or postnatally, depending on their karyotype. The loss of an X chromosome (45, X) leads to streak gonads with complete follicular depletion, whereas those with mosaicism, such as (45, X/46, XX), may undergo spontaneous puberty.[4,15] Fragile X syndrome is caused by a mutation in the *FMR1* gene (fragile X messenger ribonucleoprotein), one of the most common single-gene defects leading to POI in women with a (46, XX) karyotype.[10,15] The condition is characterized by a cytosine-guanine-guanine (CGG) trinucleotide repeat expansion, and the extent of its impact on OR is correlated with the length of the CGG sequence.[2,10,15]

Other single-gene defects associated with POI include mutations in growth factor genes, such as the *BMP-15* gene (bone morphogenetic protein 15), which plays a role in folliculogenesis and oocyte maturation.[2,10,15] Among autosomal single-gene mutations, the most notable are those affecting transcription factors, such as the *FOXL2* gene (forkhead box L2), which is associated with autosomal dominant blepharophimosis, ptosis, and epicanthus inversus syndrome; the *FIGLA* gene (folliculogenesis-specific basic helix-loop-helix); and the *NOBOX* gene (newborn ovary homeobox).[2,10,15] Another genetic condition linked to POI is classic galactosemia, where over 80% of affected girls develop POI, regardless of adherence to a strict galactose-restricted diet.[10] Although idiopathic POI, by definition, has no identifiable cause, it is believed to have an underlying genetic component.[10] If a standard genetic workup, including karyotyping and known gene mutation testing, is negative, Genome-Wide Association Studies (GWAS) may be employed to identify lesser-known genetic mutations.[10,15] For example, mutations in the *AMH* gene and its receptor gene *AMHR2* (anti-Müllerian hormone receptor type 2), along with defects in transcription factors regulating these genes, have been identified as causes of idiopathic POI.[2]

*Autoimmune diseases*

Autoimmune diseases are more prevalent in patients with POI than in the general population.[4,10,12,15] However, the pathogenesis of autoimmune POI remains poorly understood.[10] The most commonly associated autoimmune disorders include Addison's disease, Hashimoto's thyroiditis, systemic lupus erythemato-

sus, type 1 diabetes, celiac disease, Takayasu arteritis, Behçet's disease, myasthenia gravis, inflammatory bowel diseases, Sjögren's syndrome, and multiple sclerosis.[4,9,10,12,15] Among these, Addison's disease, myasthenia gravis, and autoimmune polyglandular syndrome type 1, which is caused by mutations in the *AIRE* gene, show the strongest correlations with POI.[15] There is a correlation between histologically diagnosed oophoritis and circulating adrenal or anti-ovarian antibodies.[10] However, the diagnostic validity and accuracy of antibody assays remain unestablished, leaving their specificity and pathogenic role uncertain.[10] Although endocrine abnormalities and impaired follicular growth due to autoimmunity have been hypothesized, the precise mechanisms underlying these processes remain unknown and require further investigation.[4,10]

*Psychological factors*

Several studies suggest that psychological well-being and mental stress can significantly impact OR.[3,9,17,18] Anxiety, depression, and other negative emotions have frequently been linked to reduced fertility. Hardy et al.[17] demonstrated that abnormal AMH levels, whether above or below the age-adjusted ranges, were linked to chronic abdominal pain and elevated urinary cortisol levels. Furthermore, serum AMH levels were significantly reduced in female rats exposed to chronic stress.[3,9] Research on occupational factors revealed that women engaged in physically demanding jobs, particularly those involving heavy lifting or night shifts, had lower ovarian reserves.[19] Psychological stress may impact the female reproductive system through the hypothalamus-pituitary-adrenal axis and the sympathetic-adrenomedullary pathway.[18] As a result, growing follicles may be lost due to oxidative damage to ovarian follicular cells, leading to a decrease in AMH levels.[18] It is also important to note that infertility itself represents a significant source of stress for patients, particularly women. Infertility-induced stress can negatively affect the outcomes of fertility treatments.[18]

*PCOS*

Anti-Müllerian hormone concentrations in both serum and follicular fluid are 2 to 4 times higher in women with PCOS compared to healthy counterparts.[2,4] In contrast to healthy women, a significant decline in AMH levels in PCOS patients is only observed after the age of 40.[3] Elevated serum AMH levels in PCOS may result from both an increased number of small antral follicles and AMH overexpression.[2] Studies suggest that this overexpression may be linked to hyperandrogenism.[2,4] Despite elevated AMH levels, histopathological examination of the ovaries in women with PCOS revealed a similar number of primordial follicles compared to the control group.[3]

*Neoplasms*

Anti-Müllerian hormone levels are elevated in 76–93% of women diagnosed with granulosa cell tumors.[3,20] This marker can be detected at an early stage of the disease, even before clinical symptoms appear. Additionally, AMH is a highly sensitive and specific marker for detecting recurrence in patients with folliculomas who have undergone oophorectomy.[3,4,20]

On the other hand, the determination of AMH concentrations in the serum of patients with epithelial ovarian cancer is not considered useful. Studies have examined AMH concentrations in ovarian cancer patients in relation to clinicopathological features, such as the pathological subtype of the tumor, FIGO (The International Federation of Gynecology and Obstetrics) stage, and tumor grading.[20] However, no significant correlations were observed between serum AMH levels and these factors.[20] Additionally, no association was found between serum AMH concentrations and the five-year survival rate.[20]

***Exogenous factors influencing AMH levels and OR***

*Environment*

Organic compounds that are ubiquitous in drinking water, food, food packaging, cosmetics, paints, or on frying pans, as well as other substances, may contribute to a decrease in OR.[9,10,21] Exposure to poly- and perfluoroalkyl substances, known as perfluoroalkyl and polyfluoroalkyl substances (PFAS), leads to a reduction in the number of follicular cells and may contribute to the development of infertility.[9] The effect of PFAS is dose-dependent and results in a decrease in E2 and progesterone levels in serum.[9] The toxicity of 3-monochloropropanediol esters also affects ovarian function by regulating follicular development and increasing the expression of inflammatory factors.[9] Chronic exposure to propylparaben or bisphenol A indicates potential toxicity to the ovaries.[9] Fenvalerate, a pesticide widely used in modern agriculture, inhibits follicle expansion by disrupting steroidogenesis.[9] Recent research has also shown that perfluorooctanoic acid, a substance commonly found in Teflon pans, is elevated in follicular fluid in patients with DOR and affects the composition of follicular fluid.[9] Phthalates, used in cosmetics (cosmetics, toiletries, food packaging), medical products, containers, toys, or building materials, have been identified as endocrine-disrupting chemicals and are considered potential risk factors for POI.[9,10,21] Numerous studies have shown a reduction in the ovarian follicle count after exposure to phthalates in mice of different ages.[10,22]

*Diet*

Some studies suggest that proper nutrition can influence AMH levels, OR, and the timing of menopause. However, these factors remain unclear due to inconsistencies in

findings, and the relationship between diet and OR has not been definitively proven.[1]

The greatest potential is attributed to a diet rich in antioxidants. Increased accumulation of reactive oxygen species (ROS) is one of the better-known causes of ovarian failure and decreased OR.[12,23] It is suggested that unexplained infertility may be caused by an imbalance between the levels of ROS and antioxidants.[24] The increase in free radical production, coupled with a reduced amount of antioxidants leads to oxidative stress.[12,23,24] Therefore, there is growing attention to mitochondrial function in the context of fertility. Mitochondrial dysfunction leads to increased accumulation of free radicals, which amplifies defense mechanisms and may cause apoptosis in granulosa cells and tissue damage, resulting in infertility and even POI.[12,23] The intake of antioxidant compounds, both through nutrition and supplements (described in more detail below), aims to protect against ROS accumulation, inhibit apoptosis, and reduce oxidative stress.[12,23]

In one study by Hu et al.[25] using animal models, protective effects of natural compounds with antioxidant activity, such as phenols, flavonoids, polyphenols, and alkaloids, in POI were demonstrated. Among flavonoids, quercetin received particular attention. It improved the quality of oocytes and embryos by affecting proliferation and apoptosis and reducing oxidative stress in granulosa cells.[25] Treatment with other natural products like icariin, resveratrol, and curcumin significantly increased AMH levels in animal models of POI.[25] Another important antioxidant is vitamin C, which plays a key role in collagen synthesis in the extracellular matrix of the corpus luteum.[24] These results suggest that the intake of antioxidants contributes to the improvement of ovarian function, the restoration of follicle count, and consequently an increase in AMH levels.[24,25]

In a prospective study conducted on women aged 20–50 years, dairy consumption was inversely correlated with the annual decline in AMH levels and a lower risk of rapid AMH decline, regardless of baseline age, body mass index (BMI), and total caloric intake.[1] Additionally, the consumption of berries and the total calcium intake level were inversely correlated with the annual decline in AMH. In this study, other dietary factors were not associated with the rate of AMH decline.[1]

A review by Prieto-Huecas et al.[14] analyzed the impact of nutritional status on OR. A high BMI was correlated with a decrease in OR.[14] Overweight and obesity negatively impact ovarian function. According to the literature review, women with a high BMI exhibit lower AMH and AFC levels compared to those with a normal BMI. Additionally, obesity and overweight may negatively impact oocyte quality, contributing to an increased rate of infertility.[14]

In a cohort study involving 296 premenopausal women (aged 35–45 years), AMH levels were positively correlated with total carbohydrate intake and inversely proportional to total fat intake.[26]

One cross-sectional study involving 234 adult women from an infertility clinic in Iran showed that serum AMH levels were negatively correlated with the consumption of fast food and saturated fats. Additional adjustments for BMI and physical activity did not change the results. This study, however, did not show a significant association between the consumption of fruits, vegetables, dairy products, and salt with AMH levels among adult women without PCOS.[27]

In an animal study, Hohos et al.[28] demonstrated that higher levels of n-3 docosahexaenoic acid, docosapentaenoic acid, and eicosapentaenoic acid in serum were positively associated with the number of primary follicles. It was found that omega-3 fatty acids may contribute to improving OR by reducing inflammation and oxidative stress.[27,28]

*Supplementation*

Supplements that may affect the level of the AMH hormone include coenzyme Q10, vitamin D, hehydroepiandrosterone (DHEA), selenium, and vitamin E. The mechanism of action of these supplements primarily relies on their antioxidant effects.[12,23,24,25,29]

Coenzyme Q10

Coenzyme Q10 (CoQ10) is an essential component of the mitochondrial electron transport chain, playing an important antioxidant role.[29] One study conducted on rat models assessed the protective effect of CoQ10 on ovaries and OR.[23] Cisplatin was used as a source of oxidative stress in ovarian tissue, while CoQ10 acted as an antioxidant.[23] The AFC significantly increased during the use of CoQ10 in combination with cisplatin, while the number of atretic follicles decreased significantly. These findings suggest that CoQ10 may be effective in protecting OR and preventing ovarian damage related to oxidative stress. AMH levels in the group receiving the combination of Cisplatin + CoQ10 were higher than in the group receiving cisplatin alone, but this difference was not statistically significant.[23] It is believed that CoQ10 supplementation may protect the ovaries by improving mitochondrial function, counteracting both mitochondrial and physiological ovarian aging.[23] Another study conducted on women confirmed the positive impact of CoQ10 supplementation among those with reduced OR undergoing gonadotropin stimulation during ART.[29] The intervention in the study group included oral administration of 200 mg CoQ10 three times a day for 60 days. The results showed that CoQ10 improved ovarian response to stimulation, as well as improved oocyte and embryo quality in young patients with poor prognosis and reduced OR.[29]

Vitamin D

Both animal and human studies provide evidence highlighting the significant role of vitamin D in female reproductive physiology.[7] It has been shown that elements in the promoter of the *AMH* gene respond to vitamin D, which explains its effect on *AMH* gene expression.[7,30] Nonetheless, the results of studies investigating the relationship between serum vitamin D and AMH levels are inconclusive.[7,30] It is also possible that vitamin D increases AMH levels without affecting the number of ovarian follicles.[30] However, vitamin D is a relatively safe and affordable supplement, with growing evidence suggesting its potential benefits for multiple aspects of human reproduction including increased pregnancy and live birth rates following ART, as well as a reduced risk of miscarriage and related complications.[7] Vitamin D deficiency causes a 75% decrease in fertility in rats and increases the risk of fetal growth disorders.[7] One study found that AMH levels significantly decreased after vitamin D supplementation in women with PCOS, whereas it significantly increased in women without PCOS.[7] Other studies have demonstrated that, in patients with vitamin D deficiency, supplementation led to a significant increase in AMH serum levels.[30] Dennis et al.[31] examined the effects of high-dose vitamin D supplementation, finding that AMH levels in women receiving vitamin D3 gradually increased over the course of a week. The results confirmed a positive relationship between vitamin D and AMH in healthy young women.[27,31]

Selenium and vitamin E

Selenium and vitamin E are cofactors of antioxidant enzymes, including glutathione peroxidase, and play an important role in removing ROS from the ovaries.[12] Glutathione peroxidase is one of the most important antioxidants preventing ROS production in the ovaries.[12] It has been demonstrated that selenium accumulates in granulosa cells of healthy and large follicles, but is not present in small and atretic follicles.[12] In a study by Delkhorramy et al.[32], plasma selenium levels were found to be lower in patients with idiopathic POI compared to healthy, fertile women.[32] Vitamin E is another vital component of the cellular antioxidant system. Its deficiency accelerates the peroxidation of membrane lipids, leading to faster cell destruction. The antioxidant effects of selenium and vitamin E enhances each other.[12] Vitamin E functions as a cofactor for glutathione peroxidase, and the enzyme's activity is dependent on adequate selenium levels. Therefore, a deficiency in either vitamin E or selenium leads to dysfunction of this enzyme.[12] In a study involving 70 participants, 35 women in the treatment group received 200 mcg of selenium and 400 IU of vitamin E, while 35 women in the control group received a placebo. AMH and AFC levels were measured in both groups after 12 months. Before the intervention, AMH levels did not differ significantly between the groups.. After the intervention, there was a significant increase in AMH levels and the number of antral follicles in the selenium + vitamin E supplementation group compared to the placebo group.[12]

Dehydroepiandrosterone (DHEA)

Studies suggest that women with POI have lower androgen levels compared to healthy women.[33] Conversely, women with higher levels of androgens in the blood tend to have a higher small AFC, suggesting that androgens contribute to their development.[33,34] Research indicates that DHEA supplementation increases AMH in women with DOR. Beneficial effects of supplementation are also observed in terms of AFC, E2, inhibin B, and FSH levels.[33,35] In a rat model of DOR, DHEA administration partially reduced the atresia rate of follicles.[36] Following supplementation, the treated animals showed a markedly increased number of primary and growing follicles compared to the untreated group. Despite androgen supplementation, the follicle count remained lower than in control rats without DOR.[36] Another study demonstrated that four months of DHEA therapy significantly improved OR parameters, such as AMH and FSH levels on day 2 of the cycle.[37] However, no significant improvement in AFC was observed. DHEA therapy in this study improved the hormonal profile of all patients with poor OR, but improvement in fertilization was only observed in patients under 38 years of age.[37] It is suggested that DHEA supplementation may impact OR by stimulating the maturation of primary follicles to pre-antral ones, increasing the expression of androgen and FSH receptors in the ovaries.[33] Supplementation may also improve the number of in vitro embryos in some patients.[33] It is important to note that DHEA supplementation is particularly effective in women with low endogenous DHEA levels. Supplementation of DHEA is probably ineffective if endogenous DHEA levels are normal.[33]

Despite the substantial body of literature on DHEA use in patients with DOR, much of the evidence is still insufficient to draw definitive conclusions.[34] The ESHRE guidelines do not recommend any treatment for premature ovarian insufficiency, aside from oocyte donation, and emphasize the limited scientific evidence supporting the effectiveness of androgen supplementation[13,33] Nonetheless, infertility centers have already started androgen treatment for patients with reduced OR in an attempt to improve reproductive outcomes.[34]

*Contraception*

The scientific literature suggests that contraception may affect AMH levels, although the data remain conflicting. Studies suggest that women using oral contraceptives

(OC) tend to have a lower average AMH level compared to those not using them.[3,4,9] For instance, in a cohort study involving 863 women (228 women using OC and 504 women not using OC, serum AMH levels were 29.8% lower in those using OC compared to those not using them.[38] Nevertheless, the total follicle count, including primary follicles, remained unaffected.[38] AMH levels can still be measured while using hormonal contraception to asses OR, but the findings from this study should be taken into consideration when analyzing results. Measuring AMH during the use of OC may not serve as a fully reliable marker of OR.[3]

*Iatrogenic factors – radiotherapy, chemotherapy, surgical treatment*

Exposure to radiotherapy, chemotherapy, or surgical treatment can lead to a reduction in OR. Cancer treatment, often with gonadotoxic effects, are a known cause of POI.[2,3,12] The majority of studies have demonstrated that AMH levels are not detectable in women who have undergone cancer treatment and received pelvic radiotherapy or chemotherapy with alkylating agents.[2] AMH is considered an invaluable marker of OR in women at risk of ovarian damage from these treatments, enabling preventive measures like cryopreservation.[3] AMH concentration measurements, both before and after chemotherapy, can also help assess the toxic effects of specific chemotherapeutic agents on the ovaries.[3] Cyclophosphamide, a commonly used chemotherapy drug, is particularly well-studied for its negative impact on reproductive health, influencing both the risk of POI and the age of menopause onset.[15,41] Another clinical-control study confirms the hypothesis that exposure to alkylating agents and pelvic radiation is linked to a decrease in OR, as indicated by higher FSH levels and lower AMH and AFC levels compared to the control group.[15,40]

*Physical activity*

While a sedentary lifestyle generally negatively impacts reproductive function, excessive physical activity can also have adverse effects on reproductive health. Studies have shown that women who engage in intense training regularly tend to have lower AMH levels compared to those leading a sedentary lifestyle. The negative impact on AMH levels is more pronounced with longer durations of intense sports activity and more frequent weekly training sessions. In contrast, moderate physical activity, such as brisk walking or cycling, is considered optimal for maintaining reproductive health and supporting healthy AMH levels.[26]

*Smoking*

Smoking is widely recognized as one of the most significant risk factors for reduced OR and premature menopause.[3,12,15,21,26] However, not all studies confirm this relationship.[26] For instance, a cross-sectional study by Dölleman et al.[42] suggested that smoking is associated with lower AMH levels, regardless of the dose, though this effect appears to be reversible.[26,42] In a retrospective study, Barrier et al.[43] found that AMH levels were significantly lower in smokers compared to non-smokers, with the impact being more pronounced in those who smoked daily. The degree of AMH reduction was also found to depend on the total smoking dose, measured in pack-years. Further research is needed to clarify the precise relationship between smoking and OR indicators.[3,26]

## Conclusion

Anti-Müllerian hormone is a crucial marker of OR, widely used in infertility clinics to assess eligibility for ART. For many women struggling with conception, an abnormal AMH result can lead to significant stress and anxiety. Unfortunately, the causes of reduced OR and abnormal AMH levels relative to age remain unclear in many cases. Primary prevention strategies emphasize lifestyle modifications, such as following a diet rich in antioxidants, avoiding smoking, and minimizing exposure to toxins. Additionally, genetic predisposition screening is recommended. Studies suggest that appropriate supplementation may be effective in many cases. It is essential to raise awareness among women regarding the factors influencing OR, as early diagnosis can facilitate timely interventions, including preservation of fertility. It is important to note that AMH serum levels should not be considered in isolation, and reliance on a single measurement is not recommended. Given the significance of AMH, the various factors influencing its levels, and the impact of AMH decline on both reproductive and non-reproductive health, further research is essential.

Declarations

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***Author contributions***

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***Conflicts of interest***

The authors declare no conflict of interest.

***Data availability***

No datasets were generated or analyzed during the current study.

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Not applicable.

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**REVIEW PAPER**

# Antimicrobial activity of ozonated oils and their applications in medicine – a narrative review

Taras Pyatkovskyy , Olena Pokryshko

I. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine

**ABSTRACT**

**Introduction and aim.** Ozonated oils have emerged as promising agents due to their potent antimicrobial properties and broad therapeutic potential across various medical fields. This review aims to evaluate the antibacterial activity of ozonated oils, focusing on their applications in dermatology, dentistry, ophthalmology, gynecology, and proctology, while addressing their safety profiles and limitations.

**Material and methods.** This review compiles and evaluates peer-reviewed publications on the antibacterial properties and therapeutic uses of ozonated oils. The authors conducted a comprehensive literature review by searching through the largest scientific databases using Google Scholar, Google Patents, Scopus, Web of Science, and PubMed within the last 15 years.

**Analysis of the literature.** Ozonated oils exhibited strong antibacterial effects against a wide range of microorganisms, including those resistant to antibiotics. Their efficacy has been confirmed in treating conditions such as onychomycosis, oral and vaginal candidiasis, and MRSA skin infections, among others. Clinical studies highlight their ability to reduce symptoms and improve outcomes in ophthalmic applications as well. Despite their effectiveness there are limitations which include a lack of long-term safety data, insufficient number of studies conducted on human subjects.

**Conclusion.** Ozonated oils have a lot of potential as antimicrobials that can be used in a variety of medicinal fields. However, further clinical trials are needed to establish their long-term safety and efficacy.

**Keywords.** bacterial biofilms, ozonated vegetable oils, ozonide, oxidation, pathogen inactivation

## Introduction

Ozone is widely known for its antibacterial properties, which are attributed to its strong oxidative ability.[1,2] As a powerful oxidant, it can damage microbial cell walls, membranes and interfere with their essential cellular functions by proteins damage and loss of organelle functions.[3–5] In its gaseous form, ozone is commonly used to disinfect surfaces in food industry and medicine.[1,6,7] Aqueous ozone, created by dissolving ozone gas in water or by water electrolysis, effectively eliminates bacteria, fungi, and viruses on various surfaces and in liquid environments.[8–10] This allows its utilization in food processing and for wound irrigation.[11–13] The use of ozone in these forms has paved the way for the development of ozonated oils, which offer a more stable and localized application for medical purposes. These oils are a distinct family of ozone-based antibacterial agents, they are relatively new and have attracted a lot of interest lately due to their versatility and broad-spectrum effectiveness in a range of medicinal applications. Additional interest to the antimicrobial activity of such natural products is caused by the increased distribution of multidrug resistant microorganisms.[14] Olive oil has been used for centuries in cosmetic and medical applications.[15] Its

**Corresponding author:** Taras Pyatkovskyy, e-mail: pyatkovskyy@tdmu.edu.ua

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beneficial properties are now further enhanced through ozonation. Produced by infusing vegetable oils with ozone gas, these oils possess strong oxidative properties, making them effective against microorganisms.[16] Their antibacterial action and possible therapeutic advantages are caused by their bioactive components, which include ozonides and peroxides.[14] The use of ozonated oils has expanded across several medical disciplines due to their ability to promote healing, reduce inflammation, and combat infections.

Despite their promising potential, the application of ozonated oils is not without limitations. Issues such as the stability of ozonated compounds, potential toxic byproducts, and the lack of standardized formulations remain challenges that require further investigation. Understanding these limitations is crucial for the safe and effective use of ozonated oils in clinical practice.

## Aim

The aim of this narrative review is to synthesize and evaluate the current publications on the antimicrobial activity of ozonated oils. The review highlights their applications against bacteria (e.g., *Enterococcus faecalis, Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*), fungi (e.g., *Candida* spp., dermatophytes), and viruses (e.g., *Herpesviridae*). Additionally, their safety in medical applications as well as limitations and gaps in the literature are discussed to guide future research.

## Material and methods

Google Scholar, Google Patents, Scopus, Web of Science and PubMed were used as web resources to search for relevant literature. The search was conducted using the keywords "ozonated oil", "ozonized oil" and "ozonide" in combination with "antimicrobial activity." Articles were selected based on their relevance to the antimicrobial properties of ozonated oils and medical applications. Both in vitro and in vivo studies were considered. Preference was given to peer-reviewed journal articles published within the last 15 years, although older studies were included when necessary. This review builds upon the author's previous work[17], expanding the scope to include recent advancements and additional perspectives on the antimicrobial activity of ozonated oils and their applications in medicine. Since this paper is a narrative review, PRISMA guidelines were not applied, as no systematic search strategy or meta-analysis was conducted.

## Analysis of the literature

### *Applications of ozonated oils in dermatology*

At this time, the market offers a variety of ozonated oils designed for topical use. Additionally, a variety of cosmetic formulations are currently on the market that emphasize the inclusion of ozonated oil as one of their constituents. These formulations include cream, shampoo, shower gel, aftershave balm, body lotion, emulgel, toothpaste, makeup remover, intimate wash, and lip balm.[14] The therapeutic potential of ozonated oils extends beyond skincare to medical applications in dermatology. Their antimicrobial and wound-healing properties make them valuable in treating various dermatological conditions. Huang et al. demonstrated the topical application of ozone in treatment of viral skin infection. Although their study did not directly evaluate the antiviral activity of ozonated oil, it demonstrated the clinical efficacy of ozonated oil and water application in patients with herpes zoster. Specifically, the results revealed a significant difference between the group treated with oral valacyclovir plus topical 2% mupirocin and the group receiving oral valacyclovir combined with topical ozone application. The ozone-treated group exhibited significantly faster clinical improvement without any side effects.[18] Silva et al. evaluated the antimicrobial and antibiofilm activity of ozonated vegetable oils against methicillin-resistant *S. aureus* (MRSA) strains isolated from diabetic foot ulcers. They demonstrated that ozonated oils had moderate to high potential to remove adhered bacterial cells and showed a high ability to eliminate 24 hour old biofilms. According to the results of their study, most MRSA strains were inactivated by ozonated oil at a concentration of 4.24 mg/g.[19] Song et al. demonstrated the antistaphylococcal activity of camellia oil with a peroxide index (PI) of 2000–2200 in vitro, as well as the resolution of MRSA skin infections following the topical application of ozonated water and ozonated camellia oil.[20] In addition to their well-documented antimicrobial properties, ozonated oils have also demonstrated significant antifungal activity, making them valuable in the treatment of dermatological conditions caused by fungal infections. The study conducted by Ouf et al. investigated the efficacy of ozonated oil against five dermatophyte species commonly responsible for superficial infections of the skin, nails, and hair. Their findings revealed that ozonated oil was more effective than gaseous ozone. Among the tested strains, *Microsporum gypseum* and *Microsporum canis* were the most susceptible, while *Trichophyton interdigitale* and *T. mentagrophytes* showed relative resistance.[21] In a phase III clinical trial involving 400 outpatients with onychomycosis, Menéndez et al. reported that the topical application of ozonated sunflower oil was more effective in treating onychomycosis than ketoconazole. After one year of follow-up, the experimental and control groups showed relapse rates of 2.8% and 44.4%, respectively.[22]

### *Applications of ozonated oils in dentistry*

Beyond their applications in dermatology, ozonated oils have also shown promise in dentistry and oral care, where their antimicrobial and healing properties can address a variety of conditions. These oils are in-

creasingly being explored as adjuncts to traditional dental treatments, offering potential benefits for managing infections and promoting oral health. In 2016, O'Malley proposed, and later patented in 2017, the incorporation of micro-encapsulated ozonated oils into a two-part oral care system. This system can be integrated into various dental and oral hygiene products, such as dental floss, toothbrush bristles, chewing sticks, comforters, denture bases, plastic retainers, and orthodontic devices.[23]

The efficacy of ozonated olive oil against *Streptococcus mutans*, the primary causative agent of dental caries, was examined by Nardi et al. Two commercial mouthwashes containing ozonated olive oil were tested in vitro. Both formulations successfully inactivated the principal pathogen of dental caries, effectively overcoming the salivary dilution effect in the oral cavity.[24] Pietrocola et al. compared the effectiveness of a commercial ozonated oil with two chlorhexidine digluconate-based agents. The study revealed a relatively moderate antiseptic effect for ozonated oil, which was more pronounced against Gram-negative bacteria than Gram-positive ones, and demonstrated its ability to inhibit dental plaque formation. However, the antibacterial activity of ozonated oil was found to be lower than that of the tested chlorhexidine-based agents.[25] Crastechini et al. investigated 493 *Candida* spp. isolates to evaluate the in vitro antifungal activity of ozonated olive oil and its in vivo effects on oral *Candida* spp. levels in patients with prosthetic stomatitis. The antifungal activity was tested against *Candida albicans* and five non-albicans species (*C. dubliniensis, C. guilliermondii, C. krusei, C. parapsilosis,* and *C. tropicalis*). In vitro, ozonated oil demonstrated antifungal activity against all tested *Candida* species and exhibited anti-biofilm activity against *C. albicans*. In vivo, 30 patients were treated with ozonated oil, while another 20 received sodium bicarbonate for 14 days. Both treatments significantly reduced oral candidiasis levels compared to baseline. After seven days of treatment, all patients in both groups showed complete remission of prosthetic stomatitis. According to the study's findings, ozonated oil could be a useful substitute for biofilm control in individuals suffering from prosthetic stomatitis.[26] Elshinawy et al. compared the antimicrobial and anti-biofilm activities of ozonated olive oil, chitosan, and silver nanoparticles, both individually and in combination, against the endodontic pathogens *E. faecalis*, *S. mutans*, and *C. albicans*. Ozonated oil demonstrated an 86% and 79% reduction in biofilms, respectively. The combination of ozonated oil and chitosan nanoparticles was the most effective, achieving a significant killing effect with a 6-log reduction in viable cells.[27]

***Applications of ozonated oils in ophthalmology***

A variety of microorganisms, including bacteria, viruses, and fungi, can cause ocular infections. Among bacterial pathogens, *Staphylococcus* spp., including *S. aureus*, and *P. aeruginosa* are the most common culprits behind acute and chronic eye infections.[28] Representatives of *Adenoviridae* and *Alphaherpesvirinae* families may cause such eye infections as conjunctivitis and keratitis.[29] Fungal eye infections can affect any part of the ocular surface and, in severe cases, spread to internal ocular structures, leading to endophthalmitis.[28]

Numerous studies have highlighted the use of ozonated oils in ophthalmology. Spadea et al. reported the successful application of a commercial product containing ozonated oil in liposomes and hypromellose for treating blepharitis, conjunctivitis, keratitis, and corneal ulcers in both humans and animals. Additionally, they demonstrated the antibacterial effects of ozonated oil against streptococci and enterococci.[30] Studies conducted by Pérez-Santonja et al. demonstrated the in vitro bactericidal activity of liposomal ozonated oil against *S. aureus*, *P. aeruginosa*, and *Porphyromonas gingivalis*. They also reported its effectiveness in treating recurrent conjunctivitis and corneal ulcers caused by MRSA and *P. aeruginosa* in animals.[28] Research completed by Cagini et al. confirmed that using ozonated oil-containing eye drops in combination with topical tobramycin 0.3% and dexamethasone 0.1% eye drops, administered four times daily, effectively reduced the signs of conjunctivitis and shortened the duration of the viral infection.[29] Celenza et al. reported that clinical *Candida* spp. isolates were susceptible to a commercial eye lubricant containing ozonated oil. They suggested that its antifungal activity was due to mitochondrial dysfunction and the accumulation of reactive oxygen species.[31] Since bacterial biofilms pose a significant challenge in both general medicine and ophthalmology, some researchers have investigated the anti-adhesion and anti-biofilm activities of ozonated oil-based eye drops. For example, Gentili et al. evaluated the effectiveness of a commercial eye drop preparation containing ozonated sunflower oil liposomes against *S. aureus*, MRSA, *Staphylococcus epidermidis*, *P. aeruginosa*, and *E. coli*. Their study demonstrated that the preparation exhibited substantial microbicidal and anti-biofilm activity, with the ozonated sunflower oil liposomes effectively preventing bacterial adhesion to human corneal cells.[32]

***Applications of ozonated oils in gynecology and proctology***

The application of ozonated oils in gynecology and proctology remains an emerging area of research, with limited studies directly addressing both their antibacterial properties and rectal use. Ozonated olive oil has been reported to be effective against *C. albicans* in the treatment of vaginal mucosa and has demonstrated antifungal activity in vitro.[33] Tara et al. reported that ozonated olive oil appears to be as effective as clotrimazole in the treatment of vulvovaginal candidiasis, both in reducing clinical symptoms and achieving laboratory-confirmed results.[34]

The potential of ozonated olive oil for rectal administration is demonstrated by the available data. For instance, Gültekin et al. demonstrated in their study the successful rectal application of ozonated olive oil in a rat model of acute radiation proctitis, showing promising therapeutic effects.[35] Additionally, several studies have confirmed the antibacterial activity of ozonated oils against pathogens that may be associated with vaginal and rectal infections. In particular, Yekanipour et al. demonstrated that commercially obtained ozonated oil exhibited bacteriostatic and bactericidal effects on *Neisseria gonorrhoeae* at concentrations of 3.12 PI and 6.25 PI, respectively.[36] While the laboratory-obtained data suggest a possible role for ozonated oils in managing rectal conditions, there is not enough clinical studies to validate their efficacy and safety in human proctological applications.

***Safety and limitations of ozonated oils***

To responsibly incorporate ozonated oils into medical practice, it is essential to thoroughly assess their safety. This ensures the effective use of these promising agents, maximizing their benefits while preserving patient health. Unfortunately, high-quality literature on the potential risks and side effects of ozonated oils on human tissues is limited. In one such study, researchers tested commercial ozonated olive and sunflower oils against *C. albicans*, *E. faecalis*, *S. aureus*, and *E. coli*, while also evaluating their toxicity on keratinocytes and epithelial cells. The results demonstrated significant microbicidal effects with no observed cytotoxicity.[37] Elshinawy et al. reported that, among the tested substances – ozonated olive oil, chitosan, and silver nanoparticles – ozonated olive oil exhibited the least cytotoxic effect on normal human fibroblasts.[27] Comparatively, De Oliveira et al. tested ozonated neem oil against laboratory and clinical strains of *E. faecalis*, *Enterococcus faecium*, *S. aureus*, *P.aeruginosa*, *E. coli*, and *Klebsiella pneumoniae*, as well as on two human cell lines: HaCaT (Immortalized Human Keratinocytes) and HCEC (Human Corneal Epithelial Cells). They reported greater antimicrobial activity of the ozonated neem oil compared to literature data for other ozonated oils, with no cytotoxicity observed at the concentrations tested.[38] In contrast, Radzimierska-Kaźmierczak et al. reported some cytotoxicity. They evaluated refined and ozonated olive oil for their antimicrobial properties against *E. coli*, *S. aureus*, *C. albicans*, and *Aspergillus brasiliensis* using the agar diffusion method, as well as for cytotoxicity using the MTT assay on two normal cell lines (LLC-PK1 and HaCaT) and two cancerous cell lines (Caco-2 and HeLa). Their findings showed that the ozonated oil exhibited a weak inhibitory effect against the tested microorganisms and a slight cytotoxic effect on HaCaT cells at concentrations of 312 and 1250 μg/mL.[33] Another study evaluated adverse drug reactions (ADRs) associated with a commercial ozonated sunflower oil in a Phase IV open clinical trial for the treatment of tinea pedis. The authors reported that out of the 2,165 patients who completed the trial, only six experienced ADRs, all of which were rated as mild by the study participants.[39] Similarly, Lu et al. evaluated the combination of laboratory-prepared ozonated water and commercial ozonated oil in a clinical trial for the treatment of tinea pedis. Their results demonstrated that this combination was effective in treating tinea pedis, as confirmed by mycological examination, and revealed no side effects.[40] Despite the growing amount of evidence supporting the in vitro effectiveness of ozonated oils, there remains a critical need for more comprehensive clinical trials to establish their safety and efficacy in medical applications. Significant gaps persist in the existing literature, including the lack of long-term safety data and a limited number of studies conducted on human subjects. Addressing these gaps is essential to ensure that ozonated oils can be responsibly and effectively integrated into clinical practice.

***Study limitations***

This review has several limitations. First, as a narrative review, it does not include a systematic search strategy, which may introduce selection bias. Second, while many *in vitro* and animal studies suggest promising antimicrobial properties of ozonated oils, human clinical trials remain limited, making it difficult to generalize findings. Third, differences in ozone concentration and oil type across studies may contribute to variability in reported effectiveness. Future systematic reviews and meta-analyses should aim to consolidate standardized data on ozonated oil applications.

## Conclusion

The antibacterial properties of ozonated oils and their wide range of uses in dermatology, dentistry, ophthalmology, gynecology, and proctology are explored in this review. Ozonated oils have demonstrated significant potential as effective agents against a wide range of microorganisms. They confirmed to be as good options for addressing microbial resistance to antibiotics, dealing with bacterial biofilms and enhancing patient outcomes. The reviewed papers highlight the many uses of ozonated oils, especially their effective use in the treatment of gynecological and proctological illnesses, mouth infections, ophthalmic diseases, and dermatological conditions. Additionally, clinical research indicates that ozonated oils have good safety profiles with very few reported side effects. However, these conclusions should be interpreted cautiously due to the limited availability of high-quality, long-term safety data and the relatively small number of human studies conducted to date,

which should be addressed in the future research.

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Conceptualization, T.P. and O.P.; Methodology, T.P.; Writing – Original Draft Preparation, T.P.; Writing – Review & Editing, O.P.; Supervision, O.P.

***Conflicts of interest***
The authors declare no conflict of interest.

***Data availability***
No datasets were generated or analyzed during the current study.

***Ethics approval***
Not applicable.

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**CASE REPORT**

# Transient ischemic attacks – the role of arterial spin labelling

Sreehari Dinesh, Dulari Gupta, Vivek Murumkar, Sankar Prasad Gorthi

Ringgold Standard Institution – Neurology, Bharati Vidyapeeth (Deemed to be University), Dhankawadi, Pune, Maharashtra, India

**ABSTRACT**

**Introduction and aim.** Transient ischemic attacks are usually diagnosed by clinical criteria. (1) Arterial spin labelling (ASL) is a noninvasive technique based on magnetic labeling of hydrogen ions in arterial blood with high sensitivity for the detection of oligemic areas in the corresponding cerebral hemisphere to the clinical phenotype. The aim is to demonstrate hypoperfusion using Arterial spin labelling techniques in TIA cases with the objective of intervening with appropriate methods to stop the stroke progression.

**Description of the cases.** The authors analyzed 90 cases of TIA in order to ultimately select four cases of clinical TIA with Arterial spin labelling to test the proof of concept.

Case 1. 47-year-old male with transient right-sided limb weakness and corresponding ASL hypoperfusion.

Case 2. 57-year-old male with recurrent transient ischemic symptoms and ASL showing hypoperfusion in the right parietal lobe.

Case 3. 73-year-old male with a high risk TIA and right parietal hypoperfusion on ASL that evolved into an infarct.

Case 4. 30-year-old female with a sensory TIA and hypoperfusion in the right cerebral hemisphere on ASL.

The hypothesis is that Arterial spin labelling will be able to demonstrate a penumbra in brain tissue in TIA cases which suggests likely progression to stroke and help in appropriate intervention to stop progression in real time. The following sequences were used during the brain MRI: diffusion-weighted imaging, fluid attenuated inversion recovery, apparent diffusion coefficient, and ASL sequences after written informed consent. Philips Ingenia 3Tesla machine obtains a 3D pseudocontinuous ASL sequence with a color coded map is obtained by Philips Ingenia 3 Tesla machine with a magnet weight of 4600 kg, 90-degree flip angle, and total duration of 3 minutes and 19 seconds.

The authors present a series of 4 cases in which patients had clinical TIA and had hypoperfusion on ASL sequence corresponding to the clinical manifestation depicting the penumbra. ASL hypoperfusion was assessed visually and cerebral blood flow (CBF) data was averaged to develop a visual CBF map.

**Conclusion.** Arterial spin labelling is a novel marker for hypoperfusion that indicates brain parenchyma under threat due to either stenosis in vessels of the cerebral circulation or embolic phenomenon.

**Keywords.** arterial spin labeling, magnetic resonance imaging, stroke, transient ischemic attack

## Introduction

Transient ischemic attacks are usually diagnosed by clinical criteria.[1] Arterial spin labelling (ASL) is a non-invasive technique based on magnetic labelling of hydrogen ions in arterial blood with a high sensitivity for the detection of oligemic and hypoperfused areas in the corresponding cerebral hemisphere to the clinical phenotype. ASL pulse sequences fall into three main kinds that are frequently found in clinical Magnetic resonance imaging (MRI) scanners: pulsed ASL (PASL), pseudo-continuous ASL

**Corresponding author:** Sreehari Dinesh, e-mail: sreehari4657@yahoo.co.in

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(pCASL), and continuous ASL (CASL). In frequent clinical applications, 3T pCASL is recommended as the preferred ASL technique by the ASL consensus ISMRM 2014 guideline.[2] Furthermore, ASL approaches are being developed and include vessel-encoded pCASL (ve-pCASL), acceleration-selective (AccASL), velocity-selective ASL (VS-ASL) and time-encoded ASL (te-ASL).[3]

## Aim

The authors present 4 cases of clinical TIA in which neuroimaging showed hypoperfused areas in the ASL sequence. The aim is to demonstrate hypoperfusion using ASL techniques in TIA cases with the objective of intervening with appropriate methods to stop the stroke progression.

## Description of the cases

The authors have evaluated four cases of TIA with ASL in a total of 90 cases of TIA to test the proof of concept. The hypothesis is that ASL will be able to demonstrate a penumbra in brain tissue in TIA cases which suggests likely progression to stroke and help in appropriate intervention to stop progression in real time. The following sequences were used during the Brain Magnetic Resonance Imaging: Diffusion weighted imaging (DWI), Fluid attenuated inversion recovery (FLAIR) sequence, apparent diffusion coefficient imaging and ASL sequences after written informed consent. A 3D pseudocontinuous ASL sequence with color coded map was done using the Philips Ingenia 3Tesla machine with a magnet weight of 4600kg, 90-degree flip angle and total duration of 3 minutes and 19 seconds. Clinical TIA syndromes older than 18 years were included, and we excluded cases of ischemic and hemorrhagic stroke, stroke mimics, and secondary and venous strokes.

***Case 1.***

A 47-year-old male, known hypertensive in treatment, presented acute onset right upper limb and lower limb, associated with slurring of speech slurred and difficulty in comprehension that resolved in 10 minutes spontaneously. In the emergency room, he suddenly developed similar weakness and slurring of speech. One week before presentation, he had a sudden onset of confusion and weakness in the right upper limb while driving which resolved in minutes. At presentation, his heart rate was 54 beats per minute, blood pressure 180/100 mm Hg. He had an ABCD2 score of 4 with weakness and dysarthria of the right upper and lower extremities, facial weakness, and mild aphasia which resolved completely resolved 10 minutes later. The investigations were normal except for electrocardiography which showed inversions of the T wave in the inferior and lateral leads with sinus bradycardia. Troponin I level was less than 0.01 and echocardiography was normal. The brain showed no acute infarct, but ASL showed hypoperfusion of the left MCA territory (middle cerebral artery), which was consistent with the patient's symptoms. Magnetic resonance angiography revealed no intracranial or extracranial stenosis and the repeat ASL showed a reversal of hypoperfusion 24 hours later (Fig. 1).

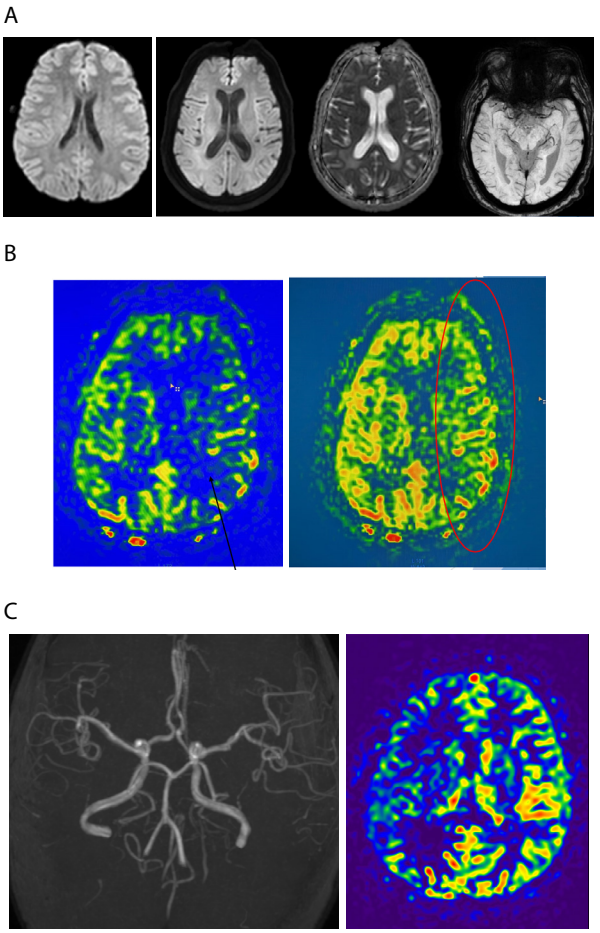



**Fig. 1.** A: Case 1- A 47-year-old male who presented weakness and language abnormality, his MRI, FLAIR, ADC image and Susceptibility weighted imaging showing no acute infarct, B: ASL sequence depicting hypoperfusion in the left MCA territory (black arrow and red circle), C: MR angiogram of brain that was normal and ASL sequence after 24 hours revealed normal perfusion

***Case 2.***

A 57-year-old male, a known case of hypertension presented with acute onset weakness and a tingling sensation of left upper limb and the lower limb with heaviness of the tongue which resolved spontaneously in 10 minutes. On presentation, his blood pressure was 140/80 mm Hg. Initially he had left upper and lower limb drift, dysarthria, and facial weakness which resolved to only dysarthria 10 minutes later and he then had complete resolution of symptoms after 15 minutes, making his ABCD2 score 4. Blood investigations were normal. Echocardiography showed mild concentric left ventricular hypertrophy.

Magnetic resonance imaging of the brain revealed no acute infarction, but ASL showed hypoperfusion in the right parietal lobe. CT angiography revealed right Internal Carotid artery stenosis. Repeat ASL done 24 hours later was normal (Fig. 2).

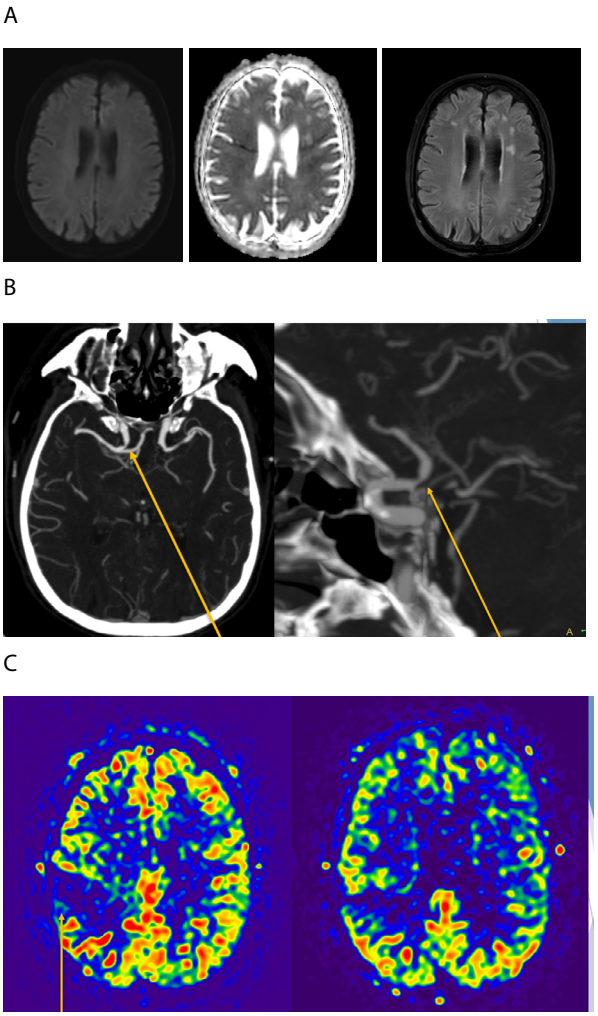



**Fig. 2.** A: Case 2: A 57-year-old hypertensive male with acute left-sided paraesthesisas and weakness of the extremities, his brain MRI diffusion, ADC image and FLAIR showing no acute infarction, B: axial and sagittal CT images depicting Right ICA intracranial part (yellow arrow), C: ASL sequence initially showing hypoperfusion (yellow arrow) and followed by normal perfusion

*Case 3.*

A 73-year-old male, a known case of diabetes and hypertension with ischemic heart disease and coronary angiogram suggestive of critical triple vessel disease, underwent coronary artery bypass surgery. On postoperative day 2 he developed acute onset weakness of the left upper and lower extremities, deviation of angle of mouth to the right side, and speech loss. The symptoms lasted for 40 minutes, and then there was complete recovery. On examination, his blood pressure was 190/90 mm Hg. Initially had weakness and dysarthria in the left upper and lower extremities, plantar response to the left extensor, facial weakness, inattention, and confusion which resolved 40 minutes later, making his ABCD2 score 7. Magnetic resonance imaging of the brain did not show acute infarct, but ASL revealed hypoperfusion in the right MCA territory. Repeat MRI of the brain 24 hours later showed an infarct of the MCA territory in the right parietal region with luxury perfusion in the ASL sequence (Fig. 3).

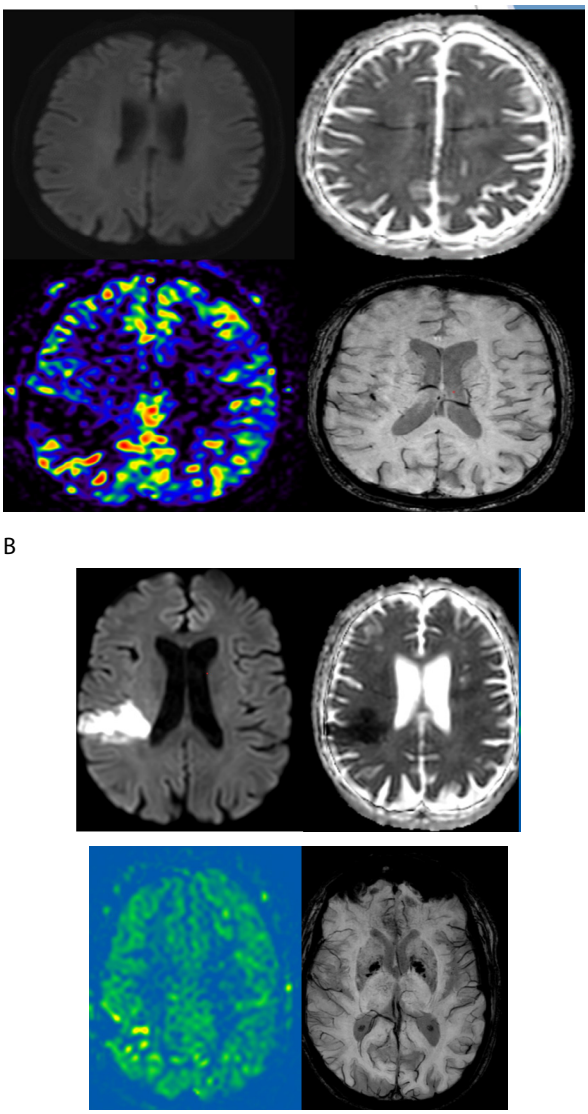
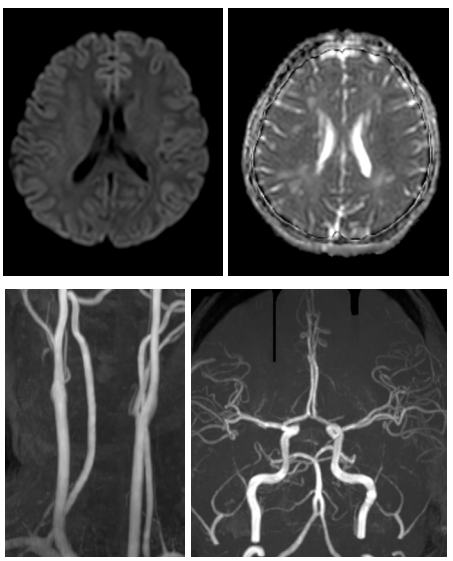



**Fig. 3.** A: Case 3: A 73-year-old male after coronary artery bypass surgery developed acute left sided weakness, his brain diffusion and ADC showing no acute infarct and ASL suggestive of hypoperfusion in the right parietal region with a small clot visualized on susceptibility-weighted imaging in the right parietal region, B: MRI of the brain 24 hours later: diffusion-weighted imaging and ADC showing infarct of the right MCA territory in the right parietal region, ASL shows luxury perfusion in the corresponding area, no clot is seen on susceptibility-weighted imaging

Carotid Doppler revealed atherosclerotic plaques in bilateral common carotid arteries and right internal carotid artery without any significant luminal compromise. Electrocardiography had pathological Q waves in the inferior and anterior leads. Echocardiography showed basal mid distal lateral wall apex basal mid posterior wall, basal septum akinetic, moderate left ventricular systolic dysfunction, and an ejection fraction of 30%.

A



B

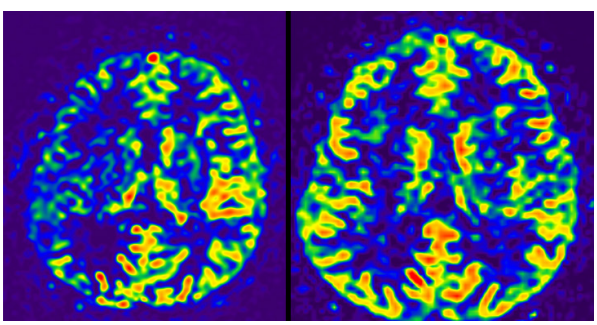


**Fig. 4.** A: A 30-year-old woman after emergency caesarean section for preeclampsia developed left sided paraesthesias, her brain with angiography with no acute infarct nor vessel stenosis, B: ASL showing hypoperfusion in the right cerebral hemisphere at presentation and normal perfusion 24 hours later

***Case 4.***

A 30-year-old antenatal mother underwent an emergency lower segment caesarean section due to preeclampsia. On the second postoperative day, she developed sudden onset paresthesia of the left upper and lower extremities associated with acute severe bifrontal headache. The symptoms lasted for the next 40 minutes and then there was complete recovery making her ABCD2 score 2. MRI of the brain with angiography had no acute infarct nor evidence of any vessel stenosis but ASL sequence showed hypoperfusion in the right MCA territory. Repeating ASL 24 hours later was normal (Fig. 4). Echocardiography did not reveal left ventricular dysfunction. The possibility of TIA and postpartum reversible cerebral vasoconstriction syndrome was investigated and treated, and rest of the postpartum period was uneventful.

## Discussion

In our study, we had four cases where the ASL sequence revealed evidence of a penumbra tissue in high-risk TIA. Two of these cases had arterial occlusion that was later diagnosed on vascular imaging. In another study of 116 cases, Zaharchuk et al. found that the sensitivity and specificity of arterial spin labeling in the diagnosis of perfusion abnormalities in TIA was 55.8% and 90.7%, respectively.[4] ASL perfusion increased the detection of ischemia in patients with TIA and was most useful in conjunction with DWI.[5]

Comparing pCASL, CASL, and dynamic susceptibility contrast (DSC) MRI in ICA Stenosis highlights the plausibility of ASL-based measurements and provides additional insights by mapping perfusion territory shifts.[6,7]

In acute ischemic stroke, the concept of determining the salvageable penumbra and imaging the mismatch region is most important. DSC imaging was used to categorize patients into clinically significant categories (ie, those with a mismatch, without a mismatch, or with reperfusion). Compared to that ASL is a suitable method for image tissue perfusion and collaterals owing to its noninvasive nature and sensitivity to delayed arterial arrival. Research indicates that ASL can predict the regional distribution of collaterals, demonstrating strong concordance in individuals with arterial stenoocclusive disease.[8,9]

ASL also has clinical applications in various other fields of medicine. In Alzheimer's disease, ASL has shown a reduction in CBF in a posterior parietal distribution, including the precuneus, posterior cingulate, angular gyrus, and superior parietal gyrus.[10-12] Taylor et al. found posterior hypoperfusion in the posterior cingulate and areas of visual association areas in Lewy body dementia.[13]

ASL can also help in locating a potential epileptogenic focus in epilepsy patients. During the acute peri-ictal period, CBF is typically increased due to pathologic neuronal activity, while in the chronic interictal period, CBF is typically reduced as the epileptogenic region is less functional compared to normal brain tissue.[14-18]

Posterior reversible encephalopathy syndrome can show higher CBF in the afflicted areas of ASL, lending support to the hypertension-hyperperfusion idea. However, it can indicate lower CBF as well, which promotes the vasoconstriction-hypoperfusion concept.[19,20]

ASL can also help with the follow-up of arteriovenous malformations by exposing the location of the nidus, allowing for longer follow-up and more objective monitoring of nidus obliteration after radiosurgery or partial embolization.[21] Compared to CT perfusion (CTP) studies, the range of ischemic penumbra determined by ASL-CBF and DWI mismatch was consistent with CTP.[22]

The limitation of our study is that our sample size was very small as this was done to test proof of concept. This novel concept warrants further research in larger samples to establish the efficacy of ASL in identifying penumbra tissue in the brain.

## Conclusion

ASL is a surrogate marker for hypoperfusion that indicates brain parenchyma under threat due to stenosis in vessels of cerebral circulation or embolic phenomenon. ASL is a useful imaging technique for diagnosing high-risk TIA and minor stroke.

## Declarations

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***Author contributions***

Conceptualization, S.P.G.; Methodology, S.P.G., D.G., V.M. and S.D.; Validation, S.P.G., D.G. and V.M.; Formal Analysis, S.D.; Investigation, V.M. and S.D.; Resources, S.D.; Data Curation, S.D.; Writing – Original Draft Preparation, S.D.; Writing – Review & Editing, V.M. and D.G.; Visualization, V.M.; Supervision, S.P.G.; Project Administration, S.P.G.

***Conflicts of interest***

There are no conflicts of interest.

***Data availability***

The data used and/or analyzed during the current study are open from the corresponding author on reasonable request.

***Ethics approval***

The authors reported that they acquired the necessary informed consent form from the patients, who consented to the publication of their photo and other clinical information. Patients were informed that confidentiality would be ensured.

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**CASE REPORT**

# Secondary chondrosarcoma of the iliac bone in a young woman – a rare case report and review of the literature

Therese David [1], Swetha Lakshmi Narla [1], Annapurneswari Subramanyan,[1] Kathiresan N. [2]

[1] Department of Histopathology, Apollo Hospitals, Chennai, India
[2] Department of Surgical Oncology, Apollo Hospitals, Chennai, India

**ABSTRACT**

**Introduction and aim.** Chondrosarcomas are malignant cartilage-forming tumors, with secondary cases rarely arising from osteochondromas. This report presents a case of secondary chondrosarcoma developing from an undiagnosed pelvic osteochondroma in a young female, emphasizing the importance of early detection and timely intervention.

**Description of the case.** A 28-year-old woman with hip and back pain underwent magnetic resonance imaging, revealing a lesion in the left iliacus muscle with sacroiliac extension. Biopsy confirmed a chondroid neoplasm, and positron emission topography-computed tomography showed minimal metabolic activity. Due to the extensive soft tissue component and recurrence risk, wide local excision was performed. Histology confirmed well-differentiated secondary chondrosarcoma, WHO grade 1 arising from an undiagnosed pre-existing osteochondroma.

**Conclusion.** The described case highlights the critical role of radiological and histopathological evaluation, timely surgical intervention, and multidisciplinary management for optimal patient outcomes.

**Keywords.** chondrosarcoma, iliac bone, malignant transformation, osteochondroma

## Introduction

*Chondrosarcomas* are locally aggressive or malignant tumors that form a cartilaginous matrix, and make up around one-fifth of all primary malignant bone tumors. Conventional primary chondrosarcomas arise without a benign precursor, while secondary types can be central (from enchondromas), peripheral (from osteochondromas), or periosteal (on the bone surface near the periosteum). Its global incidence varies, ranging from less than 10% in India and Saudi Arabia to over 45% in Finland, Slovenia, and the Netherlands.[1]

Chondrosarcoma mainly affects middle-aged to older adults and is more common in males. It typically arises in the pelvic bones, femur and humerus, with rare occurrences in the trunk, skull, facial bones, hands and feet. Periosteal chondrosarcoma primarily affects the metaphysis of long bones, with a predilection for the humerus and distal femur.[2]

The evolution of osteochondroma into chondrosarcoma is a relatively infrequent yet well-documented occurrence, often leading to the development of low-grade tumors, although higher-grade variants can also arise. This transformation is more prevalent in adults, particularly those with conditions like multiple hereditary exostoses (MHE), with only rare instances reported in pediatric patients.[3] Osteochondromas represent a significant portion of benign bone tumors, comprising 20–50% of such cases and are among the most com-

**Corresponding author:** Swetha Lakshmi Narla, e-mail: drswetha.gmc2k2@gmail.com

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mon bone neoplasms. They frequently manifest in the metaphysis and metaphyseal equivalents, with the femur being the primary site of occurrence (30% of cases), while the pelvis, scapula, and spinal involvement are less common.[4] Malignant progression to chondrosarcoma, a complication seen in approximately 1% of solitary osteochondromas, is typically identified by persistent lesion growth post-skeletal maturity and a hyaline cartilage cap thickness of more than 1.5 cm.[5]

## Aim

Progression of an osteochondroma in the pelvis of a young female is an uncommon occurrence, particularly without prior imaging, making the diagnosis unexpected. A prompt biopsy and appropriate management of the lesion are crucial for a thorough and effective treatment strategy.

The aim of the study was to present a case of secondary chondrosarcoma developing from an undiagnosed pelvic osteochondroma in a young female, emphasizing the importance of early detection and timely intervention

## Description of the case

We present a 28-year-old Indian woman who presented with hip and backache. Notably, the severity of her symptoms prompted her to seek medical attention only recently. Clinical evaluation revealed no palpable tenderness or limitations in range of motion. Bilateral lower extremity strength was symmetric, and reflexes were within normal limits. Laboratory findings indicated a normal complete blood count (CBC). Upon observation of concerning symptoms, a pelvic magnetic resonance imaging (MRI) was conducted, which revealed a well-defined lesion within the left iliacus muscle exhibiting T1 isointensity and T2 hyperintensity, with a fusiform extension into the left sacroiliac joint with calcification and patchy peripheral and central septal enhancement. Differential considerations were proposed, including benign neurogenic tumor like schwannoma or intramuscular myxoma.

A preliminary ultrasound-guided biopsy of the lesion was done, which was reported as a chondroid neoplasm. A cartilaginous neoplasm extending into soft tissue was high on the list of differential diagnoses, even though prior imaging confirming the presence of an osteochondroma was unavailable.

Subsequently, a positron emission tomography–computed tomography (PET-CT) fusion study was done for staging and showed minimal metabolic activity within the lesion, accompanied by a discernible mass effect on the left psoas muscle *(Fig. 1)*. Both imaging modalities also revealed polycystic ovarian morphology in both ovaries.

After appropriate preoperative tests, including negative results for viral markers screening (HIV, HBsAg, and Anti-HCV), the patient was deemed fit and taken up for surgery.

Given the extensive soft tissue component and the risks of recurrence and metastasis at a young age, wide resection was chosen as the treatment approach.

Gross examination disclosed a nodular, well-circumscribed lesion within the soft tissue measuring 4×3.5×2.2 cm. The cut surface was myxoid and gray-tan, with focal gritty areas *(Fig. 2)*.

Microscopically, the lesion comprised of neoplastic chondroid tissue with minimal increase in cellularity arranged as nodules and lobules separated by both thin and thick fibrous septae and exhibiting focal cystic change. The atypical chondrocytes showed nuclear enlargement and occasional binucleation. The mitotic rate was observed to be 2–4 per 10 high-power fields. Additionally, portions of a pre-existing osteochondroma were identified, with the lesion arising from the bony trabeculae. (Fig. 3). A diagnosis of well-differentiated secondary chondrosarcoma WHO grade 1 was rendered.

The patient was counselled regarding the diagnosis and the importance of regular follow-up for monitoring and management of their condition.

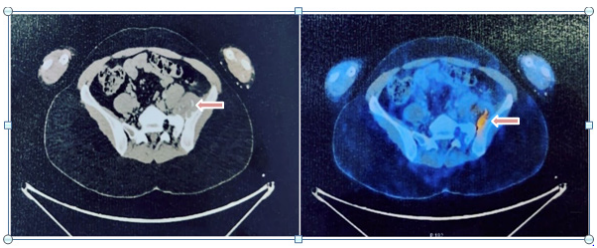

**Fig.1.** PET-CT FUSION study revealing a well-defined lesion with minimal metabolic activity within the substance of left iliacus muscle with fusiform extension into the left sacroiliac joint

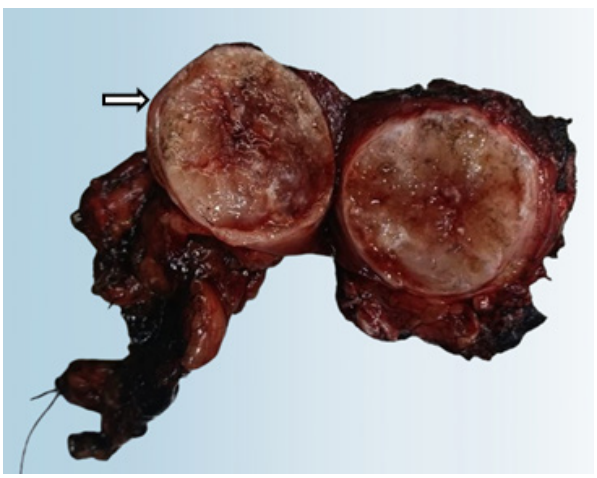

**Fig. 2.** Macroscopic image showing a well-circumscribed lesion in the soft tissue with myxoid appearance

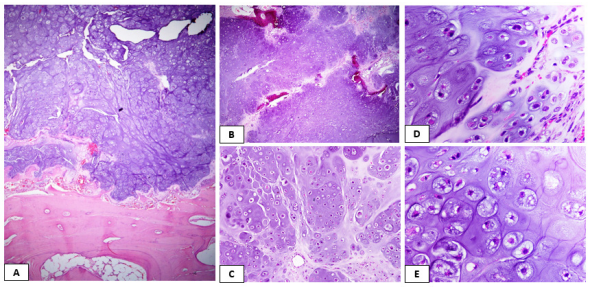


**Fig. 3**. Microscopic images showing A: Hematoxylin and eosin stain (H&E), 40×, bony trabeculae with thick lobulated cartilaginous cap composed of atypical cartilaginous tumor causing scalloping of the cortex, B: H&E, 40×, hyaline cartilage matrix entrapping the pre-existing lamellar bone, C: H&E, 100×, neoplastic lobules with increased cellularity and occasional binucleation, D: H&E, 400×,cells exhibiting mild nuclear pleomorphism and vacuolated cytoplasm

## Discussion

Osteochondromas commonly affect the femur, with distal lesions being predominant, followed by the tibia and humerus. At the same time, those originating from flat bones are rare, with less apparent medullary continuity on radiographs.[6] The transformation of an osteochondroma into chondrosarcoma is a rare phenomenon, particularly in cases not associated with syndromes like multiple hereditary exostoses. This transformation becomes even more uncommon when considering solitary osteochondromas. Indeed, documented cases of secondary chondrosarcoma originating from solitary pelvic osteochondromas are rare. To date, few cases and series have been reported in medical literature, underscoring the exceptional nature of such occurrences and the need for further research to understand the underlying mechanisms.[7,8]

Few studies have suggested that estrogen contributes to cartilage metabolism and human growth, with its involvement in chondrosarcoma, particularly through estrogen receptor alpha, indicating a role in tumor proliferation.[8] Additionally, some studies indicate that the active estrogen-signaling pathway may not have a significant role in the development and progression of chondrosarcoma.[9] The hypothesis regarding the possible role of PCOS (polycystic ovary syndrome) in the progression of chondrosarcoma requires further in-depth research.

Alarming signs such as sudden pain without trauma, recent increase in tumor size, along with a thick cartilaginous cap may suggest malignant degeneration, needing confirmation. From a management perspective, pelvic chondrosarcoma presents surgical challenges because of its size and its proximity to critical structures. Wide-margin surgical resection is recommended for high-grade cases, while curettage alone or in combination with adjuvant therapy may be adequate for low-grade lesions.

Local recurrence is common, especially in pelvic secondary chondrosarcoma, affecting 10-20% of patients.[7,10] Despite these challenges, comprehensive approaches can lead to favorable outcomes in patients undergoing pelvic resection for chondrosarcoma.[11]

Tsuda et al.[12] analyzed 51 cases of secondary chondrosarcomas arising from osteochondromas, with a median age of 36 and a 6.9-year follow-up. In their study, the pelvis was most commonly affected (59%), with 69%, 25%, and 6% showing grade I, II, and III tumors, respectively. Preoperative biopsy accurately predicted the final grade in 27% of cases. They reported a 10-year disease-specific survival rate of 89.4%, noting a higher rate of local recurrence in pelvic tumors (37% vs. 19% in limb tumors). Wide or radical resection was linked to improved local recurrence-free survival.

Righi et al.[13] retrospectively (1943 to 2019) analyzed 214 cases of secondary peripheral chondrosarcomas from solitary osteochondromas. The median patient age was 38 years, with a male-to-female distribution of 66.4% to 33.6%. They reported a 17.3% local recurrence rate and a 5.1% metastasis rate. High histologic grade was the only factor linked to worse 5-year and 10-year overall survival, highlighting the importance of accurate histological assessment and long-term follow-up for this rare chondrosarcoma variant.

Ahmed et al.[14] conducted a study on 107 patients diagnosed with secondary chondrosarcoma, originating either from a solitary osteochondroma (61 cases) or multiple hereditary exostoses (46 cases). Compared to primary chondrosarcoma, these patients were generally younger by one to two decades and showed a male predominance, with tumors more commonly affecting flat bones. Radiologically, malignant transformation was marked by irregular margins, uneven mineralization, and the presence of a soft tissue mass. Histologically, the majority of tumors were well-differentiated, with only ten cases classified as Grade 2.

At the microscopic level, differentiating between enchondroma and atypical cartilaginous tumor relies on several growth patterns. Key features that suggest malignancy include the tumor permeating into the surrounding lamellar bone, causing bone destruction and forming resorption spaces known as Howship's lacunae. Additionally, the lack of new layers of lamellar bone forming around the edges of cartilage nodules, a process called encasement, further supports a malignant diagnosis. Additionally, a myxoid matrix comprising more than 20% of the tumor is also an indicator of malignancy.[15]

Radiology alone cannot reliably differentiate between benign and malignant cartilaginous neoplasms. Some studies suggest that PET-CT could potentially help distinguish between low- and high-grade chon-

drosarcomas based on standardized uptake values.[16] In this case, the PET-CT showed minimal metabolic activity, which corresponded to the low-grade nature of the chondrosarcoma. Due to the absence of prior radiological evidence of osteochondroma in this case, the diagnosis of a chondroid neoplasm in the preoperative biopsy of the lesion was crucial in guiding surgical decisions. Due to the predominant soft tissue component, a pre-existing osteochondroma was confirmed only in the resection specimen. This underscores the importance of both preoperative and postoperative histopathological examination in guiding further management plans.

However, the absence of long-term follow-up remains a limitation. Additionally, further research into the molecular and hormonal pathways involved in the progression of secondary chondrosarcomas is essential to improve diagnostic accuracy and develop targeted therapeutic strategies.

## Conclusion

Presented case report of a 28-year-old woman diagnosed with secondary chondrosarcoma, which developed from a previously unrecognized osteochondroma of the iliac bone highlight the importance of both radiological and histopathological examination, appropriate surgical intervention and close follow-up. It should be emphasize the need for a comprehensive, interdisciplinary approach involving orthopedic, oncological, and pathological expertise.

## Declarations

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***Author contributions:***

Conceptualization, T.D., S.L.N., A.S. and K.N.; Methodology, T.D. and S.L.N.; Validation, T.D., S.L.N., A.S. and K.N.; Formal Analysis, T.D., S.L.N., A.S. and K.N.; Investigation, T.D., S.L.N. and A.S.; Resources, T.D. and S.L.N.; Data Curation, T.D., S.L.N., A.S. and K.N.; Writing – Original Draft Preparation, T.D.; Writing – Review & Editing, S.L.N., A.S. and K.N.; Visualization, T.D. and S.L.N.; Supervision, S.L.N., A.S. and K.N.; Project Administration, S.L.N.

***Conflicts of interest***

The authors have no conflicts of interest to declare.

***Data availability:***

The data that support the findings of this study are available from the authors.

***Ethics approval:***

Institutional Ethical Committee approval has been obtained (AMH-C-S-051/06-24).

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**LETTER TO THE EDITOR**

# Depression scores among pet dog owners

Hinpetch Daungsupawong [1], Viroj Wiwanitkit [2]

[1] Private Academic Consultant, Phonhong, Lao People's Democratic Republic
[2] Department of Research Analytics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India

**Dear Editor,**

We would like to discuss on "Low depression scores among pet dog owners – a comparative cross-sectional study from Anuradhapura Sri Lanka".[1] This study looks at the important and understudied problem of dog companionship and its effects on psychological and cardiovascular health in South Asia, namely Sri Lanka. The scientists used a cross-sectional methodology to compare pet dog owners to non-pet dog owners, which was effective in identifying early differences in depression and cortisol outcomes. Dog ownership may reduce stress and improve mental health, according to significant findings on depression scores (which are lower among pet dog owners) and the negative relationship between pet bonding ratings and cortisol levels. However, several key study findings warrant further investigation and discussion.

First, this study is cross-sectional design makes it more difficult to prove causation. Although having a pet has been linked to better psychosocial health, can owning a dog actually lower depression, or is it just that dog owners are more likely to have moderate depression? To investigate the direction of this association, longitudinal research might be helpful. The results may also be impacted by additional variables like socioeconomic position, cultural views on dogs, and pre-existing mental health issues. Our conclusions might be improved with a more sophisticated comprehension of these variables. Second, the study might be extended to look at a wider range of physiological indicators of stress and cardiovascular health, even though the authors emphasize the link between pet bonding and cortisol levels. Blood pressure, heart rate variability, and cholesterol levels, for instance, may offer more information about the cardiovascular advantages of pet keeping. Furthermore, qualitative information about participants' emotional experiences and perspectives of their pet-owner relationship may enhance the results and offer a more thorough comprehension of psychosocial causes.

Future studies should also look at how owning a dog affects the physical and emotional well-being of various South Asian groups over the long run. A more thorough understanding might be obtained by comparing the psychosocial impacts of pet ownership in urban and rural areas, as well as cultural variations in pet ownership. Furthermore, examining any possible gender disparities in the connection between pet ownership and health may provide crucial information on how men and women differ in their interactions with their animals.

This study leaves room for future research on the beneficial health effects of dog companionship in Sri Lanka, especially with longitudinal studies that incorporate different physiological measures, cultural and gender differences, and other factors.

**Corresponding author:** Hinpetch Daungsupawong, e-mail: hinpetchdaung@gmail.com

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## Declarations

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

***Conflicts of interest***
The authors declare no conflict.

***Data availability***
There is no new data generated.

Ethics approval
Not applicable.

## References

1. Rathish D, Rajapakse J, Weerakoon K. Low depression scores among pet dog owners – a comparative cross-sectional study from Anuradhapura Sri Lanka. *Eur J Clin Exp Med*. 2024;22(4):785-800. doi: 10.15584/ejcem.2024.4.14

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**CORRIGENDUM**

# Corrigendum: Interleukin-13 as a potential biomarker in the management of pediatric asthma – a longitudinal study

Prasanna Raju [1] , Subash Sundar [1] , Preethi Suresh [1] , Leela Kakithakara Vajravelu [2] , Vivekanandhan Aravindhan [3]

[1] Department of Pediatrics, SRM Medical College Hospital and Research Center, SRMIST, Chennai, India
[2] Department of Microbiology, SRM Medical College Hospital and Research Center, SRMIST, Chennai, India
[3] Department of Genetics, University of Madras, Chennai, India

**A Corrigendum on**

Interleukin-13 as a potential biomarker in the management of pediatric asthma – a longitudinal study by Raju P, Sundar S, Suresh P, Vajravelu LK, Aravindhan V. *Eur J Clin Exp Med*. 2025;23(1):15–20. doi: 10.15584/ejcem.2025.1.3.

In this paper, the affiliations were published incorrectly. The authors apologize for any inconvenience that it may have caused.
The affiliations should be corrected as follows:

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[1] Department of Pediatrics, SRM Medical College Hospital and Research Center, SRMIST, Chennai, India
[2] Department of Microbiology, SRM Medical College Hospital and Research Center, SRMIST, Chennai, India

After:
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Raju P, Sundar S, Suresh P, Vajravelu LK, Aravindhan V. Corrigendum: Interleukin-13 as a potential biomarker in the management of pediatric asthma – a longitudinal study. *Eur J Clin Exp Med.* 2025;23(2):531. doi: 10.15584/ejcem.2025.2.29.

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**CORRIGENDUM**

# Corrigendum: A family screening of CD19 gene mutation by PCR-RFLP

Mehmet Ali Karaselek [1], Hasan Kapaklı [1], Şükrü Nail Güner [1], Ercan Kurar [2], Serkan Küççüktürk [3], Sevgi Keleş [1], İsmail Reisli [1]

[1] Department of Pediatric Allergy and Immunology, Meram Medical Faculty, Necmettin Erbakan University, Konya, Turkey
[2] Department of Medical Biology, Meram Medical Faculty, Necmettin Erbakan University, Konya, Turkey
[3] Department of Medical Biology, Medical Faculty, Karamanoğlu Mehmetbey University, Karaman, Turkey

## A Corrigendum on

A family screening of "CD19" gene mutation by PCR-RFLP by Karaselek MA, Kapaklı H, Güner ŞN, Kurar E, et al. *Eur J Clin Exp Med*. 2022;20(2):141–145. doi: 10.15584/ejcem.2022.2.1.

Following the publication of our original article, we became aware of the omission of a key reference in the Material and methods. We regret this oversight and hereby provide the missing citation in this Corrigendum. Moreover, a grammatical error was present in the Aim section at the time of the publication of the above-mentioned article.

In the first paragraph of the Material and methods/ Patients , it states: "The patient (P1), mother (P2), father (P3), sister (P4), brother (P5), the patient identified in 2006 (P6), newborn baby of P6 (P7) and mother of P6 (P8) were included in the study. The studies were performed with 2 healthy controls (C1 and C2). "

These sentences should be revised to include the reference below: "The patient (P1), mother (P2), father (P3), sister (P4), brother (P5), the patient identified in 2006 (P6), newborn baby of P6 (P7) and mother of P6 (P8) were included in the study. The diagnosis of newborns with RFLP was optimized in a previous study. This study also evaluated its usability for family screening.[17] The studies were performed with 2 healthy controls (C1 and C2)."

In the first paragraph of the Material and methods/ Flow cytometry , it states: "Peripheral lymphocyte subgroup study of the patient and family members were performed."

These sentences should be revised to include the reference below: "Peripheral lymphocyte subgroup study of the patient and family members were performed as previously described.[17]"

In the first paragraph of the Material and methods/ PCR and PCR-RFLP, it states: "Polymerase chain reaction (PCR) primers covering mutation regions in CD19 gene were designed (forward primer: 5'-CCTGAGGAGGAAAAGAAT-3' and reverse primer: 5'-GGAAACAGTAAGTGCAAGGCATA-3')."

These sentences should be revised to include the reference below: "PCR and RFLP were performed as previously described.[17] Polymerase chain reaction (PCR) primers covering mutation regions in CD19 gene were designed (forward primer: 5'-CCTGAGGAGGAAAAGAAT-3' and reverse primer: 5'-GGAAACAGTAAGTGCAAGGCATA-3')."

17. Efe H, Karaselek M, Kapaklı H, et al. The Use of RFLP Method in the Diagnosıs of CD19 Deficiency. *Genel Tıp Derg*. 2021;31(4):365-368.

In the Aim, it states: "Therefore, in this study, it was aimed to determine CD19 gene mutation by PCR-RFLP which is a cheap, reliable and fast method."

Karaselek MA, Kapaklı H, Güner ŞN, Kurar E, Küççüktürk S, Keleş S, Reisli İ. Corrigendum: A family screening of CD19 gene mutation by PCR-RFLP. *Eur J Clin Exp Med.* 2025;23(2):532–533. doi: 10.15584/ejcem.2025.2.30.

These sentences should be revised below: "Therefore, this study aimed to perform family screening for CD19 gene mutation using PCR-RFLP, which is a cheap, reliable and rapid method."

The authors apologize for this error and confirm that it does not affect the scientific conclusions of the article in any way. The original article has been updated.

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— CARE guidelines for clinical case reports
— CONSORT guidelines for clinical trials
— PRISMA guidelines for systematic reviews and meta-analyses
— SPIRIT for clinical trials
— STARD guidelines for studies of diagnostic accuracy
— STROBE guidelines for observational studies

## Manuscript preparation

Your paper should consist of the following parts. Title page should be supplied as a **separate** file.

**Research manuscripts** should comprise:

— Title page: Title, Author list, Affiliations, Abstract, Keywords.
— Research manuscript sections: Introduction, Aim, Materials and Methods, Results, Discussion, Conclusions.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions,

Conflicts of Interest, Data Availability, Ethics Approval, References.

Research manuscript sections:

— *Introduction*

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

— *Material and methods*

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

— *Results*

Results should be clear and concise. The section may be divided into subsections, each with a concise subheading. Tables and figures central to the study should be included in the main paper. Do not use the term "significant" unless p-values are provided. Show p-values to 2 or 3 decimal places. The Results section should be written in past tense.

— *Discussion*

This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature.

— *Conclusions*

Summarize the work's findings, state their importance, and possibly recommend further research.

**Review manuscripts** should comprise:

— Title page: Title, Author list, Affiliations.
— Abstract, Keywords, Literature review sections.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions, Conflicts of Interest, Data Availability, References.

Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.

**Case reports** should comprise:

— Title page: Title, Author list, Affiliations.
— Abstract, Keywords. Case reports should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions, Conflicts of Interest, Data Availability, Ethics Approval, References.

Requirements for case reports submitted to Eur J Clin Exp Med:

— Patient ethnicity must be included in the Abstract under the Case Presentation section.
— Consent for publication is a mandatory journal requirement for all case reports. Written informed consent for publication must be obtained from the patient (or their parent or legal guardian in the case of children under 18, or from the next of kin if the patient has died).

## Language Style

Manuscripts must be submitted in English (American or British usage is accepted, but not a mixture of these).

## Title page

These sections should appear in all manuscript types:

**Title:** The title of your manuscript should be concise and informative. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.

**Author list and affiliations:** Authors' full first and last names must be provided. For each affiliation provide the details in the following order: department, institution, city, country. If available, the e-mail address of each author should also be provided. At least one author should be designated as *corresponding author*, and his or her email address and other details should be included at the end of the affiliation section.

**Abstract:** The abstract should be a total of about 250 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts: *Introduction and aim:* Place the question addressed in a broad context and highlight the purpose of the study; *Material and methods*: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. *Results:* Summarize the article's main findings; and *Conclusion*: Indicate the main conclusions or interpretations. The abstract should not contain any undefined abbreviations or unspecified references.

**Keywords:** Three to six pertinent keywords need to be added after the abstract in alphabetical order. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

## Back matter

**Supplementary materials**: Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.

**Acknowledgments:** Thank all of the people who helped with the research but did not qualify for authorship. Acknowledge anyone who provided intellectual assistance, technical help, or special equipment or materials.

**Funding statement:** All sources of funding of the study should be disclosed.

**Author contributions**: Authors must supply an Author Contribution Statement as described in the *Author contributions statements* section.

**Conflicts of interest**: Authors must supply a competing interests statement. For more details please see *Competing interests policy*.

**Data availability:** Authors must include a Data Availability Statement in all submitted manuscripts; see *Availability of materials and data* section for more information.

**Ethics approval:** Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

**References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, Reference Manager or Zotero to avoid typing mistakes and duplicated references.

## References style

In-text citations and references should be prepared according to the American Medical Association (AMA) style. Each item should be listed in numerical order.

### In-text citations

Each reference should be cited in the text using superscript arabic numerals. These superscript numbers should be outside periods. If you are citing sequential references, these should be indicated with a hyphen. Nonsequential references should be separated with commas. There should not be a space between numbers. For example: The degree of respiratory muscles fatigue depends on the applied exercise protocol and the research group's fitness level.[1,2] The greatest load with which a patient continues breathing for at least one minute is a measure of inspiratory muscles strength.[3] Diabetes mellitus is associated with a high risk of foot ulcers.[4-6]

### Sample Reference

In listed references, the names of all authors should be given unless there are more than 6, in which case the names of the first 3 authors are used, followed by "et al.". If the source does not have any authors, the citation should begin with the title.

To find the proper abbreviation of journal go to the National Library of Medicine PubMed Journals Database at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Journals.

Page number(s) should be inserted in full (for example: use 111–112, not 111–2).

The following are examples of individual citations made according to the required rules of editing and punctuation:

— **Article from a journal, number of authors from 1 to 6**

Author AA, Author BB, Author CC. Title of article. *Accepted Abbreviated Journal Title*. Year;Volume(Issue):Page-Page. doi (if available)

Lee JC, Seo HG, Lee WH, Kim HC, Han TR, Oh BM. Computer-assisted detection of swallowing difficulty. C*omput Methods Programs Biomed*. 2016;134(2):72-78. doi: 10.1016/j.cmpb.2016.07.010

Morris A. New test for diabetes insipidus. *Nat Rev Endocrinol*. 2019;15(10):564-565. doi: 10.1038/s41574-019-0247-x

— ***Article from a journal, number of authors* more *than 6***

Author AA, Author BB, Author CC, et al. Title of article. *Accepted Abbreviated Journal Title*. Year;Volume(Issue):Page-Page. doi (if available)

Gonzalez ME, Martin EE, Anwar T, et al. Mesenchymal stem cell-induced DDR2 mediates stromal-breast cancer interactions and metastasis growth. *Cell Rep*. 2017;18:1215-1228. doi: 10.1016/j.celrep.2016.12.079

Jordan J, Toplak H, Grassi G, et al. Joint statement of the European Association for the Study of Obesity and the European Society of Hypertension: obesity and heart failure. *J Hypertens*. 2016;34:1678-1688. doi: 10.1097/HJH.0000000000001013

— **Websites**

Author AA (if indicated). Webpage title. Name of Website. URL. Published or Updated date. Accessed date.

Cholera in Haiti. Centers for Disease Control and Prevention Web site. http://www.cdc.gov/haiticholera/. Published October 22, 2010. Updated January 9, 2012. Accessed February 1, 2012.

Address double burden of malnutrition: WHO. World Health Organization site. http://www.searo.who.int/mediacentre/releases/2016/1636/en/. Accessed February 2, 2017.

— **Book**

Author AA, Author BB. *Title of Work*. Location: Publisher; Year:Page-Page

Doane GH, Varcoe C. *Family Nursing as Relational Inquiry: Developing Health– Promoting Practice*. Philadelphia, PA: Lippincott Williams & Wilkins; 2005:25-28.
London ML, Ladewig PW, Ball JW, et al. *Maternal & Child Nursing Care*. Upper Saddle River, NJ: Pearson Education; c2011:101-103.
— Chapter in a book
Chapter Author AA. Title of chapter. In: *Name of Book*. Edition Number. Editor AA, ed. Location: Name of Publisher; Year:Page-Page.
Grimsey E. An overview of the breast and breast cancer. In: *Breast Cancer Nursing Care and Management*. 2nd ed. Harmer V, ed. Chichester, UK: Wiley-Blackwell; 2011:35-42.
NOTE: The Editorial Board requires consistent and carefully made references prepared according to the above-mentioned AMA standards. Otherwise, the work will be sent back to the authors.

## Preparing figures, schemes and tables

File for Figures and Schemes must be provided during submission and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption should be placed directly below the figure (not on the figure itself) or above the table. All figures, schemes, and tables should be numbered following their number of appearance (Figure 1, Scheme 1, Figure 2, Scheme 2, Table 1, etc.).

Tables should present new information rather than duplicating what is in the text. Readers should be able to interpret the table without reference to the text.

All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Tables must be provided in an editable format in appropriate place in the main text. Tables provided as jpeg/tiff files will not be accepted. Do not submit your tables in separate files.

## Abbreviations

The journal requires using only standard abbreviations. Common abbreviations such as DNA and RNA do not require definitions. Abbreviations should be defined in parentheses the first time they appear in the abstract, main text and in figure or table captions and used consistently thereafter. Ensure consistency of abbreviations throughout the article. Use the following abbreviations for measurement units: gram (g), litre (L), milligram (mg), kilogram (kg), seconds (s), minutes (min), and hours (h). Do not add 's' to indicate plural forms of units. Keep abbreviations to a minimum.

## SI Units

SI Units (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.