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¬**Discussion:** Emphasize the new and important aspects of the study, conclude, and relate the observations to other relevant studies. Link the conclusions with the goals of the study.
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Conflicts of Interest

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References should be in Vancouver style where they should be numbered consecutively in the order of their appearance in the text tables or figures with Arabic numbers between two parentheses at the end of each paragraph before the full stop.

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Research Article

Maternal Serum Ferritin, C-Reactive Protein, and Procalcitonin Levels for Predicting Subclinical Intra-Amniotic Infection in Preterm Premature Rupture of Membrane

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Abstract

Background: The preterm premature rupture of the membrane is linked to various perinatal problems, including chorioamnionitis.

Objectives: To evaluate the use of serum ferritin, C-reactive protein, and procalcitonin as early indicators for predictions of subclinical intra-amniotic fluid infection.

Methods: A case-control study was conducted at Baghdad Teaching Hospital from January to October 2021. A convenient sample of 90 singleton pregnant women with a live fetus between 24 - 36 weeks of gestation were divided into three groups: Group 1 (controls) included 30 women with intact membranes and no signs of labour seen in the outpatient obstetrics clinic in Baghdad Teaching Hospital; Group 2 included 30 women with preterm premature rupture of membrane (PPROM) but without chorioamnionitis; and Group 3 included 30 women with PPROM and chorioamnionitis. The second and third groups were collected from the labour room in Baghdad Teaching Hospital.

Results: There was no significant difference in the levels of C-reactive protein between the study groups. Serum ferritin and Procalcitonin levels were normal in all of the participants, with a significant difference in the level of Procalcitonin between group 2 (PPROM with chorioamnionitis) and group 3 (PPROM without chorioamnionitis).

Conclusion: Procalcitonin might be used to detect the presence of chorioamnionitis. Serum ferritin and C-reactive protein had no role in the detection of chorioamnionitis among patients with preterm premature membrane rupture.

Keywords: C-reactive protein; Chorioamnionitis; Procalcitonin; Preterm premature rupture of membrane; Serum ferritin.

Introduction:

The World Health Organization defined Preterm birth as any birth occurring before 37 completed weeks of gestation or within 259 days after a woman's last menstrual cycle. Preterm delivery is the main cause of mortality in children under the age of five, accounting for around 35% of newborn infant deaths and 16% of all deaths (1).

Preterm birth is a global issue with 15 million children delivered prematurely each year (2). However, discrepancies in gestational age, preterm definitions, and data collection and reporting methods complicate estimations. The incidence rates are higher in developing countries than in developed countries (3, 4). About 30-35% of preterm births are caused by maternal or fetal factors in which labour is induced or the infant is delivered via cesarean section, 40-45% are due to spontaneous preterm births with intact membranes, while preterm premature rupture of the membrane (PPROM), regardless of vaginal or cesarean delivery, accounts for 25-30% of preterm births (5). PPROM is the rupture of the amniotic membranes (amnion and chorion) before the 37th week of gestation, and it complicates about 1% of deliveries (6, 7).

Chorioamnionitis is an acute inflammation of the placental membranes and chorion produced by infection of polymicrobial bacteria that ascend following membrane rupture. Clinical chorioamnionitis refers to the presence of certain clinical indicators, whereas subclinical chorioamnionitis refers to the absence of specific clinical signs (8). Early and definitive diagnosis of subclinical chorioamnionitis is critical for preventing maternal and newborn death and morbidity, especially in situations of PPROM. Some biochemical biomarkers with high diagnostic accuracy and the ability to detect subclinical chorioamnionitis early in

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pregnancy would be extremely valuable in clinical practice (9).

Serum ferritin can be considered as an indicator for infections in PPROM. The elevated ferritin levels could indicate an acute phase response to a subclinical genital tract infection or inflammation and a latent infectious process that is associated with preterm delivery and causes tissue damage (10).

C-reactive protein (CRP) is an acute-phase protein. During an infection, the liver produces CRP in response to interleukin-6 synthesis. Although maternal serum CRP levels increase somewhat with gestational age, this measure is nevertheless utilized as a predictor of intrauterine infection, particularly in PPROM instances, despite reports suggesting its benefits are inconsistent (11).

Procalcitonin (PCT) is a peptide precursor of calcitonin, but the biological function and induction are different from that of calcitonin. It consists of 116 amino acids(12). The production of PCT is elicited by endotoxin or mediators released in bacterial infections, and this production correlates with the severity and extent of the infection (13). As the PPROM is associated with an inflammatory process, PCT can be used as a good indicative marker of infection for preterm labour (1).

The study aims to evaluate the use of serum ferritin, CRP, and PCT as an early indicator for predictions for subclinical intra-amniotic infection.

Patients and Methods:

An analytic case-control study was conducted at Baghdad Teaching Hospital during the period from the 1st of January to the 1st of October 2021.

The study was approved by the Scientific Council of Gynecology and Obstetrics of the Iraqi Board of Medical Specializations. Women were asked to participate voluntarily after an adequate explanation about the study's aim and methods. All participants were assured of anonymity and confidentiality of information.

Sampling method and inclusion criteria:

A convenient sampling method was used to select 90 singleton pregnant women with a live fetus between 24 to 36 weeks of gestation who were sub-divided into three groups, 30 members each:

Group 1 (control group): Included 30 women at preterm gestation without labour, and with intact membranes, the sample was collected from the outpatient clinic of obstetrics in Baghdad Teaching Hospital.

Group 2: Included 30 patients with PPROM but without chorioamnionitis.

Group 3: Included 30 patients with PPROM and chorioamnionitis.

The second and third groups were collected from the labour room in Baghdad Teaching Hospital.

Exclusion criteria:

- Women who had medical or obstetrical diseases including diabetes mellitus, hypertension, chronic kidney disease, chronic liver disease, cancer, heart disease, infectious disease, and antepartum haemorrhage.
- Evidence of intrauterine growth restriction (IUGR) or congenital abnormalities of the fetus
- Consumption of non-steroidal anti-inflammatory drugs (NSAIDs), or immunosuppressant drugs such as steroids.

Data collection:

A structured questionnaire form was used for data collection. The gestational age was calculated by the date of the last menstrual period, early ultrasound, or both. To confirm the diagnosis of PPROM, a warm speculum was inserted in the vagina under aseptic conditions to detect the pooling of clear fluid in the posterior fornix of the vagina or leakage of fluid from the cervical os with the woman in dorsal position. Five milliliters of venous blood samples were taken from each participant by the researcher and sent to the Teaching Laboratories in the Directorate of the Medical City where they were centrifuged for 10 minutes at 3500 rpm. After that, the serum was separated and stored at −20 to −80°C and sent to a private laboratory to detect the levels of serum ferritin, CRP and Procalcitonin.

Statistical analysis:

The data was entered and analyzed by the Statistical Package for Social Sciences (SPSS) version 22. Descriptive statistics were presented as frequencies and percentages and were applied to explain the characteristics of participants. The mean values of the study parameters in the study group were compared using the t-test and the associations between the variables were tested using the Chi-Square test. A P-value of less than 0.05 was considered statistically significant.

Results:

Table 1 shows that there was no significant association between CRP tests and the presence of PPROM with chorioamnionitis. The sensitivity was 60%, specificity 64%, positive predictive value (PPV) 70%, and negative predictive value (NPV) 53%. Similarly, there was no significant association between CRP tests and the presence of PPROM without chorioamnionitis. Sensitivity was 48%, specificity 48%, PPV 43%, and NPV 35%.

Table 1: Distribution of C-reactive protein test in the controls and the PPROM with, and without chorioamnionitis groups

Groups	CRP		Total (100.0%)	P-value
	Positive- N (%)	Negative- N (%)		
PPROM with chorioamnionitis	21 (70.0)	9 (30.0)	30	0.066
Controls	14 (46.7)	16 (53.3)	30	
Total	35 (58.3)	25 (41.7)	60	
PPROM without chorioamnionitis	13 (43.3)	17 (56.7)	30	0.795
Control	14 (46.7)	16 (53.3)	30	
Total	27 (45.0)	33 (55.0)	60	

The serum ferritin levels were normal in the three study groups with no significant difference in their mean values between control group and PPROM with chorioamnionitis groups and without chorioamnionitis groups respectively, Table 2.

Table 2: Mean± SD serum ferritin level in the three study groups

Groups	N	Serum ferritin (ng/mL)		P-value
		Mean	±SD	
Control	30	48.64	57.952	0.620
PPROM with chorioamnionitis	30	55.60	49.819	
Control	30	48.64	57.952	0.683
PPROM without chorioamnionitis	30	42.97	48.643	

Table 3 shows that all groups had a normal mean level of procalcitonin. When the ANOVA test was applied, a significant difference was detected between the mean values of PCT in the three study groups.

Table 3: Mean ±SD serum Procalcitonin level in the three study groups

Groups	N	Procalcitonin (ng/ml)		P-value
		Mean	±SD	
Controls	30	0.21	0.039	0.011
PPROM with chorioamnionitis	30	0.23	0.028	
PPROM without chorioamnionitis	30	0.20	0.029	

Significant association according to ANOVA and Post Hoc test

As a significant difference in the level of PCT between the study groups was found, the Receiver Operating Characteristic (ROC) Curve analysis for medical diagnostic test evaluation was done to estimate a cut-off point between normal and abnormal values with better sensitivity and specificity (Figure 1).

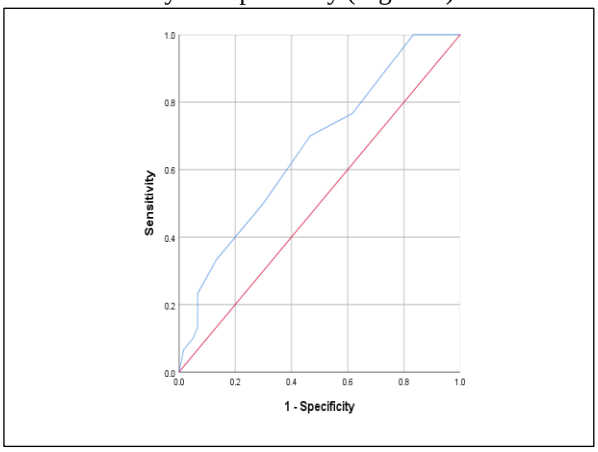

Figure 1: Roc curve analysis for diagnostic evaluation of procalcitonin

According to the ROC test, the better cut-off point was 0.21 ng/ml with 70% sensitivity and 60% specificity, with a significant association between the test results and the presence or absence of chorioamnionitis, (P <0.05), Table 4.

Table 4: Distribution of procalcitonin according to the cut-off point of 0.20 ng/ml

Procalcitonin	Groups N (%)		Total N (%)	P-value
	PPROM* With chorioamnionitis	PPROM* without chorioamnionitis		
Positive	21 (70.0)	12 (40.0)	33 (55.0)	**0.018**
Negative	9 (30.0)	18 (60.0)	27 (45.0)	
Total (100.0%)	30	30	60	

Sensitivity=70%. Specificity=60%. ***Preterm premature rupture of membrane***

Discussion:

The mother and the fetus are at risk of problems if chorioamnionitis is not detected early (14). This study is one of the studies that tried to evaluate the diagnostic value of positive maternal CRP, serum ferritin, and procalcitonin in association with maternal clinical chorioamnionitis.

The initial finding of the current study was the absence of a significant link between CRP test results and the existence of chorioamnionitis in the study groups. An earlier study by Wiwanitkit in Thailand revealed that the overall diagnostic activity showed the values of sensitivity, specificity, PPV, and NPV of 72.8%,

76.4%, 23.6%, and 27.2%, respectively (15). Balciuniene et al found the values of the same indicator to be 84%, 77%, 74%, and 86% respectively (16). A systematic review by Martinez et al concluded that of the eight studies reviewed, three studies concluded that CRP was a useful diagnostic tool for chorioamnionitis while the other five studies concluded the opposite (17). The discrepancy in the results of CRP and its diagnostic effectiveness in the diagnosis of chorioamnionitis might be related to the participant's condition and may affect the level of CRP, the accuracy of the investigation, and the methodology of these studies.

In the current study, there was no significant difference between the study groups regarding the mean of serum ferritin. In contrast, Valappil et al found that serum ferritin was significantly higher in PPROM cases when compared to the control group of women with the same gestational period (10). Khattab *et al.* concluded that serum ferritin levels may serve as a marker of infection among women with premature rupture of membranes (18). The difference in these results could be due to the prevalence of iron deficiency in different populations.

In the current study, all the participants had normal PCT levels, but the mean was significantly higher in patients who had PPROM with chorioamnionitis than those without chorioamnionitis. The same finding was reported by Şen C *et al.* who found that the mean procalcitonin values among PPROM with chorioamnionitis patients were significantly higher than those among PPROM without chorioamnionitis (19), while other studies concluded that serum PCT is a poor predictor for clinical or pathological chorioamnionitis (20). With a cut-off value of 0.05ng/mL, the sensitivity of PCT was 54%, and the specificity was 79%, with positive and negative predictive values of 60% and 75%, respectively (21). Bakar et al concluded that low or average PCT does not rule out bacterial infections, particularly in localized infections like chorioamnionitis, because PCT's sensitivity and specificity in the diagnosis of chorioamnionitis are low (22). The discrepancy in the results of inflammatory markers between different studies might be related to the prevalence of other asymptomatic infections that may impact the results.

Limitations:

1. Small sample size.
2. Short data collection time.
3. Long distance between sample collection place and private laboratory.
4. Relatively high investigation cost.

Conclusion:

Procalcitonin might be used to detect the presence of chorioamnionitis. Serum ferritin and CRP had no role in the detection of chorioamnionitis among patients with preterm premature rupture of the membrane.

Authors' declaration

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's Approval-Ethical Clearance: The project was approved by the local ethical committee in the Scientific Council of Gynecology and Obstetrics of the Iraqi Board of Medical Specializations according to the code number (55) on (8th of November 2020)

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Authors' contributions

Study conception & design: (Maad M. Shallal). Literature search: (Balsam N. Ibrahim). Data acquisition: (Balsam N. Ibrahim). Data analysis & interpretation: (Balsam N. Ibrahim). Manuscript preparation: (Balsam N. Ibrahim). Manuscript editing & review: (Maad M. Shallal).

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مستويات الفيريتين في مصل الأم والبروتين سي التفاعلي والبروكالسيتونين للتنبؤ بالعدوى داخل السلى تحت الإكلينيكي في تمزق الغشاء المبكر قبل الأوان

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الخلاصة
الخلفية: يرتبط تمزق غشاء الحمل قبل الأوان بالعديد من المضاعفات في الفترة المحيطة بالولادة بما في ذلك إلتهاب المشيمة والسلى.
الهدف: لتقييم إستخدام الفيريتين في مصل الدم وبروتين سي التفاعلي والبروكالسيتونين كمؤشرات للتنبؤات بعدوى السائل الأمنيوسي.
الطريقة المنهجية: تم إجراء دراسة تحليلية للحالات والشواهد في مستشفى بغداد التعليمي خلال الفترة من 1 كانون الثاني إلى 1 تشرين الأول 2021. تم تسجيل عينة ملائمة من 90 إمرأة حامل بجنين واحد حي في عمر حمل من 24 إلى 36 أسبوعًا.
النتائج: لم تجد الدراسة فرقا ذا دلالة إحصائية في نتائج بروتين سي التفاعلي بين مجموعات الدراسة. كان لدى جميع المرضى مستوى طبيعي من البروكالسيتونين والفيريتين في مصل الدم.
الإستنتاج: يمكن استخدام البروكالسيتونين للكشف عن وجود التهاب المشيمة والسلى. لم يكن للفيريتين في مصل الدم وبروتين سي التفاعلي دور في الكشف عن التهاب المشيمة والسلى بين المرضى الذين يعانون من تمزق الغشاء المبكر قبل الأوان.
الكلمات المفتاحية: الفيريتين في مصل الدم، البروكالسيتونين، بروتين سي التفاعلي، التهاب المشيمة والسلى.

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Research Article

Assessment of the Impact of Apremilast on Levels of IL-17, IL-23, and Lipids in Obese Psoriatic Patient

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Abstract

Background: Psoriasis is an immune-mediated inflammatory disease with unknown aetiology that may be associated with the defect in proliferation and differentiation of the keratinocytes related to inflammatory cell infiltration. According to published reports, it is universal in occurrence; its prevalence in different populations varies from 0.1% to 11.8%. Receiving Apremilast resulted in a strong reduction in interleukin 17 and interleukin 23, as well as reduced expression of other inflammatory cytokines and improvement of psoriatic lesions.

Objectives: This study aimed to assess the impact of Apremilast on levels of IL-17, IL-23, and lipids in obese psoriatic patients.

Methods: Thirty obese patients with psoriasis were included in this prospective interventional study to measure serum levels of lipid profile, IL-17, and IL-23, before and after receiving Apremilast treatment. A t-test was used to compare between means.

Results: The mean age of the participants was 38 years. The most common age group was 30–40 years. The levels of IL-17 before the administration of Apremilast were 225.55 ± 7.70 pg/mL. After six months of treatment with Apremilast, a statistically significant reduction was seen, with the value decreasing to 183.41 ±2.33 pg/ml. IL-22 levels before the administration of Apremilast were measured to be 76.42 ± 4.03 pg/mL. After six months of treatment with Apremilast, these levels exhibited a non-significant decrease to 67.15 ± 5.40 pg/ml. Modest alterations were noted in the lipid profile.

Conclusion: The use of Apremilast is effective in decreasing IL-17 levels, which have pro-inflammatory effects; this leads to improvement in psoriatic lesions. Moreover, receiving Apremilast in obese psoriatic individuals led to a reduction in TG levels and an elevation in HDL-C levels. Additionally, a rise in TC levels and LDL-C was seen.

Keywords: Apremilast; IL-17; IL-23; Obese; Psoriasis.

Introduction

Psoriasis is an immune-mediated inflammatory disease with unknown etiology that may be associated with the defect in proliferation and differentiation of keratinocytes associated with inflammatory cell infiltration particularly consisting of T-lymphocytes, macrophages, and neutrophils (1). Psoriasis is a common disease characterized by highly proliferating keratinocytes and extensive leukocyte infiltration (2). These skin lesions are typically covered with dry, fragile, loosely attached, silvery, or greyish-white scales with a micaceous appearance (3).

The most characteristic lesions consist of red, scaly, well-demarcated plaques (4). It is universal in occurrence; its prevalence in different populations varies from 0.1% to 11.8%, according to published reports (5). Psoriasis is a common disease with unknown etiology (6). It is characterized by inflammation, autoimmune responses, and abnormal proliferation of skin cells (7). The disease involves inflammation and scaling of the skin as epidermal cells come to the surface prematurely before Completing their maturation process (8). Apremilast phosphodiesterase-4 inhibitors (PDE-4 inhibitors) inhibit the degradation of cAMP, thus increasing the concentration of cAMP and ultimately reducing the expression of pro-inflammatory mediators, including IFN-γ, TNF-α, and IL-2, IL-12, and IL-23 (9).

In the pharmacodynamic analyses in patients with moderate to severe psoriasis, Apremilast demonstrated partial inhibition of key cytokines that regulate inflammation in psoriasis, including IL-23 and IL-17 (10). Furthermore, it led to a decrease in the infiltration of myeloid dendritic cells and T lymphocytes into the epidermis and dermis of psoriatic lesions (11). Additionally, IL17- plays a pivotal role in the pathogenesis of psoriasis. Dysregulation in the production of IL-17 induces chronic inflammation and autoimmune disorders (12). The induction of immunological responses at skin surfaces is mostly dependent on IL17- cytokines, in particular on IL-17A. As a result, neutrophils migrate into the surrounding tissue (13, 14). The IL-17/23 axis is central to psoriasis pathogenesis, and the efficacy of monoclonal antibodies targeting IL-17

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supports the importance of this cytokine in the disease. Inhibiting antibodies against IL-17A have shown success in treating psoriasis (15, 16). In addition, IL-23 is involved in the pathophysiology of psoriasis, and by inhibiting the function of IL-23, inflammation that produces psoriasis symptoms can be reduced (17).

Previous studies have demonstrated that during phase 3 clinical trials, modest elevations in total cholesterol, HDL cholesterol, and triglyceride levels were detected following 24-52 weeks of Apremilast administration. Nevertheless, it should be noted that these increments were deemed to lack clinical significance (18). According to Gualtierotti et al. (2019), Apremilast has beneficial effects on the metabolic profile, in their case study, they reported that Apremilast was associated with an improvement in the lipid profile of the patient (19). In addition to improvements in psoriatic disease activity, Apremilast has been reported to be associated with weight loss (20).

Although Apremilast has been employed as a treatment for psoriasis, the existing body of research, pertaining to its effects on inflammatory cytokines such as IL-17 and IL-23 in patients, remains limited. Hence, the primary objective of this research effort was to examine the impact of Apremilast on the levels of IL-17 and IL-23, along with the lipid profile, in the serum of obese patients with psoriasis.

Patients, Materials, and Methods

This prospective interventional study was conducted at the Dermatology Center, Medical City in Baghdad, Iraq, between November 2021 and December 2022. Participants in the current study were informed about the aim of the study, the nature of the illness, course prognosis, and Apremilast treatment as well as its potential complications by a Dermatologist. Also, formal consent was obtained from each patient before starting the study. Ethical approval No. 145 on 10-11-2022 was obtained from the Development Department at the Medical City Directorate in Baghdad, Iraq.

A total of 30 psoriatic patients (19malles and 11 females) attending the outpatient clinic were enrolled in the study. These patients received Apremilast (Aprezo®), administered twice daily after food (approximately 12 hours apart) without food or drink restrictions and with titration over the first week to mitigate gastrointestinal side effects, started with 10 mg morning dose with a daily increment of 10 mg until day 6 when the recommended dose (30 mg bid) is reached which is continued at thereafter for six months. Of the 30 patients enrolled, 6 did not complete the study for various reasons.

Blood samples were obtained from all patients before receiving Apremilast at the beginning of the study. A second sample was obtained from each patient after they had been receiving Apremilast for 24 weeks.

These samples were employed for the purpose of quantifying IL17-, IL23-, TC, HDL-C, and TG. The levels of IL17- and IL23- were quantified using commercially available human ELISA kits obtained from ELK Biotechnology-China. The lipid profile tests were performed using a colorimetric assay kit supplied by Linear Company.

The BMI of each patient was calculated according to the international standard equation "[BMI = weight (kg) / (height (m))2]". Baseline BMI was calculated and monitored monthly.

The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference between the two groups in study parameters, the t-test was used to compare between means, Chi-squared test was used to compare between percentages. The P value ≤0.05 is considered significant (21).

Results

Demographic characteristics of participants

In the present study, data showed that nineteen (63.3%) of the participants were males and eleven (36.7%) were females. Moreover, cigarette smokers were only four (13.3%) out of 30 participants, while twenty-six (86.7%) were non-smokers. In addition, the average age of the group was 38 years. The most common age group was 30-40 years (60% of participants) followed by the >40 years' age group (26.6% of participants) (Table 1).

Table 1: Demographic characteristics of the study sample

	No.	Percentage
Sex		
Male	19	63.33
Female	11	36.67
Total	30	100%
P-value	---	0.074 NS
Smoking		
Yes	4	13.33
No	26	86.67
Total	30	100%
P-value	---	0.0001 **
** ($P \leq 0.01$)		
Age group (year)		
<30	4	13.33
30-40	18	60.00
>40	8	26.67
Total	30	100%
P-value	---	0.0052 **

** ($P \leq 0.01$), NS: Non-Significant

Interleukins-17 and Interleukins-23 (IL-23): In the current study, prior to receiving Apremilast, the mean IL-17 levels were 225.55 pg/ml. After six months of treatment, the mean IL-17 level decreased significantly to 183.41 pg/ml ($p < 0.01$). In addition, the mean IL-23 level prior to Apremilast was 76.42 pg/ml. After six months of treatment, the mean IL-23 level decreased to 67.15 pg/ml. However, this decrease was not statistically significant ($P>0.05$), as illustrated in Table 2.

Table 2: Comparison between Interleukins-17 and Interleukins-23 levels before and after receiving Apremilast

Group	Mean ± SE	
	IL-17 (pg / ml)	IL-23 (pg / ml)
Patients: Before treatment	225.55 ±7.70	76.42 ±4.03
Patients: After treatment	183.41 ±2.33	67.15 ±5.40
P - value	0.0001	0.166
	(p < 0.01).	

IL-17: Interleukin-17, IL-23: Interleukin-23.

Lipid profile: In the present study, before the administration of Apremilast, the mean levels of total cholesterol were recorded as 152.86 mg/dl. Following a period of six months of treatment, the observed value rose to 167.17 mg/dl. However, this observed rise did not reach statistical significance (p>0.05). The average triglyceride level prior to the administration of Apremilast was recorded as 190.80 mg/dl. Following the intervention, the observed value reduced to 150.39 mg/dl; however, this reduction did not yield statistically significant results (p> 0.05). Moreover, HDL cholesterol before the administration of Apremilast was recorded as 22.17 mg/dl. After a period of six months, there was a notable increase to 32.82 mg \ dl, with statistical significance shown by a *P*-value of less than 0.01. Ultimately, the mean of LDL-C levels prior to the administration of Apremilast was recorded as 92.53 mg/dl, which subsequently rose to 104.36 mg/dl following the completion of the treatment. However, the observed increase did not reach statistical significance (P> 0.05). as shown in Table 3.

Table 3: Comparison between lipid profile data before and after receiving Apremilast

Group	Mean ± SE of Lipid profile (mg/dl)			
	Cholesterol	Triglyceride	HDL-C	LDL-C
Patients: Before treatment	152.86 ±5.62	190.80 ±19.12	22.17 ±1.04	92.53 ±4.94
Patients: After treatment	167.17 ±4.71	150.39 ±16.02	32.82 ±4.78	104.36 ±7.14
p-value	0.649	0.126	0.010	0.166
	* (p-< 0.05), ** (p-< 0.01).			

HDL-C: High Density Lipoprotein cholesterol, LDL-C: Low Density Lipoprotein cholesterol.

Body mass index Participants in the current study were considered obese according to international standards (22). However, their mean BMI after 6 months of treatment with Apremilast did not significantly differ from that before treatment (P>0.05; Table 4).

Table 4: Comparison of participants' BMI before and after treatment with Apremilast for 6 months

Group	BMI (Mean ± SE) (kg/m^2)
Patients: Before treatment	32.97 ±1.07
Patients: After treatment	30.48 ±1.14
p-value	0.349

Discussion:

Psoriasis is a chronic disease of the immune system that is characterized by increased inflammation cytokine production, including IL-17 and IL-23. To the best of our knowledge, this is the first study to assess the effects of Apremilast on IL-17 and IL-23 in psoriatic patients in Iraq.

The findings showed that after six months of Apremilast treatment, there was a decrease in the serum levels of IL-17 and IL-23. These findings point to its efficacy in reducing inflammation. According to *Strober et al. (19*), this study was consistent with their previous findings, which also showed a decrease in mean levels of IL-17. Apremilast significantly decreased plasma IL-17A, IL-17F, and IL-22 levels among patients with moderate plaque psoriasis (10). Additionally, this further corroborates the Apremilast involvement in modulating IL-17-related pathways. It also plays a role in modulating the release of inflammatory cytokines by immune and non-immune cells (23). In T cells, Apremilast inhibited various T-cell-derived cytokines, including IL-2, IL-5, IL-13, and IL-17 (24).

In addition, this study was parallel with *Parab et al. (2022),* which found that Apremilast decreased the production of IL-12 and IL-23; however, in patients with mild plaque psoriasis who were innocent to systemic therapy, it did not significantly change IL-23 levels (25). Also, another study by *Ilowite et al*. (*2016*), showed that Apremilast is an oral targeted PDE-4 inhibitor that affects many inflammatory mediators involved in psoriasis and psoriatic arthritis. These mediators include inducible TNFα, IL-23, and IL-10 expression, which are all decreased, and IL-10, which is increased (26).

This study elucidated novel effects of Apremilast on blood lipids, demonstrating its efficacy in beneficially modulating lipids. Specifically, Apremilast elevated HDL-C while decreasing circulating TG in psoriatic patients. According to a recent study, found an increase in HDL-C serum, as well as a progressive and constant decrease in TC, LDL-C, and TG over the first month of receiving Apremilast (27, 28). Moreover, according to *Blum et al. (2019),* Apremilast may provide additional advantage due to its ability to increase lipolysis, decrease hepatic triglyceride synthesis, and improve cholesterol flux, all of which can lead to improvements in the lipid profile (29).

Moreover, according to Ferguson LD et al. (2022), There were reductions in weight and BMI with Apremilast treatment across all time points compared with baseline, with a mean weight loss of 2.2 kg and a mean BMI decrease of 0.8 kg/m2 by the end of the study (30).

Conclusion

Apremilast as monotherapy is effective in reducing the inflammatory cytokines and is effective in decreasing IL17- and IL23- levels, which have pro-inflammatory effects, this leads to improvement in psoriatic lesions. Additionally, Apremilast exhibits

favorable modulation of the lipid profile, decreasing triglycerides and increasing HDL cholesterol. Though total and LDL cholesterol increased

Limitation:
The study was conducted at a single centre, the Dermatology Centre, Medical City in Baghdad, and the study had a limited sample size of 30 patients initially, with 6 participants withdrawing before completion.

Authors' declaration:
We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's Approval-Ethical Clearance: The project was approved by the local ethical committee in (the College of Medicine, University of Baghdad) according to the code number (145 on 1/11/2023). In addition, approval of the Baghdad Hospital was obtained. While verbal agreement was obtained from patients to participate.
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Authors' contributions:
Study conception & design: (Haitham M. Saad, Adil A. Noaimi, Halla Gh. Mahmood). Literature search: (Haitham M. Saad, Adil A. Noaimi, Halla Gh. Mahmood). Data acquisition: (Haitham M. Saad, Adil A. Noaimi, Halla Gh. Mahmood). Data analysis & interpretation: (Haitham M. Saad, Adil A. Noaimi, Halla Gh. Mahmood). Manuscript preparation: (Haitham M. Saad, Adil A. Noaimi, Halla Gh. Mahmood). Manuscript editing & review: (Haitham M. Saad, Adil A. Noaimi, Halla Gh. Mahmood).

Adil A. Noaim is an **Editorial board member** but did not participate in the peer review process other than as an author.

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تقييم تأثير أبريميلاست على مستويات الإنترلوكين-17 والإنترلوكين-23 والدهون لدى مرضى الصدفية المصابين بالسمنة

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الخلاصة

خلفية البحث: الصدفية هو مرض التهابي مناعي مجهول السبب قد يرتبط بخلل في تكاثر وتمايز الخلايا الكيراتينية المرتبطة بتسلل الخلايا الالتهابية. هوا مرض عالمي الحدوث؛ ويتراوح معدل انتشاره بين المجموعات السكانية المختلفة من 0.1% إلى 11.8%، وفقا للتقارير المنشورة. أدى تلقي ابريميلاست إلى انخفاض كبير في IL-23 وIL-17A، كما أدى إلى انخفاض التعبير عن السيتوكينات الالتهابية الأخرى وتحسين من مرض الصدفية
الهداف: الغرض من هذه الدراسة هو تقييم تأثير ابريميلاست على مستويات IL-23 , IL-17 والدهون لدى الأشخاص المصابين بالصدفية و يعانون من السمنة المفرطة.
الطرق: تم تضمين ثلاثين مريضا يعانون من السمنة والصدفية في هذه الدراسة التدخلية المستقبلية لقياس المستويات المصلية لملف الدهون، والإنترلوكين-17 والإنترلوكين-23، قبل وبعد تلقي العلاج بأبريميلاست. تم استخدام اختبار "اختبار T" للمقارنة بين المتوسط
النتائج: كان متوسط عمر المشاركين 38 عاما. وكانت الفئة العمرية الأكثر شيوعا هي (30-40 عاما). وكانت مستويات الإنترلوكين-17 قبل إعطاء أبريميلاست 225.55 ±7.70 بيكوغرام/مل. بعد ستة أشهر من العلاج بأبريميلاست، لوحظ انخفاض ذو دلالة إحصائية، حيث انخفضت القيمة إلى 183.41 ±2.33 بيكوغرام/مل. تم قياس مستويات الإنترلوكين-23 قبل إعطاء أبريميلاست بـ 76.42 ±4.03 بيكوغرام/مل. بعد ستة أشهر من العلاج بأبريميلاست، أظهرت هذه المستويات انخفاضا غير ملحوظ إحصائيا إلى 67.15 ±5.40 بيكوغرام/مل (P > 0.05). كما لوحظت تغيرات طفيفة في ملف الدهون (P > 0.05).
الاستنتاجات: أدى استخدام ابريميلاست إلى انخفاض ملحوظ إحصائيا في مستويات السيتوكينات الالتهابية IL-17 و IL-23, أدى استخدام عقار ابريميلاست لدى الأشخاص المصابين بالصدفية الذين يعانون من السمنة المفرطة إلى انخفاض مستويات الدهون الثلاثية(TG) وارتفاع مستويات البروتين الدهني عالي الكثافة (HDL) . بالإضافة إلى ذلك، لوحظ ارتفاع في مستويات الكوليسترول الكلي(TC) والبروتين الدهني منخفض الكثافة (LDL) .
الكلمات المفتاحية: الصدفية, السمنه, أبريميلاست، IL-17, IL-23.

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Research Article

The Significance of Albumin Concentration and Some of Its Altered Forms in Iraqi Patients with Chronic Hepatitis B Virus

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Abstract:

Background: The liver synthesizes albumin, a pivotal protein that accounts for approximately 60-65% of total plasma proteins. During ischemic attacks linked to oxidative stress, reactive oxygen species, and acidosis, albumin's properties undergo alterations. This leads to the generation of ischemia-modified albumin, characterized by a diminished metal-binding capacity, particularly for transition metals like copper, nickel, and cobalt.

Objectives: This study aimed to assess the significance of serum albumin and ischemia-modified albumin concentrations in Iraqi individuals with hepatitis B virus

Methods: A case-control study, including 50 patients with hepatitis B recruited from a Gastroenterology hospital in the Medical City/ Baghdad/ Iraq, was conducted from January to February 2023. The patients' group included males and females, ages 18 to 77 years, with a mean value of 44 years. Meanwhile, the study group consisted of 50 sex-matched normal healthy individuals. Albumin concentration was determined in the serum samples using a Biosystems kit, and ischemia-modified albumin concentration was measured through the albumin cobalt binding test. The ischemia-modified albumin/ [albumin] ratio and ischemia-modified albumin index were then calculated.

Results: lower serum albumin concentration was measured in the patients' group, while the mean value of ischemia-modified albumin concentration in the patients' group and healthy control was 0.466 ± 0.114 absorbance unit & 0.395 ± 0.070 absorbance unit, respectively, with a statistically significant increase ($P < 0.001$). The ischemia-modified albumin ratio in the hepatitis B patients and control groups was 0.172 ± 0.073 and 0.117 ± 0.050, respectively, showing a significant increase ($P < 0.001$). Additionally, the ischemia-modified albumin index in the patients and the control groups were 0.491 ± 0.167 & 0.390 ± 0.131, respectively, with a statistically significant increase ($p<0.001$) in the patients group.

Conclusion: In the patients' group with hepatitis B, serum albumin concentration decreased, while the levels of ischemia-modified albumin, ischemia-modified albumin ratio, and ischemia-modified albumin index increased. The elevation in ischemia-modified albumin, ischemia-modified albumin ratio, and ischemia-modified albumin index was more prominent in younger patients and those with albumin concentrations less than 4g/dl. Moreover, the prevalence of hepatitis B is higher in men compared to women.

Keywords: Albumin concentration; Chronic hepatitis B Virus; Ischemia modified albumin; Ischemia modified albumin index; Ischemia modified albumin ratio.

Introduction

Hepatitis B virus (HBV) infection is one of the widespread and most important public health problems worldwide (1). Globally, about 2 billion people have been infected with the hepatitis B virus, and about 5% of them have chronic infections (2). As a marker of active HBV infection; the seroprevalence of hepatitis B surface antigen (HBsAg), was previously reported to be 3.61% worldwide, this indicates that a substantial number of people are chronically infected with this virus (3). Due to the consequences of HBV infection, it is estimated that each year about 600,000 people die. In addition, according to statistics from the Iraqi Ministry of Health, the number of Iraqi individuals who were infected with viral hepatitis B in 2022 was 2040(4). Human Serum Albumin (HSA), a crucial protein synthesized in the liver, serves essential roles such as maintaining osmotic pressure & transporting various metabolites in the bloodstream (5). Additionally, HSA exhibits the ability to bind specific metals, including copper, cobalt, and nickel, via its amino-terminal end. It undergoes some alterations in its structure (post-translation modification). Under normal physiological conditions, such alteration is minimal (6) This leads to the imposition of a new concept known as effective albumin concentration, a term, that indicates that albumin concentration is reduced under some conditions such as disease, but also, its quality as well altered. These alterations are due to several reversible and nonreversible changes, which lead to changes in albumin properties as well as the structure and production of different albumin isoforms (7). Several

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factors including ischemic attacks associated with excess production of reactive
oxygen species (ROS). the presence of oxidative stress (OS), and acidosis (8) development induces such alteration and thus the production of such different isoforms (9) (10), in which IMA is considered the most important isoform (11) and (12). This isoform was reported as an oxidative stress marker (13). Under conditions of ischemic attacks associated with excess production of reactive oxygen species (ROS), oxidative stress, and acidosis development,certain alterations in albumin properties occur (14). Among these alterations, the N-terminal sequence (Asp1-Ala2-His3-Lys4) of (HAS) is highly susceptible to certain biochemical modifications and degradation induced by oxidative stress. This leads to a reduction in the affinity of the N-terminal sequence towards cobalt, resulting in a variant of albumin known as ischemia-modified albumin (IMA) (9). Various models have been proposed to explain the formation of IMA, according to one of them, the α-amino group of Asp1 exhibits nucleophilic properties, leading to a nucleophilic attack on the peptide bond between Ala2 and His3, which results in the cleavage and release of a cyclic dipeptide. This truncated albumin is unable to bind transition metal ions (14). Another model suggests that during ischemia and acidosis, release of Cu^{2+} from weak binding sites occurs, and in the presence of reducing agents, such as ascorbic acid, free Cu^{2+} is converted to Cu^{+}, which reacts with O_2 to generate superoxide radicals. The albumin N-terminus scavenges these ions, forming hydrogen peroxide (H2O2) and subsequent production of hydroxyl-free radicals. This process causes damage to HSA, leading to the removal of two, or three of the amino acids at the N-terminal end and the release of Cu^{2+}. This chain reaction is repeated resulting in a rapid increase in IMA concentration following an ischemic attack (14) Recently the parameters: ischemia-modified albumin ratio (IMAR) and ischemia-modified albumin index (IMA index), have been introduced to compensate for the albumin concentration effect (15). In this context, this study aimed to compare the significance of albumin concentration, [IMA], IMAR, as well as IMA index in Iraqi patients with hepatitis

Materials and Methods:

A case-control study consisted of 50 patients with hepatitis B and 50, age and gender-matched healthy individuals as control. Blood samples were collected from patients who were attending Gastroenterology Hospital in the Medical city/ Baghdad/ Iraq, during the period from January to February 2023. The study Participants were both males and females, whose ages ranged from 18 to 77 years with a mean value equal to 44 years. Patients who had any type of infection other than chronic virus B, or had any other disease such as heart disease, diabetes mellitus, liver cirrhosis, or alcohol drinkers, smokers, and drug users were excluded from the study. The individual diagnosis testing kit (cat. No: vc010503, Sure Biotech (USA) co., ltd) comprises a test cassette, a dropper, a buffer, and a package insert. The cassette's test line region is precoated with antibodies against Hepatitis B surface antigen HBsAg. During the testing process, the serum specimen (or the whole blood) interacts with particles coated with anti-HBsAg antibodies. Through capillary action, the mixture migrates upward on the chromatographic membrane, reacting with anti-HBsAg antibodies on the membrane to produce a visible colored line. The presence of this colored line in the test region signifies a positive result, while its absence indicates a negative result. As a procedural control, a colored line always appears in the control line region, confirming that the correct specimen volume has been added. The diagnosis was also confirmed by the specialist at the same hospital from where the blood samples were collected for detection of the viral RNA in the blood using a PCR device and some biochemical enzymatic parameters including alanine aminotransferase (ALT) activity, aspartate aminotransferase (AST) activity, and alkaline phosphatase (ALP) activity. Blood samples of the patients and healthy individuals were subjected to centrifuge to obtain serum to conduct laboratory tests including measurement of the concentration of albumin and IMA. The albumin concentration was measured using the Biosystems kit (cat. No: b012139 Biosystem S.A. Costa and Spain) based on the reaction of bromocresol green with albumin and the formation of a colored product which was measured at ƛ= 630nm. A laboratory procedure based on the method mentioned (16) was used to determine the concentration of serum IMA. In this procedure, a volume (120 microliters) of cobalt dichloride reagent was added to the serum (35 microliters), and then the mixture was incubated for five minutes. During this incubation period, binding between the cobalt and the N- N-terminal of unmodified albumin occurred. To remove the unbound cobalt, a volume (35 microliters) of dithiothreitol reagent (DTT) was added resulting in color development. The change in the color was followed using ƛ= 480 nm and the IMA value was expressed in absorbance units (ABSU). IMA ratio (IMAR) was calculated as follows (17). IMAR = IMA absorbance/Alb. concentration While the IMA index was calculated as follows [16]: Individual [IMA] × individual Albumin concentration

$$\text{IMA index} = \frac{\text{Individual [IMA]} \times \text{individual Albumin concentration}}{\text{Median albumin concentration}}$$

Statistical Analysis: The GraphPad prism 9.5.1 (733) program (t-test, One-Way ANOVA, and Pearson correlation) was used to analyze the obtained results and to perform the correlation relationships, respectively. Throughout this work, the obtained results were reported as a mean value ± standard deviation. The differences were considered highly significant if ($p < (0.001)$ ***), and significant where ($p = (0.002)$ **) and ($p = (0.033)$ *)

Results:

The general characteristics of the individuals enrolled in the current study are shown in Table 1.

Table 1: Demographic characteristics and laboratory data of the studied groups

		Control group	Patient group	P value
Number		50	50	
Age/year	Total range (18-77)	41.800± 14.400	44.000± 15.307	<0.999
	range (18-50)	33.733± 6.313 (n=30)	34.867± 8.476 (n=30)	0.559
	range (51-77)	63.615±7.206 (n=9)	65.111± 6.900 (n=9)	0.632
Male	Percentage (number)	34 (17)	79.5 (31)	
	Age range /year	44.180± 9.665	48.118±14.115	0.351
Female	Percentage (number)	66 (33)	20.5 (8)	
	Age range	49.125±19.838	43.000±18.134	0.530
ALT (U/L)		19.940±15.330	46.350± 49.850	<0.001
AST (U/L)		23.130± 9.070	36.550± 32.230	0.008
ALP (U/L)		195.820± 59.900	129.228±72.660	<0.001
(PCR)		-	+	

The values were expressed as mean value ± S.D.

ALT: alanine aminotransferase activity. AST: aspartate aminotransferase activity. ALP: alkaline phosphatase activity. PCR: Polymerase Chain Reaction Test.

As it is clear from the above results the levels of ALT, and AST activities were significantly elevated (P<0.001, P=0.008) respectively in the patients as compared with the controls, while those of ALP activity were reduced. The changes in these enzymatic activities were used to confirm the infection of the patients' group with hepatitis B and this was based on (18). The levels of albumin concentration in the patients' group and the healthy controls were 2.985± 0.891 g/dl and 3.694± 0.972 g/dl respectively with significantly lower concentrations in the patients (P<0.001), while the levels of IMA, IMAR, and IMA index were 0.466± 0.114 and 0.395± 0.070, 0.171± 0.073 and 0.117± 0.777, 0.491± 0.167 and 0.390± 0.131 with a significant elevation (P<0.001) in the patients as compared with the controls. Both controls and patients with hepatitis B groups were separately divided, based on serum albumin concentration, into two groups: those with [Albumin] < 4g/dl and those with [Albumin] > 4g/dl. The obtained results are shown in **Table** 2.

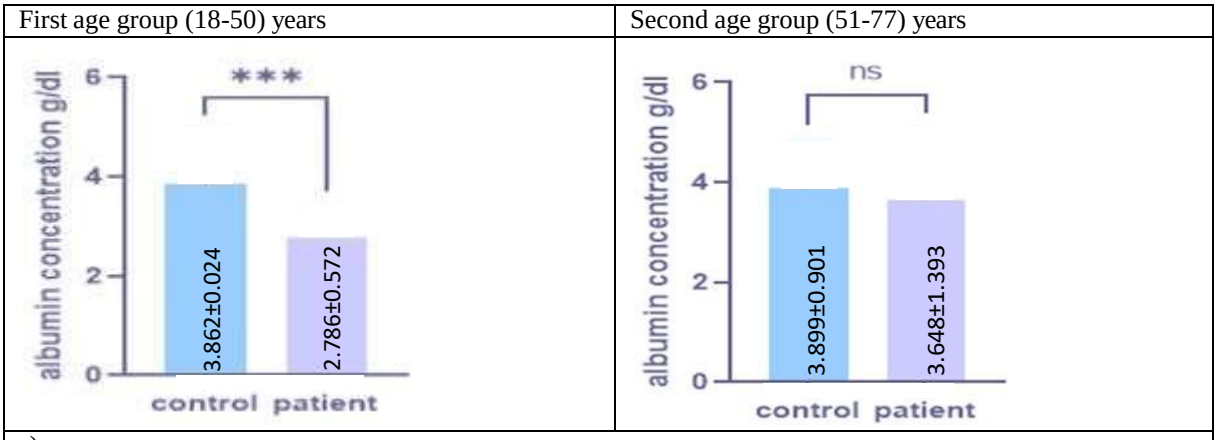
Table 2: Comparison of the levels of IMA, IMAR, and IMA index between patients and control groups according to albumin concentration.

	Albumin concentration < 4 g/dl		P value	Albumin concentration>4 g/dl		P value
	Control group	Patient group		Control group	Patient group	
IMA (ABSU)	0.388± 0.062	0.470± 0.121	<0.001	0.377± 0.045	0.436± 0.046	0.077
IMAR	0.124± 0.025	0.184± 0.071	<0.001	0.085± 0.016	0.091± 0.013	0.484
IMA index	0.331± 0.076	0.454± 0.137	<0.001	0.451± 0.049	0.748± 0.126	0.001

The values were expressed as mean value ± S.D.

The above results illustrated that the level of IMA, IMAR, and IMA index were significantly higher (P<0.001) in the patient group as compared with that in the controls. When the albumin concentration was < 4 g/dl there were no significant variations in IMA and IMAR levels between the controls and patients when the albumin concentration was >4g/dl, except in the IMA index which was statistically increased (P=0.001).

In this study, the controls and the patients were divided into two groups based on age. The first group consisted of 30 people aged between 18 and 50 years, and the second group consisted of 9 people aged between 51 and 77 years. The results of the IMA, IMAR, and IMA index are shown in Figure 1.



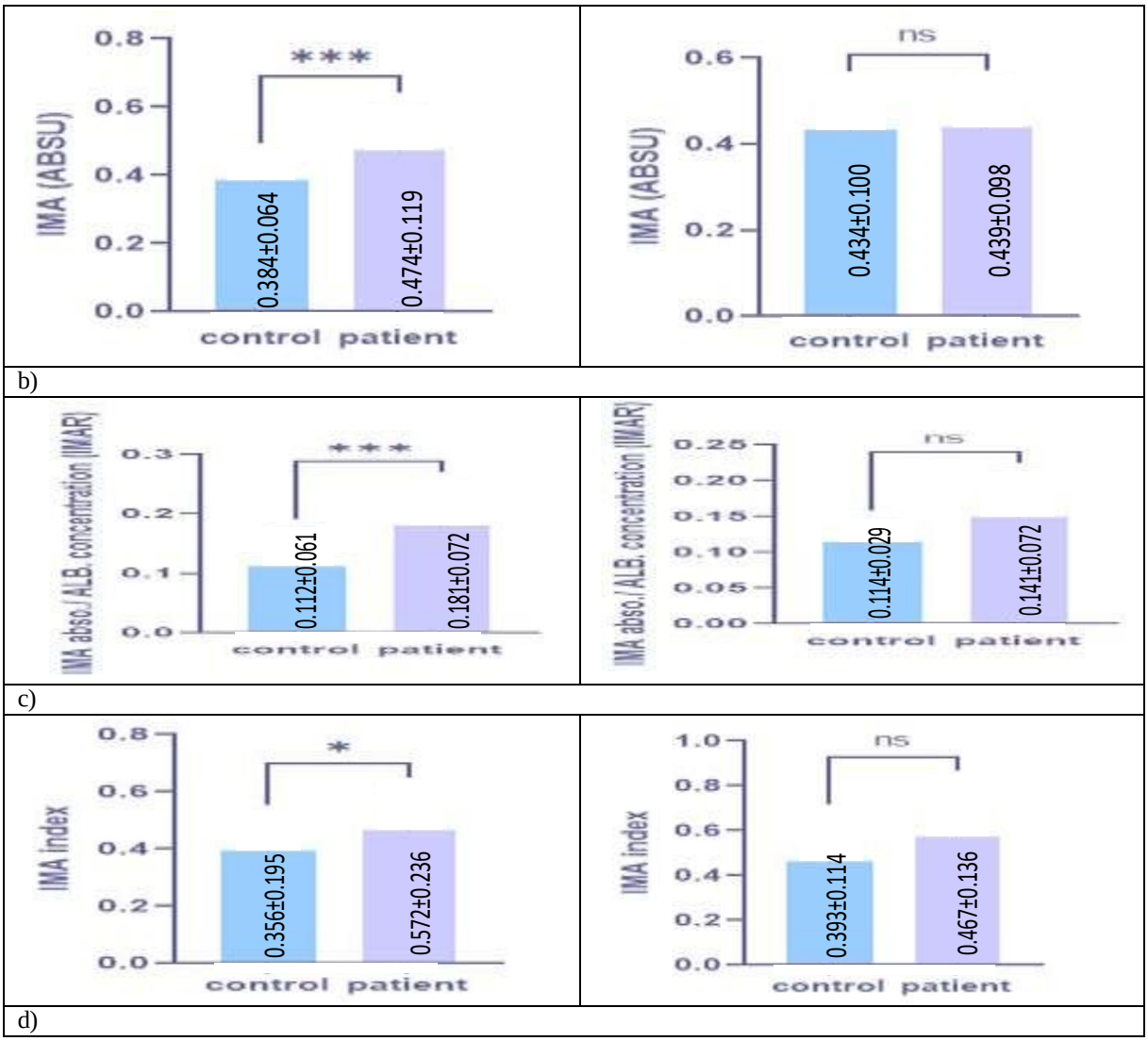


Figure 1: Comparison of the: a): albumin concentration, b): IMA, c): IMAR, and d): IMA index in the hepatitis B patients according to age. ns refer to non-significant. *: The difference is significant at P <0.05 level. ***: The difference is highly significant at the P<0.001 level.

It can be observed from these results that the level of albumin concentration was significantly lower (P<0.001), while the levels of IMA, IMAR, and IMA index were higher in patients as compared with the controls (P<0.001) for IMA, IMAR, and P=0.028) for IMA index in the first age group (18- 50 year). In the meantime, there were no observed significant variations in albumin concentration, IMA, IMAR, and IMA index (P=0.644, P=0.236, P=0.158, and P=0.657) respectively in the second age group (51- 77 years). The patients' group was divided based on gender and the obtained results of all measured parameters are shown in Table 3.

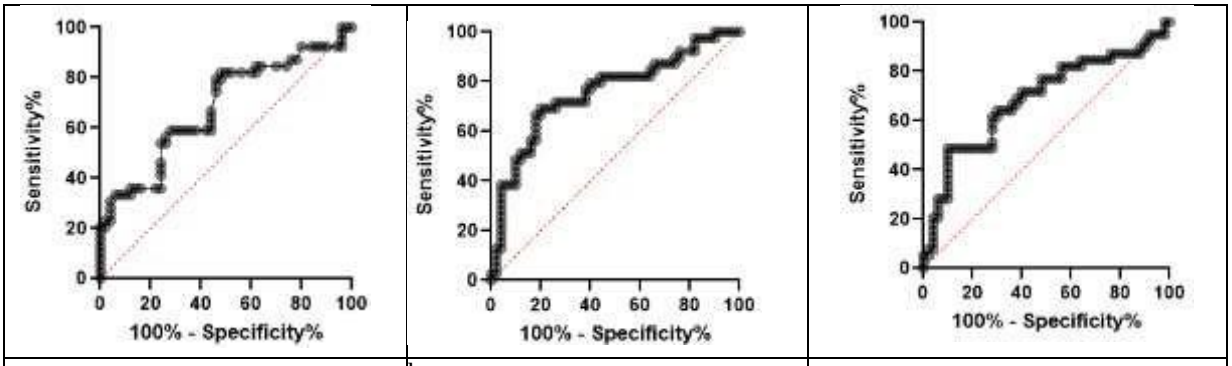
Table 3: Comparison of the albumin concentration, IMA, IMAR and IMA index in the hepatitis B patients according to their gender distribution.

	Male	Female	P value
Percentage (Number)	79.5% (n=31)	20.5% (n=8)	
Age/ year	48.118± 14.115	43.000±18.134	0.569
Albumin (g/dl)	3.143± 0.833	2.368± 0.830	0.024
IMA (ABSU)	0.472± 0.113	0.454± 0.133	0.568
IMAR	0.162± 0.060	0.216± 0.101	0.032
IMA index	0.525± 0.155	0.384± 0.181	0.168

The values were expressed as mean value ± S.D.

As it is clear from the above results in the hepatitis B female, the albumin concentration decreased significantly (P= 0.024) in comparison with that of male patients and the IMAR significantly increased as compared with that of male patients (P= 0.032). Meanwhile, there was no observed significant variation in IMA and IMA index as shown in Table 3

Receiver operating characteristic (ROC) curves analysis for IMA, IMAR & IMA index in the chronic hepatitis B patients' group, and the computed area under the curve (AUC) was found to be 0.6851 (95% CI: 0.5722– 0.7980) for IMA and its specified cut-off value of > 0.4550 revealed 43.59% sensitivity and 76% specificity. However, ROC analyses for IMAR revealed that the computed AUC was 0.7579 (95% CI: 0.6539– 0.8620), and for the specified cut-off value was > 0.1644 with the calculated sensitivity and specificity were 41.03% and 90%, respectively. Furthermore, the ROC analyses for the IMA index revealed that the computed AUC was 0.6877 (95% CI: 0.5725– 0.8029), and for the specified cut-off value of >0.7055 and the calculated sensitivity and specificity were 10.26% and 96%, respectively. The curves for IMA, IMAR, and IMA index are summarized in Figure 2.



a. b. c.

Figure 2: ROC curves (a) for IMA in the patients (n=39). (b): for IMAR in the patients (n=39). (c): for IMA index in the patients (n=39).

The effect of the entecavir (Figure 3) treatment on the level of albumin, IMA, IMAR, and IMA index was tested using 11 patients, who were under this Table **4**. These results showed that the entecavir drug had non-significant effects on the level of IMA, IMAR, and IMA index.

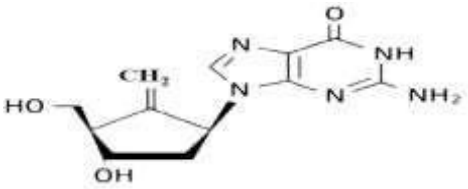


Figure 3: The chemical structure of entecavir.

treatment at a dose of 0.5 mg once a day orally and compared with those without treatment and results were shown in

Table 4: Comparison of IMA, IMAR, IMA index, and Albumin level in patients with treatment and those without treatment.

	Control group	Patient without treatment	Patient with treatment	P value between control and patient without treatment	P value between control and patient with treatment	P value between patient with treatment and without treatment
Number	11	11	11			
Age (year)	48.818±17.429	47.363±14.580	46.909±16.040	0.837	0.792	0.946
Albumin concentration mg/dl	3.837±0.553	2.828±1.036	2.772±0.497	0.010	<0.001	0.873
IMA (ABSU)	0.415±0.070	0.464±0.117	0.441±0.087	0.247	0.448	0.610
IMAR	0.113±0.026	0.189±0.099	0.163±0.041	0.019	0.002	0.440
IMA index	0.438±0.115	0.461±0.185	0.439±0.134	0.611	0.826	0.753

Values were expressed as mean value ± S.D.

Discussion:

The present study covered the variations in the albumin and IMA concentration in hepatitis B infected Iraqi patients, Human serum albumin is the most abundant circulating protein in the plasma (19)

The hypoalbuminemia recorded in the present study may be due to the decrease in the synthesis of albumin as a result of liver parenchymal failure (12), (13) Meanwhile, synthesis of this protein, which is one of the phase proteins, has been reported to be inhibited

by the presence of acidosis (chronic, but not acute), and by proinflammatory cytokine (20), (21).

Furthermore, the balance between albumin synthesis, catabolism, its intravascular and interstitial compartments intestinal exchange, or its renal loss, determines its concentration in the blood plasma (22), (7). The observed increase in the level of IMA in HBV Iraqi patients in the present research may be a result of the presence of chronic oxidative stress in hepatitis B patients. The measured elevation in IMA concentration agreed with (18) who reported in their studies in different HCV patients and agreed with (21) in their study on chronic liver disease in Turkish patients and with coronary collateral circulation in Chinese patients (7). Also, the measured elevated IMA in the present study patients agreed with the results of a study about acute ischemic stroke (7). Furthermore, Jagiełło (2012) reported that the high concentration of IMA might indicate chronic oxidative stress in chronic hepatitis C infection associated with metabolic complications (13). The reported high IMA level in HBV patients in the present study may be due to the increased formation of free radicals which causes oxidative damage to albumin N-terminal residues. Moreover, it may be due to changes in the liver microenvironment resulting from an inflammation caused by the viral infection. IMA was suggested to be a parameter that assesses albumin function (14).

Furthermore, it was reported to be affected by the level of albumin, hence it was suggested that IMAR and IMA index are more valuable indicators than IMA alone (13). Therefore, they were introduced as biochemical parameters to eliminate the albumin-level effect (23). In the current study, these two parameters were found to be elevated in HBV Iraqi patients. IMAR and IMA were also reported to reflect liver excretory function (14) and the observed elevation in IMAR not only reduces the effective circulating volume that is associated with the decreased albumin concentration in blood plasma but also indicates a reduction of the toxic metabolites such as bile acids, fatty acids, tryptophan… etc., removal from the blood because of the impairment in albumin binding capacity (25). Such reduction in albumin binding capacity results in the circulation of the waste products in their free forms which lead to their random reactions, instead of being delivered for clearance in a specific site. Both this deficiency in albumin's functional capacity to remove different toxins, as well as act as an efficient antioxidant predispose to liver function decompensation as a result of the disturbance in the live (25) (26).

The results of variations in the measured biochemical parameters according to the gender of the patients agreed with the result of (27) in their study on Nigerian patients with hepatitis C and (22) in their study on coronary disease. The higher prevalence of HBV among the males compared with females included in the current research may be explained by the higher possibility of men's exposure to viral infection than women. This finding could be in general, due to the fact that men are being employed to perform many activities outside their households (27). In this study, in order to look up if the variations in the measured biochemical parameters were affected by the patient's age, he present research results indicated that this type of infection was higher in the younger ages than in the elderly ones. These results agreed with the results of (28) in their study on Pakistani Patients with hepatitis B and are comparable with what was reported in central Nigeria patients with hepatitis B (29). The application of entecavir as a treatment regimen had no effect on the present study's measured parameters. This drug is known to affect HBV replication (30) The obtained results with this type of treatment may be due to the short period used for the treatment, which resulted in a non-observed effect of this type of treatment on either albumin concentration, or its measured related parameters in the current studied patients, or it may be due to that the impairment in the function of albumin in HBV patients was irreversible.

Limitations: The numbers of females and males were unequal, and there was an unbalanced distribution of age. This lead**s** to considering the present study results regarding these factors, as a pilot one which led to the statistical power of these factors on the present obtained results being limited

Conclusion:

The non-significant variations were obtained when the measurement results were analyzed according to gender, elderly age, and the effect of entecavir treatment were based on comparative samples of a small population, the number**s** of female**s** and males were unequal, as well as unbalanced distribution of age. This lead**s** to considering the present study results regarding these factors, as a pilot one which lead to the statistical power of these factors on the present obtained results were limited. Meanwhile, the ROC analysis pointed out to high specificity of each IMA, IMAR, and IMA index with low sensitivity and IMAR may be a promising advantage for liver function tests in patients with chronic HBV.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's Approval-Ethical Clearance: The project was approved by the local ethical committee in (Gastroenterology Hospital in the Medical City/ Baghdad/ Iraq) according to the code number (CSEC/1223/0139) on (1/ 1/ 2023).

Conflicts of Interest: None

Funding: None

Author contributions:

Study conception & design: (Zahraa Faris and Hathama Razooki Hasan). Literature search: (Zahraa Faris and Hathama Razooki Hasan). Data acquisition: (Zahraa Faris). Data analysis & interpretation: (Zahraa Faris and Hathama Razooki Hasan). Manuscript preparation: (Zahraa Faris and Hathama Razooki Hasan). Manuscript editing & review: (Zahraa Faris and Hathama Razooki Hasan).

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اهمية تركيز الألبومين وبعض أشكاله المتغيرة لدى المرضى العراقيين المصابين بفايروس التهاب الكبد ب الوبائي المزمن

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الخلفية: يقوم الكبد بتخليق الألبومين، وهو بروتين محوري يشكل حوالي 60-65% من إجمالي بروتينات البلازما. أثناء النوبات الإقفارية المرتبطة بالإجهاد التأكسدي وأنواع الأوكسجين الفعالة والحامضية، تخضع خصائص الألبومين لتغييرات. تؤدي إلى توليد الألبومين المعدل بالاقفار، والذي يتميز بانخفاض قدرة الارتباط بالمعادن، خاصة بالنسبة للمعادن الانتقالية مثل النحاس والنيكل والكوبالت.
هدف الدراسة: هدفت هذه الدراسة إلى تقييم تراكيزالألبومين والألبومين المعدل بالاقفار (IMA) لدى الأفراد العراقيين المصابين بفيروس التهاب الكبد ب(HBV) .
الطرائق العمل: شملت دراسة الحالة- السيطرة 50 مريض مصاب بالتهاب الكبد الوبائي ب المزمن
تم جمع نماذج الدم منهم اثناء مراجعتهم مستشفى أمراض الجهاز الهضمي في مدينة الطب في بغداد، العراق، خلال الفترة من كانون الثاني إلى شباط من عام 2023 . وشملت مجموعة المرضى ذكور واناث تراوحت أعمارهم بين 18 سنة إلى 77 سنة بمتوسط عمر 44 سنة. تم أيضا جمع 50 افراد اصحاء متوافقين بالعمر والجنس مع مجموعة الدراسة لاستخدامهم كسيطرة. تم تحديد تركيز الألبومين في المصل باستخدام عدة Biosystem ، وتم قياس تركيز الالبومين المعدل بالاقفار في المصل من خلال اختبار ربط الكوبالت بالألبومين. وتم حساب نسبة الالبومين المعدل بالاقفار/الألبومين (و نسبة الالبومين المعدل بالاقفار) ومؤشر الالبومين المعدل بالاقفار.
النتائج: وجدت تراكيز واطئة للالبومين في مصول المرضى مما في مجموعة السيطرة وكان متوسط تراكيزال الالبومين المعدل بالاقفار في مجموعة المرضى والسيطرة الاصحاء 0.466 ± ABSU 0.114 و 0.395 ± ABSU 0.070، على التوالي، مع زيادة ذات دلالة معنويةإحصائيا (P <0.001).و كانت ال نسبة الالبومين المعدل بالاقفار في مرضى التهاب الكبد الفايروسي ب ومجموعة السيطرة مساوية الى 0.172 ± 0.073 و 0.117 ± 0.050 على التوالي، مما يدل على زيادة كبيرة أيضا (P <0.001). بالإضافة إلى ذلك، كان مؤشر الالبومين المعدل بالاقفار في المرضى ومجموعة السيطرة 0.491 ± 0.167 و 0.390 ± 0.131، على التوالي، مع زيادة ذات دلالة معنوية إحصائيا (P <0.001).
الاستنتاج: في مجموعة المرضى المصابين بالتهاب الكبد الفايروسي ب المزمن، انخفض تركيز الالبومين في الدم، في حين ارتفع مستوى الالبومين المعدل بالاقفار، نسبة الالبومين المعدل بالاقفار، ومؤشر الالبومين المعدل بالاقفار. كان الارتفاع في مؤشر الالبومين المعدل بالاقفار، نسبة الالبومين المعدل بالاقفار، ومؤشر الالبومين المعدل بالاقفار أكثر وضوحا في المرضى الأصغر سنا والذين لديهم تركيز الألبومين أقل من 4 ملي غرام / 100 مليلتر. علاوة على ذلك، كان معدل انتشار التهاب الكبد الفايروسي ب أعلى بين الرجال مقارنة بالنساء.

الكلمات المفتاحية: تركيز الألبومين، فيروس التهاب الكبد ب المزمن، الالبومين المعدل بالاقفار، مؤشر الالبومين المعدل بالاقفار، نسبة الألبومين المعدل بالاقفار.

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Research Article

Estimation of Salivary IL-6 and Calprotectin in Patients with Ulcerative Colitis

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Abstract

Background: Inflammatory bowel disease is a chronic inflammatory condition affecting the gastrointestinal tract, encompassing two primary conditions: Crohn's disease and ulcerative colitis. Calprotectin, a protein released by keratinocytes, phagocytes, monocytes, granulocytes, and vascular cells, plays a key role in the body's inflammatory response. It is recognized by toll-like receptors, which trigger pathways that lead to inflammation. The chronic nature of inflammatory bowel disease presents a significant health challenge, requiring precise methods for regular assessment and monitoring of disease activity. Elevated calprotectin levels are widely recognized as a biomarker for detecting inflammation in the gastrointestinal tract, making it an essential tool in managing inflammatory bowel disease, particularly ulcerative colitis.

Objectives: To examine whether significant differences exist in the levels of interleukin-6 and calprotectin between patients with ulcerative colitis and healthy control subjects, this study analyzes and compares these inflammatory markers across both groups. suggesting that both markers could serve as potential diagnostic tools for ulcerative colitis. Furthermore, these findings highlight saliva as a non-invasive source for evaluating inflammatory markers in patients with ulcerative colitis.

Methods: The subjects included were twenty-five patients with ulcerative colitis and twenty-five healthy individuals as the control group. All of whom ranged in age from 20-55 years, and the levels of interleukin-6 (IL-6) and calprotectin in the saliva of ulcerative colitis patients were measured using the ELISA method.

Results: When compared to the control group, the current findings indicated that both (IL-6) and calprotectin levels were significantly higher in UC patients. Moreover, this study found a significant positive correlation between IL-6 levels and age in all study groups (UC and control) and between IL-6 and calprotectin in UC patients.

Conclusion: There are higher levels of IL-6 and calprotectin in the saliva of patients with UC disease, both markers could be used as diagnostic markers for UC disease

Keywords: Calprotectin; Inflammatory Bowel Diseases; Interleukin-6; Saliva and Ulcerative Colitis

Introduction:

A chronic inflammatory ailment called inflammatory bowel disease (IBD) has been linked to cytokines in terms of its pathophysiology and etiology. Ulcerative colitis (UC) and Crohn's disease (CD) are the two most prevalent clinical forms of IBD (1). In people with a genetic predisposition to IBD, leads to inflammation and intestinal ulcers (2). There are numerous Therapeutic options available for the idiopathic chronic inflammatory disease of the colon known as ulcerative colitis (3).

One type of inflammatory bowel illness that affects the colon and the rectum is ulcerative colitis. Rarely does it affect infants and young children (4). It is still unclear what causes ulcerative colitis and how it develops. The notion that a genetic element is key in the progression of the disease, however, has received attention (5). The incidence has increased in nations that have adopted an industrialized lifestyle, which refers to regions where steps have been done to enhance the state of health globally, such as. vaccination, gastrointestinal disease prevention, processed foods, etc. Exacerbations can be life-threatening and come with problems. Severe UC is diagnosed based on clinical, biochemical, and endoscopic findings Serious UC patients need to be hospitalized (6). Interleukin-6 (IL-6) is produced in acute inflammatory responses that aid in host defense. It is involved in the processes of immune response regulation, inflammation, hematopoiesis, and cancer (7). Immune responses may be disrupted if IL-6 levels are elevated as IL-6 is involved in the regulation of lymphocyte tracking through the lymph node following developmental stimulation (7). It was stated that IL-6 promotes the change from severe to chronic inflammation by secreting the monocytes chemo-attractant protein-1 (MCP-1) (8). IL-6 and TNF are regarded as the two main mediators of the inflammatory process. These cytokines have systemic effects that include raised body temperature, enhanced lymphocyte activation, and neutrophil mobilization (9).

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It was concluded that patients with IBD have significantly elevated levels of IL-6 in their plasma (10). Calprotectin is generated by phagocytes, keratinocytes, granulocytes, monocytes, and vascular cells and causes an inflammatory (11). Calprotectin, also known as the migration inhibitory factor-related proteins 8 and 14, is an acute-phase protein that regulates neutrophil migration; its quantity corresponds with neutrophil migration and indicates the intensity of inflammation in IBD. Calprotectin levels in saliva could be employed as a predictive diagnostic as well as a measure of treatment efficacy. However, doctors must keep in mind that oral inflammation, obesity, oral candidiasis, and periodontal disease all have an impact on calprotectin secretion (12,13).

This study aimed to measure IL-6) and calprotectin levels in the saliva of UC patients as those cytokines have been previously proven to be elevated in sera of UC patients but have not been proved yet to be increased in saliva.

Materials and Methods:

Four milliliters of unstimulated saliva were taken from twenty-five (UC) patients and twenty-five healthy controls. For the purpose of performing the salivary analysis of IL-6 and calprotectin, the saliva samples were centrifuged for 10 minutes at 3500 rpm/min, and the supernatant was divided into two Eppendorf tubes and kept at -70°C. Commercial ELISA kits for human IL-6 and calprotectin (USA) were used to measure the salivary levels of each marker according to the manufacturer's instructions using a micro-plate reader and the absorbance was measured at a wavelength of 450 nm (Huma Reader HS, Germany).

Statistical analysis:

The statistical analysis was done in two categories:

Descriptive analysis, in which data was presented as minimum, maximum, mean, and standard deviation (SD) for quantitative variables, and frequency for qualitative variables. And inferential analysis: Inferential analysis was used to clarify valuable insights about the differences and relationships between different variables in the study community. We used parametric statistical analysis. This type of analysis is employed when dealing with continuous data and assumes that the data follows a normal distribution. The statistical tests used in the tables are the F-test, correlation coefficients, the chi-square test, or Fisher's exact test, as indicated by the p-values and independent t-tests.

Results:

The mean and standard deviations for the age of patients with UC and healthy people have the following comparable values (33.400 ± 1.0905, 30.280 ± 0.6321 ng\ ml), respectively, with no significant differences seen between both groups (*P*> 0.05) as presented in table (1).

Table (1): Distribution of study UC according to age

	Age (years)				
Group	NO	Mean	S.D.	Min.	Max.
Control	25	30.280	0.6321	23	48
UC	25	33.400 Y	1.0905 Y	20 Y	55 Y

(*p*-value UC = 0.46) (P-value control= 0.46)

The results in table (2) shown that UC patients have percentages of 48% and 52% for males and females, respectively, and the control group matches the patients' group as it has percentages of 60% for males and 40% for females with no significant difference has been observed between them (*P*>0.05).

Table (2): Distribution of subjects according to gender

Gender	Control	UC
Males	15 (60%)	12 (48%)
Females	10 (40%)	13 (52%)
Total	25 (100%)	25 (100%)

(*p*-value UC = 0.46) (*p*-value control = 0.46).

The mean levels of salivary interleukin-6 and calprotectin showed a higher concentration with a significant difference in the ulcerative colitis group when compared with the healthy controls ($p < 0.05$) as seen in Tables 3 &4.

Table (3): The mean levels of Il6 and calprotectin in study groups

Parameter	Group	No	Mean	S.D.	S.E.	Min.	Max.	*p* values
Calprotectin ng /ml	Control	25	169.800	18.867	3.773	132.784	198.777	0.000
	UC	25	241.871	6.830	13.660	135.592	378.897	
IL-6 pg\ml	Control	25	53.509	8.996	1.799	33.291	65.609	0.000
	UC	25	85.537	3.004	6.009	43.864	166.048	

Table (4): A comparative F-test for IL-6 and calprotectin levels among the study groups

Parameter		Sum of Squares	d.f.	Mean Square	F-test	*p*-value
Calprotectin	Between Groups	140327.097	2	70163.548	15.991	0.000
	Within Groups	315915.556	47	4387.716		
	Total	456242.653	49			
IL-6	Between Groups	20867.773	2	10433.886	21.100	0.000
	Within Groups	35603.598	47	494.494		
	Total	56471.371	49			

Furthermore, the correlation between IL-6 and each of calprotectin and age in ulcerative colitis patients was positive and statistically significant as correlation coefficient values were (r=0.614 and r=0.405), respectively, (*P* <0.01) as shown in (Table 5).

Table (5): Correlation coefficient of IL-6 with calprotectin and age in ulcerative colitis patients

UC			
Parameter		Calprotectin	IL-6
Age	r	-0.282	0.405
	P	0.172	0.039
Calprotectin	r		0.614
	P		0.001

The correlation coefficient between IL-6 and calprotectin in healthy control group was positive non-significant correlation (*P*>0.05) but the correlation between IL-6 and age was a significantly positive (r = 0.421) (p<0.05) as shown in table (6).

Table (6): Correlation coefficient of IL-6 with calprotectin and age in control group

Control			
Parameter		Calprotectin	IL-6
Age	r	0.017	0.421
	P	0.936	0.036
Calprotectin	r		0.289
	P		0.161

Discussion:

Furthermore, this work showed no significant differences in exposure rate to UC between both genders (p>0.05) whereas an epidemiological survey from East Asian countries notably Japan and China showed lower incidence in females than males (28). According to data from twelve Asian–Pacific countries, it was demonstrated a male predominance of UC from adolescence till age of 65 years, after which UC incidence rates were similar between females and males (30).

The ages of UC patients in present study ranged from 20-55 years with a mean value of 33.4±1.09 years. Similarly, the results of Nijakowski et al(2021)that was carried out in 2021 showed that the UC group has an age range between 24 -40.5 years with a mean age value of 32 years. Also, comparable results were seen by a cross-sectional study conducted at the Kurdistan center for gastroenterology and hepatology of the teaching hospital in Sulaymaniyah, Iraq, which included 101 patients who had previously been diagnosed with inflammatory bowel disease that showed that UC patients have a mean age value of 45.74years (16, 17). In general, females and males showed similar incidence of UC before age 45; however, above age 45 years, males demonstrated higher risk of UC incidence than females (29).

This study selected saliva collection as a straightforward and non-invasive approach for UC patients. It was observed that there was a statistically significant difference in salivary IL-6 levels between UC patients and the control group whereas other studies have also revealed that unstimulated saliva of IBD patients has higher levels of IL-6 (32). Other studies have previously demonstrated that IL-6 levels are increased in patients with inflamed, non-adhesive intestinal mucosa of IBD (33).

The patients with UC had higher IL-6 concentrations in their saliva. Because the cells that produce saliva are components of the digestive system, this may suggest that the inflammatory process in the intestine induces a significant release of IL-6 in the saliva (22). Another study illustrated that the activity of IBD might be estimated from the levels in saliva as well as plasma in UC patients (23).

After thorough validation of our analytical methods and protocol, the current study compared calprotectin levels in unstimulated saliva from UC patients with ongoing intestinal inflammation to controls. The calprotectin levels were substantially higher in UC patients' saliva than the control group. The findings of this investigation have been supported by another previous study which also observed significantly elevated levels of calprotectin in saliva of patients with ulcerative colitis as compared with control group (24).

Calprotectin is mostly present in neutrophils and, to a lesser extent, in other cells, calcium-binding protein in reactive macrophages and monocytes (25). Plasma calprotectin has been reported to increase 5- to 40-fold in inflammatory and infectious circumstances, and it has bacteriostatic and fungi-static characteristics. Stool contains calprotectin, and fecal calprotectin concentration is approximately six times that of normal plasma. Patients with intestinal irritation had feces with noticeably higher amounts of calprotectin (26).

This is the first study that evaluated calprotectin level in saliva. It was noted that calprotectin had a significant higher levels in UC group when compared with control group whereas previous researchers looked at calprotectin in feces and proved that fecal calprotectin levels in gastrointestinal disorders, such as gastritis, gastric ulcer, gastric carcinoma, duodenitis, ulcerative colitis, have significantly higher levels than the controls (27). Compared to the control group, the concentrations of calprotectin and myeloperoxidase in saliva were significantly lower both in CD patients and in UC patients (31).

Conclusion:

This study concluded that levels of interleukin-6 and calprotectin in saliva are higher in patients with ulcerative colitis compared to healthy individuals (control group), suggesting that both markers could serve as potential diagnostic tools for ulcerative colitis. Furthermore, these findings highlight saliva as a non-invasive source for evaluating inflammatory markers in patients with ulcerative colitis. Further research is recommended to explore the relationship between these marker levels and disease progression, which may enhance patient care and guide therapeutic strategies.

Authors' Declaration:
We here by confirm that all the Figures and Tables in the manuscript are ours. The project was approved by the local ethical committee in College of Dentistry/ University of Baghdad, Iraq.
Conflicts of Interest: None
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Authors' Contributions:
Study conception & design: (Maha A. Mahmood). Literature search, Data acquisition, Data analysis, interpretation & Manuscript preparation:(Fadhel A. Abed). Manuscript editing & review:(Maha A. Mahmood).

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تقدير الإنترلوكين اللعابي 6 وكالبروتكتين في مرضى التهاب القولون التقرحي

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الخلاصة
مرض التهاب الأمعاء هو التهاب مزمن في الجهاز الهضمي ويشمل مرض كرون والتهاب القولون التقرحي ، وقد يتسبب الالتهاب لفترات طويلة في غزو الطبقات المتعددة من جدران الأمعاء مما يؤدي إلى تلف الجهاز الهضمي.
الهدف : أجريت الدراسة الحالية من أجل توضيح ما إذا كانت العلامة التشخيصية لـ IBDوهيIL6 وcalprotectin ترتفع في اللعاب كما هو الحال في الأمصال وما إذا كانت هناك فروق ذات دلالة إحصائية في المستويات من تلك العلامات بين مرضى التهاب القولون التقرحي وموضوعات المراقبة الصحية.
طريقة البحث :أجريت الدراسة الحالية في مستشفى بغداد التعليمي ومستشفى أمراض الجهاز الهضمي من تشرين الثاني (نوفمبر) 2021 إلى أيار (مايو) 2022. شملت الدراسة خمسة وعشرين مريضا يعانون من التهاب القولون التقرحي وخمسة وعشرون من الأفراد الأصحاء يمثلون المجموعة الضابطة. تراوحت أعمار كل منهم بين 20-55 سنة .تم قياس مستويات Interleukin-6 وcalprotectinفي لعاب مرضى التهاب القولون التقرحيUCباستخدام طريقة ELISA في الدراسة الإحصاء تم إجراء التحليل الإحصائي على فئتين: التحليل الوصفي: تم تقديم البيانات على أنها الحد الأدنى والحد الأقصى والمتوسط والانحراف المعياري (SD) للمتغيرات الكمية وتكرار المتغيرات النوعية. والتحليل الاستنتاجي: تم استخدام التحليل الاستدلالي لتوضيح رؤى قيمة حول الاختلافات والعلاقات بين المتغيرات المختلفة في مجتمع الدراسة. استخدمنا التحليل الإحصائي البارامترية. يتم استخدام هذا النوع من التحليل عند التعامل مع البيانات المستمرة ويفترض أن البيانات تتبع التوزيع الطبيعي. الاختبارات الإحصائية المستخدمة في الجداول هي اختبار F ، ومعاملات الارتباط ، واختبار مربع كاي ، أو اختبار فيشر الدقيق ، كما يتضح من قيم p واختبارات t المستقلة.
النتائج :عند المقارنة بمجموعة التحكم ، أشارت النتائج الحالية إلى أن كلا من (IL-6) ومستويات calprotectin كانت أعلى بشكل ملحوظ في مرضى التهاب القولون التقرحي ($P = <0.05$) .علاوة على ذلك ، وجدت هذه الدراسة ارتباطا إيجابيا معنويا بين مستويات IL-6 والعمر في جميع مجموعات الدراسة (التهاب القولون التقرحي والتحكم (P 0.05) وبين IL-6 وcalprotectinفي مرضى التهاب القولون التقرحي (P <0.05)
الاستنتاج :خلصت هذه الدراسة إلى أن هناك مستويات أعلى من IL-6 و calprotectin في لعاب المرضى الذين يعانون من مرض UC مقارنة بالأشخاص الأصحاء مجموعة التحكم) ونتيجة لذلك ، يمكن استخدام كلا الواسمتين كواسمات تشخيصية لمرض UC.
الكلمات المفتاحية: انترلوكين -6, كالوبروتكتين , مرضى التهاب القالون التقرحي.

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Research Article

The Role of Activin A levels, Body Mass Index and Beta-Human Chorionic Gonadotropin in Ectopic Pregnancies and Missed Abortions – A Study on a Group of Iraqi Women

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Abstract

Background: Activin A (ACV-A), a member of the transforming growth factor-beta (TGF-beta) superfamily that regulates follicular growth hormone (FSH) secretion and initiates intracellular signaling pathways, is essential to reproductive regulation. ACV-A is involved in regulating cellular proliferation, differentiation, apoptosis, and homeostasis, among other biological processes. The pituitary gland, the gonads, and other organs all secrete ACV-A, which is made up of two beta A (βA) subunits.

Objectives: To compare the serum concentrations of ACV-A in women diagnosed with missed abortion (MA) or ectopic pregnancy (EP) with those of healthy controls.

Methods: The study was conducted in the gynecology departments of the Medical City - Baghdad Teaching Hospital and Al-Kut Obstetrics and Gynecology Hospital from October 2023 to January 2024. A total of 120 women aged between 18 and 45 years participated in the study; An ectopic pregnancy was diagnosed in 30 of them, 30 had a missed miscarriage, and the remaining 60 were considered a control group. ACV-A and beta-human chorionic gonadotropin (β-HCG) levels were measured by the enzyme-linked immunosorbent assay (ELIZA) method.

Results: The control group had a significantly lower mean ± SD of ACV-A (773.6 ± 130.26 pg/ml) in comparison to the EP group (1408.1 ± 219.02 pg/ml) and the MA group (1200.9 ± 199.31). In addition, patients in the ectopic group had a significantly lower mean± SD of ACV-A than patients in the missed abortion group.

Conclusion: Serum Activin A levels can be used as an indicator of ectopic pregnancy and missed abortions. A novel biomarker for evaluating women who have an ectopic pregnancy could be the level of HCG in their serum at a cutoff value of greater than 236 ng/ml.

Keywords: Activin A (ACV-A); BMI; Ectopic pregnancies; Missed abortions; β-HCG.

Introduction:

Ectopic pregnancy (EP) refers to fertilized eggs implanted outside of the uterus, typically in the Fallopian tubes (98%) (1). It may be linked to a genetic defect that results in an aberrant development (2). Women with an EP may experience vague symptoms like pain in the lower abdomen and vaginal bleeding. These symptoms frequently mimic the clinical presentation of trauma, kidney stones, and hepatitis (3). When a blastocyst embeds itself some place other than the coating of the uterus, this condition is known as an ectopic pregnancy (4). Vaginal infections, intrauterine devices (IUDs), assisted reproductive technologies (ARTs), and previous EP are additional established risk factors for EP (5). A "missed abortion" is the type of spontaneous abortion in which the embryo has already died but with a closed cervical ostium (6). The known potential causes include infections, fetal rejection by the mother's immune system, and environmental factors (7). Defects in the embryonic chromosome have been identified as the most common cause of unsuccessful pregnancies (8). Identifying the cause of the (MA) can help in speeding up the diagnostic process, giving a precise estimate of the likelihood of a recurrence, and providing comfort and direction (9). Human chorionic gonadotropin beta, (β-HCG) is detected in the maternal blood two days after implantation (10), and together with transvaginal ultrasound (TVUS) have become standard procedures in the evaluation of difficulties connected with early pregnancy (11). ACV-A is a bi functional glycoprotein that belongs to a class of growth factors called transforming growth factor - β (TGF-β). ACV-A secretion has been demonstrated by many reproductive tissues, such as the ovaries, the uterine cavity, the testicles, the endometrium, and the pituitary gland (12).

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Predicting and diagnosing preeclampsia in the second and third trimesters of pregnancy has been made easier by ACV-A (13). Numerous biologic fluids (cerebrospinal fluid, cord blood, peripheral blood and urine) showed elevated ACV-A levels early in life in fetuses and newborns who had been subjected to chronic and acute oxygen deprivation, perinatal death by asphyxia, and cerebral bleeding (14). Thus the current study established to compare the serum concentrations of ACV-A in women diagnosed with missed abortion (MA) or ectopic pregnancy (EP) with those of healthy controls.

Patients and Methods:

A case-control study was carried out in the gynecological wards of the Medical City Teaching Hospital in Baghdad and Al-Kut Hospital for Gynecology and Obstetrics from October 2023 to January 2024. A total of 120 women between the ages of 18 and 45 years participated in the study. Thirty women had an ectopic pregnancy, 30 had missed abortions, and the remaining 60 were healthy pregnant controls. The concentration of serum activin A and β-HCG was measured at Al-Kut Hospital for obstetrics and gynecology using the ELISA method. The study questionnaire included demographic characteristics such as age and body mass index classified according to the classification of the World Health Organization (15).

Blood samples were taken from the participants after obtaining their consent to the blood drawing procedure.

Laboratory tests were conducted in one of the private laboratories in Baghdad and in Al-Kut Hospital for Gynecology, where blood samples were drawn from the patients and serum ACV-A and β-HCG were measured using the ELISA method.

Statistical Analysis:

The data was analyzed using SPSS version 25.0 software. Frequencies, percentages, means and stander deviations were used to describe the data. Graphs were used to present the data. The Chi-square test was used to test associations between qualitative variables and the independent t test was used to test differences of mean between two quantitative variables.

Results:

The distribution of the cases and controls by age and BMI is shown in Table 1. The table shows that there were no statistically significant associations between these two variables in the study groups.

Table 1: Distribution of the study groups by age and BMI

Variables	Categories	Study Groups – No. (%)			Total No. (%)	p- value
		Controls	EP	MA		
Age group (Years)	≤ 20	6 (10.0)	5 (16.7)	6 (20.0)	17 (14.2)	0.96
	21-35	48 (80.0)	21 (70.0)	18 (60.0)	87 (72.5)	
	> 35	6 (10.0)	4 (13.3)	6 (20.0)	16 (13.3)	
BMI (kg/m2)	Low	4 (6.7)	1 (3.3)	2 (6.7)	7 (5.8)	0.50
	Normal	36 (60.0)	19 (63.3)	15 (50.0)	70 (58.3)	
	Overweight	18 (30.0)	10 (33.3)	12 (40.0)	40 (33.3)	
	Obese	2 (3.3)	0 (0)	1 (3.3)	3 (2.5)	
Total (100.0%)		60	30	30	120	120

Table 2 shows the mean ± SD of Activin-A and β-HCG in the three study groups. The control group had a significantly lower mean ± SD of ACV-A compared to the EP group and the MA group. In addition, the mean of the EP group was significantly lower than that of the MA group. The mean ± SD of β-HCG was lowest in the EP group followed by the MA group and the controls.

Table 2: Mean± SD of ACV-A and β-HCG in the study groups

Mean ±SD	Study Groups			Total	p-value
	Controls	EP	MA		
ACV-A a, b, c	773.6 ±130.26 (16.18)	1408.06 ± 219.02 (39.98)	1848.24 ± 222.37 (39.93)	1200.86 ± 199.31 (36.38)	<0.001*
β-HCG a, b, c	382.0 ± 80.21 (13.26)	284.5 ± 40.65 (7.42)	329.7 ± 70.94 (12.95)	337.5 ± 78.41 (9.84)	< 0.001*

a: Controls and EP, b: Controls and MA c: EP and MA, *p-value is significant

Activin-A had the largest AUC (0.766) with a cut-off level of >236, with 100% sensitivity and 67% specificity, to help distinguish EP from the other categories. β- HCG had the lowest AUC (0.688) with a cut-off level of >1027, with 93% sensitivity and 69% specificity, as shown in Table 3.

Table 3: ROC test for biochemical markers in EP patients

Test Result Variables	AUC	Cut value	off	p-value	Sensitivity	Specificity
ACV-A	0.688	>1027	0.002	100%	67%	
β-HCG	0.766	>236	0.000	93%	69%	

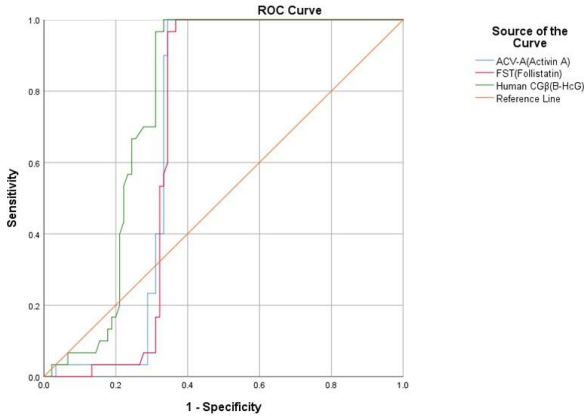

Figure 1: ROC curve for biochemical markers in the EP group

Activin-A had the largest AUC of 0.97 and a cutoff value of >1531, with 93% sensitivity and 92.2% specificity, with the objective of distinguishing patients who had MA from other patients, whereas β-HCG had the lowest AUC of 0.88 and a cutoff value of >261 and 93.3% sensitivity and 77% specificity, as shown in Table 4.

Table 4: ROC test for the biochemical markers in the MA group

Test Variables	Result	Area	Cut value	off	Sensitivity	Specificity	p-value
ACV-A		0.97	>1531		93%	92.2%	<0.001
β-HCG		0.88	>261		93.3%	77%	<0.001

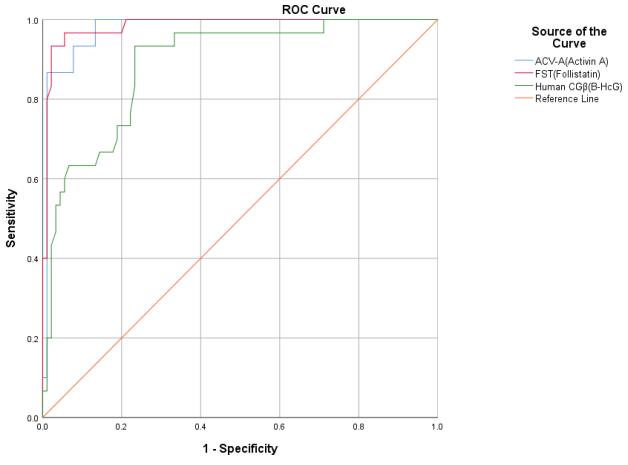

Figure 2: ROC curve for the biochemical markers in the MA group

Discussion:

The findings of the current study regarding the not-significant distribution of the three study groups by age and BMI are in agreement with that of Suliman (16) who found that age is not significantly associated with the risk of EP. Salem (17) reported non-significant differences in patient's age between controls and MA cases. In the current study the mean age of the MA group was higher than that of the controls. Zakira (18) reported a mean age of 28 years for the control group and 31 years for the MA group. Women between 25–29 years of age had the lowest chance of MA, while those 45 years old or over were the highest. The findings of the current study of the significant differences in mean ACV-A among the three groups are in agreement with those of Humadi (19) who reported that a successful pregnancy has considerably greater serum levels of ACV-A, than a failed pregnancy. It appears that cytotrophoblasts secrete ACV-A, and that aberrant decidualization with poorly implanted trophoblasts is typically seen during tubal pregnancy. Conversely, in heterogeneous ectopic pregnancies (one fetus inside the uterus), increased serum ACV-A may be detected. The current investigation found significant differences in the mean β-HCG among the three study groups. Lu (20) found that the only biomarker that is often and widely utilized in medical care is β-HCG. Although β-HCG is not sufficient to diagnose EP on its own, it can be useful in recognizing patients who need more frequent screening for early pregnancy loss. For an effective intrauterine pregnancy (IUP), there should be a minimum 53% increase in β-HCG over a period of 48 hours. But such a strategy involves multiple follow-ups over a few days for EPs, which prolongs the possibility of tubal rupture. On the other hand, the study of Daponte A (21) supports the idea that it is possible to distinguish between an IUP and a MA or EP with just one measurement of ACV-A at 6–8 weeks of gestation. More significantly, the current research shows that serum ACV-A can help to distinguish between an EP and an MA. However, there has been contradictory research looking into the use of serum ACV-A for this purpose. Serum ACV-A levels in pregnancy were shown to increase by 69-fold (with a broad range of values) from 700 ± 200 pg/mL at weeks 6-7 to a peak of 45,900 ± 54,000 pg/mL between weeks 38 and 39. It appears that this mechanism can be further impaired in EP and perhaps even more so in unsuccessful pregnancies. Lower levels of ACV-A in EPs have been compared to those in other failed pregnancies; it has been suggested that this could be because the ectopic trophoblast finds it difficult to implant correctly, which compromises the decidualization process. Additionally, some EPs may have more active trophoblasts and behave more like IUPs, while other EPs will be failing and behave more like failing MAs Daponte (21) discovered that there is a weak association between β-HCG and ACV-A in IUPs, suggesting a moderate but statistically significant association. Muttukrishna (22) found that in women with a subsequent miscarriage, there was a positive correlation between plasma ACV-A and

progesterone, estradiol, and HCG. To distinguish EP from other groups, β-HCG had the highest AUC, high sensitivity and moderate specificity. The most widely used serological marker of EP, β-HCG, is crucial for the early detection of EP. As per our experimental findings, low levels of β-HCG may raise suspicions about EP, however β-HCG by itself neither supports or disproves EP. On the other hand, Ray (23) found with a high sensitivity, specificity, and positive predictive value, ROC analyses showed that pre-treatment levels B-HcG of ≤4000 mIU/ml had a greater likelihood of successful outcome after medical management.

Receiver Operation Characteristic (ROC) and Area Under the Curve (AUC)

β-HCG is the most widely used serological biomarker of EP and is crucial for the identification of early EP. Although low levels of β-HCG can raise suspicions about EP, they do not definitively confirm or rule out the condition as suggested by Marion (24). A moderate sensitivity and specificity were obtained with the cutoff of β-HCG of 24,300 mIU/mL. Ray (23) also found a moderate sensitivity and specificity, a favorable positive predictive value of 90.3%, and a negative predictive value of 75%, ROC analyses indicated that a pre-treatment processes β-HCG levels.

Conclusions:

Serum Activin A levels can be used as an indicator of ectopic pregnancy and missed abortion. Serum β-HCG level at a cutoff value of >236, ng/ml may be a novel biomarker for the assessment of women with ectopic pregnancy.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's Approval-Ethical Clearance: The project was approved by the local ethical committee in (Place where the research was conducted or samples collected and treated) according to the code number (43) on (20/ 05/ 2024).

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Authors' Contributions:

Study conception & design: (Manal K Rasheed). Literature search: (Manal K Rasheed). Data acquisition: (Hussein Mohan Rafik & Farah Abdul Hussein Salih). Data analysis & interpretation: (Hussein Mohan Rafik & Farah Abdul Hussein Salih).Manuscript preparation: (Hussein Mohan Rafik). Manuscript editing & review: (Manal K Rasheed).

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في حالات الحمل خارج الرحم وحالات (β-HCG) وموجهة الغدد التناسلية المشيمية بيتا البشرية (BMI) ومؤشر كتلة الجسم A دراسة دور مستويات الأكتيفين الإجهاض الفائتة لدى مجموعة من النساء العراقيات

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الخلاصة
خلفية البحث: يعد Activin A (ACV-A)، وهو عضو في فصيلة عامل النمو المحول بيتا (TGF-beta) التي تنظم إفراز هرمون النمو الجريبي (FSH) ويبدأ مسارات الإشارات داخل الخلايا، ضروريًا لتنظيم التكاثر. يشارك ACV-A في تنظيم تكاثر الخلايا، والتمايز، وموت الخلايا المبرمج، والتوازن، من بين العمليات البيولوجية الأخرى. تفرز الغدة النخامية والغدد التناسلية والأعضاء الأخرى ACV-A، الذي يتكون من وحدتين فرعيتين βA (بيتا A).
الأهداف: مقارنة تركيزات Activin A في مصل الدم في النساء اللاتي تم تشخيص إصابتهن بالإجهاض الفائت (MA) والحمل خارج الرحم (EP) مع تلك الموجودة في العينة الضابطة من السيدات الأصحاء اللاتي لا يعانين من أي من هذه الحالات.
الحالات والمنهجية: جريت الدراسة في أقسام أمراض النساء في مدينة الطب - مستشفى بغداد التعليمي ومستشفى الكوت لأمراض النساء والولادة في الفترة من أكتوبر 2023 إلى يناير 2024.
وشارك في الدراسة ما مجموعه 120 امرأة تتراوح أعمارهن بين 18 و45 عامًا؛ وتم تشخيص الحمل خارج الرحم في 30 منهن، و30 لديهن اجهاض مفقود، وكانت الـ 60 المتبقيات يعتبرن كمجموعة ضابطة. تم قياس مستويات ACV-A وموجهة الغدد التناسلية المشيمية بيتا البشرية (β-HCG) بواسطة طريقة مقايسة الامتصاص المناعي المرتبط بالإنزيم (ELIZA).
النتائج: كان لدى المجموعة الضابطة متوسط أقل بكثير ± SD لـ ACV-A (773.6 ± 130.26 بيكوغرام / مل) مقارنة بمجموعة EP (1408.1 ± 219.02 بيكوغرام / مل) ومجموعة MA (1200.9 ± 199.31). بالإضافة إلى ذلك، كان لدى المرضى في مجموعة الإجهاض خارج الرحم انخفاضًا ملحوظًا مقارنة بالمرضى في مجموعة الإجهاض الفائت.
الإستنتاجات: يمكن استخدام مستويات مصل Activin A كمؤشر على الحمل خارج الرحم والإجهاض الفائت. يمكن أن يكون مستوى HCG في مصل الدم من العلامات الحيوية الجديدة لتقييم النساء اللاتي يعانين من الحمل خارج الرحم عند قيمة قطع أكبر من 236 نانوغرام / مل.
مفتاح الكلمات: اكتفين أ، هرمون موجهة الغدد التناسلية المشيماتية، مؤشر كتلة الجسم، الحمل خارج الرحم، الاجهاض الفائت.

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Research Article

Evaluation of Preptin and Other Biomarkers in Coronary Artery Disease Patients with and without Diabetes Mellitus

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Abstract

Background: Preptin is an endocrine peptide with 34 amino acids. Conjugated with insulin, it is produced by β-cells from the pro-insulin-like growth factor 2 E-peptide. However, in addition to insulin, pancreatic hormone (Preptin) is released in response to elevated blood glucose levels. Preptin's primary metabolic effect is to raise insulin synthesis, achieved through both an amplifying mechanism and a triggering route dependent on calcium signaling.

Objectives: To determine the Preptin in patients with coronary artery disease (CAD) with and without Type 2 diabetes mellitus (T2DM).

Methods: One hundred and twenty Iraqi participants between the ages of 40 and 60 years were enrolled (80 patients and 40 age-sex matched controls). The study occurred between August and December 2023 at Ibn Al-Bitar Center for Cardiac Surgery in Baghdad, Iraq. The level of Preptin in patients with CAD with and without T2DM was evaluated. The biochemical tests performed on participants included fasting blood sugar (FBS), total cholesterol (TC), triglycerides (TG), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL) high-density lipoproteins (HDL), blood urea, serum creatinine, and uric acid. The waist-to-hip ratio (WHR) and body mass index (BMI) were also computed. There was a significance level below 0.05 using the Mann-Whitney tests. A non-parametric method and Spearman's rank coefficient were used to determine the significance of correlation for the relationship between the two numerical variables. We determined the Preptin cut-off value by analyzing the receiver operation characteristic (ROC) curve.

Results: The CAD cases both with and without T2DM had a significantly higher serum Preptin than the control group. The levels of Preptin, HDL, and uric acid were significantly strongly correlated. The Preptin ROC curve showed a clear cut-off value (>601.71, >818.10, and >694.71) with the area under the curve (AUA) (0.973, 0.996, and 0.985) respectively when calculated in three groups: CAD without T2DM, CAD with T2DM, and both CAD groups together compared with the controls.

Conclusion: Preptin may serve as a predictive marker for the progression of declining heart function in people with T2DM. It also works well as a diagnostic tool to distinguish between patients with CAD and those without.

Keywords: Coronary artery disease; Lipid profile; Preptin; T2DM; Uric acid.

Introduction:

Coronary artery disease (CAD) is the cause of high rates of morbidity and mortality associated with cardiovascular diseases and is responsible for about 7 million deaths globally each year (1). An inadequate supply of oxygen and blood to the heart muscle is a hallmark of CAD. The obstruction of coronary arteries causes an imbalance between the supply and demand of oxygen. Plaques that block blood flow in the coronary artery lumen are often the cause of it (2). Numerous interrelated factors impact the pathogenesis of CAD, and its etiology is a very complex process. Thrombosis and stenosis, or mural atheroma, are the two primary causes of CAD when blood clots and stenosis are brought on by thin layers of fibrin and a collection of platelets building up on the lining. Next, regulation causes the intima to thicken. This platelet narrowing and arrangement may be due to factors other than the degradation of the elastic layer. Alternatively, stenosis may result from cholesterol becoming twisted or seeping beyond the endothelium in the barrier made of fibrin and platelets (3, 4). Variations in arterial tension can cause structural damage and degeneration of the artery's elastic layer. With the arteries becoming tiny and inflexible, this encourages the deposition of lipids and other materials that results in the development of mural atheroma, a lipid plaque produced by pro-athermic substance created due to degeneration and structural damage. Lipid plaque, smooth muscle development, and endothelial dysfunction cause the diameters of these blood vessels to narrow, which eventually leads to CAD (3, 5).

T2DM is a metabolic disease with a significant prevalence worldwide. It is primarily caused by a combination of two basic factors: The inability of insulin-sensitive tissues to respond to insulin (insulin resistance) and the defective synthesis of insulin by pancreatic β-cells (6). As a result, irregularities in any of the underlying systems might lead to a

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dysregulation of metabolism, which would then cause T2DM (7). Numerous risk factors, such as age, genetics, stress, hypertension, dyslipidemia, obesity, and inactivity, are linked to T2DM and CAD (8). Moreover, an increase in the incidence of diabetes increases the risk of CAD (9).

Preptin is an endocrine peptide that has 34 amino acids produced by β-cells in tandem with insulin. It is derived from the pro-insulin-like growth factor 2 E-peptide. Preptin is secreted in response to increased blood glucose levels together with insulin (10). Additionally, it can be released by the salivary gland, liver, kidney, and breast tissue, among other organs (11). The main metabolic impact of Preptin is to increase insulin production, which happens via a triggering pathway that depends on calcium signaling in addition to an amplifying mechanism (12).

Premature onset DM, impaired glucose tolerance, polycystic ovarian syndrome, and T2DM have all been positively connected with elevated Preptin levels, according to some studies (13). A previous study indicated that male patients with osteoporosis have been found to have lower bone mineral densities when there is a drop in the amount of circulating Preptin (14). Furthermore, it was shown that the peptide was osteogenic, lowering osteoblast apoptosis via MAP-kinase pathway-related mechanisms. Once endogenous proteases cleave Preptin at phenylalanine, it has a five-minute half-life in vivo (15). The pathophysiological effects of uric acid exerted on the cardiovascular system are responsible for the complex and difficult link between uric acid and CVD. During cardiac ischemia, xanthine oxidase activity affects the synthesis of uric acid by increasing the amount of uric acid through a compensatory rise (16). Increased blood uric acid levels can worsen lipid deposits and endothelial cell damage by increasing platelet aggregation and the release of more vasoactive substances (17). Meanwhile, uric acid precipitates and accumulates as crystals that are phagocytosed by leukocytes in the blood vessels, subcutaneous regions, joints, kidneys, and other tissue, causing damage to the heart and blood vessel intima (18).

The current study set out to measure the levels of Preptin, lipid profile (cholesterol, Triglycerides TG, High density lipoproteins HDL, low-density lipoproteins LDL, very low-density lipoproteins VLDL), fasting blood glucose (FBG), urea, creatinine, and uric acid in the sera of a group of Iraqi patients with CAD (with and without T2DM) and their controls.

Patients and Methods:

Case-control study an assessment of Preptin and its levels in CAD with and without T2DM patients was conducted. One hundred and twenty people, aged from 40 to 60 years participated in the study between August and December 2023. Eighty patients from Ibn Al-Bitar Center for Cardiac Surgery in Baghdad, Iraq, were compared to 40 healthy controls (matched for age and sex). Waist/hip ratio (WHR) and body mass index (BMI) were calculated for each participant.

Exclusion criteria included thyroid illness, osteoporosis, cancer, and polycystic ovaries (in women) for both the cases and the controls.

Laboratory testing included renal function, lipid profile, and fasting blood glucose. To quantify Preptin in the serum, Elabscience-USA provided an ELISA kit. With the use of the Kenza (240TX) Biolabo equipment and kit, biochemical markers such as FBS, TC, TG, HDL, urea, creatinine, and uric acid were analyzed.

Venipuncture was used to obtain 10 milliliters of blood, which was then put into a gel tube to separate the serum. The blood samples were centrifuged at 3000 revolutions / second to obtain the serum. Five aliquots of the serum were separated and stored at -20° C until testing.

Statistical Analysis:

The median, 25^{th,} and 75th percentiles were used to describe the study groups and to compare them. The justification for using these statistics is that the numerical variables were not normally distributed. The Mann-Whitney test was used for the analysis, with the level of significance being less than 0.05. The correlation between two numerical variables was ascertained by using the non-parametric approach and Spearman's rank coefficient. The receiver operation characteristic (ROC) curve was analyzed to ascertain the Preptin cut-off value.

Results:

The demographic characteristics and clinical features are shown in Table 1. The median age for CAD without T2DM, CAD with T2DM, and the controls were not significantly different (P>0.05). The median BMI and WHR were significantly different between the three groups.

Table (1): Medians and percentiles for demographic and anthropometric characteristics of the three study groups

Variable	CAD with T2DM	CAD without T2DM	Control	*P*-value
Age (year)	50.0 (57.0 - 46.0)	53.0 (56.0 - 48.0)	50.0 (52.0 - 43.	N.S
BMI (kg/m2)	32.0 (37.0 - 27.0) a	30.0 (35.0 - 26.0) b	25.0 (26.0 - 24.0)	0.00
WHR	0.93 (1.0 - 0.9) a	1.0 (1.0 - 0.92) b	0.82 (0.83 - 0.81)	0.00

The Mann-Whitney test was used to test the difference between two independent medians
a) CAD without T2DM Group and controls
b) CAD with T2DM Group and Controls.

*WHR: Waist to hip ratio.

Table 2 shows the median blood levels in the three study groups of FBG, cholesterol, TG, HDL, LDL, and VLDL. It shows a significantly higher level of FBG and lipid profile (cholesterol, TG, LDL, and VLDL) in the two CAD patient groups compared to the control group (p<0.001). It also shows a significantly lower median HDL in the two CAD patient groups compared to the control group (p<0.001).

Table (2): Medians and percentiles for the serum glucose and lipids of the three study groups

Variable	CAD without T2DM	CAD with T2DM	Control	*p*-value
FBG (mg /dL)	96.0 (102.0 - 86.0)a	189.0 (214.0 - 169.0)b	90.0 (94.0 - 80.0)	0.00
Cholesterol (mg/dL)	246.0 (271.0-226.0) a	151.0 (199.0 - 121.0) b	131.0 (150.0 -111.0)	0.00
TG (mg/dL)	247.0 (273.0 - 220.0) a	201.0 (242.0 - 157.0) b	90.0 (129.0 -83.0)	0.00
HDL (mg/dL)	32.0 (40.0 - 26.0) a	35.0 (44.0 - 32.0) b	48.0 (49.0 - 46.0)	0.00
LDL (mg/dL)	167.0 (192.0 - 152.0) a	72.0 (44.0 - 119.0)	55.0 (79.0 - 48.0)	0.00
VLDL (mg/dL)	48.0 (54.0 - 44.0) a	39.0 (49.0 - 32.0) b	18.0 (26.0 - 17.00	0.00

The Mann-Whitney test was used to test the difference between two independent medians.
a) CAD without T2DM group and controls.
b) CAD with T2DM group and Controls.

Median blood levels of urea, creatinine, and uric acid for the CAD with T2DM, CAD without T2DM) and control groups are shown in Table 3. Significantly higher values of kidney function tests (urea, creatinine, and uric acid) were seen in the two CAD groups (with and without T2DM) compared to the control group (p<0.001). The CAD with T2DM groups had significantly higher serum Preptin concentrations than those without T2DM and controls (p<0.001).

Table (3): Medians and percentiles for the blood urea, creatinine, uric acid and Preptin of the three study groups

Variable	CAD without T2DM	CAD with T2DM	Control	*p*-value
Urea (mg/ dL)	43.0 (44.0-41.0) a	30.0 (41.0-26.0)	30.0 (35.0-23.0)	0.00
Creatinine (mg/ dL)	1.0 (1.1- 0.9) a	0.9 (1.0 -0.7) b	0.5 (0.6 - 0.4)	0.00
Uric acid (mg/ dL)	6.0 (7.0- 5.0) a	5.0 (6.0 - 4.0) b	4.0 (4.3 - 3.45)	0.00
Preptin (pg/mL)	951.0 (995.0 - 849.0) a	1448.0 (1482.0-988.0) b	459.0 (528.0 - 409.0)	0.00

The Mann-Whitney test was used to test the difference between two independent medians.
a) CAD without T2DM group and controls.
b) CAD with T2DM group and controls.

Table 4 shows non-significant correlation between Preptin and the levels of HDL and uric acid in the control group and CAD without T2DM group, while a significant strong negative correlation was found between Preptin with HDL (r = -0.463**, p<0.01) and a non-significant positive correlation with uric acid (r = 0.349*, p>0.05) in those with CAD with T2DM.

Table (4): Correlation coefficient between Preptin and some studied parameters in the study groups

Parameter and Correlation coefficient		Group		
		CAD without T2DM	CAD With T2DM	Control
HDL (mg/dl)	Correlation coefficient (r)	0.020	-0.463**	0.091
	Sig. (2-tailed)	0.902	0.003	0.578
Uric acid (mg/ dl)	Correlation coefficient (r)	-0.174	0.349*	-0.117
	Sig. (2-tailed)	0.282	0.027	0.471

*Correlation is significant at the 0.05 level (2-tailed)
**Correlation is highly significant at the 0.01 level (2-tailed)

Evaluating the efficacy of serum Preptin concentration in distinguishing CAD with T2DM patients from CAD without T2DM patients and healthy individuals was conducted using ROC curve analysis, Table 5. The effectiveness of blood Preptin levels in distinguishing (CAD without T2DM patients), (CAD with T2DM), and (CAD with T2DM, and CAD without T2DM) compared to the controls was evaluated using the ROC curve analysis. The ROC curve for the (CAD with T2DM) group was much higher than the diagnostic tests, indicating greater validity (high sensitivity 100% and specificity 97.5%). As demonstrated by the area under the ROC curve for the (CAD with T2DM) diagnosis (0.996, p 0.001).

Table (5): Preptin ROC to distinguish between the three groups

Variable	Preptin		
	CAD without T2DM and control	CAD with T2DM and Control	All CAD and control
Area under the curve	0.973	0.996	0.985
p-value	0.001	0.001	0.001
Cutoff value	>601.71	>818.096	694.713
Sensitivity (%)	97.5	100.00	97.5
Specificity (%)	90	97.5	92.5
+ve predictive value	97.5	97.6	97.6
-ve predictive value	97.3	100.00	100.00

Figure 1 shows that Preptin shows the sensitivity of 100 and a specificity of 90.0) p > 0.001)*when* distinguishing between CAD without T2DM and controls.

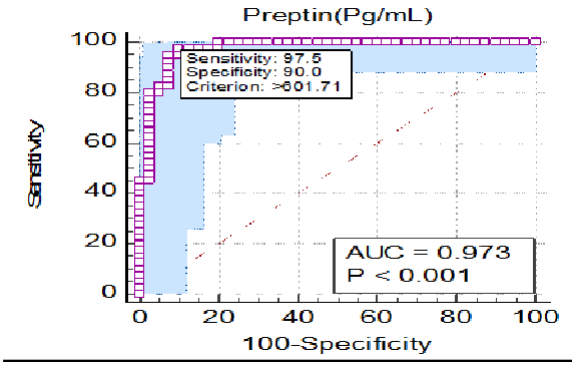

Figure (1): The ROC curve for Preptin distinguishing between CAD withoutT2DM and controls.

Figure 2 demonstrates that Preptin can differentiate between CAD with T2DM and controls with a sensitivity of 100 and a specificity of 90.0, ($p > 0.001$).

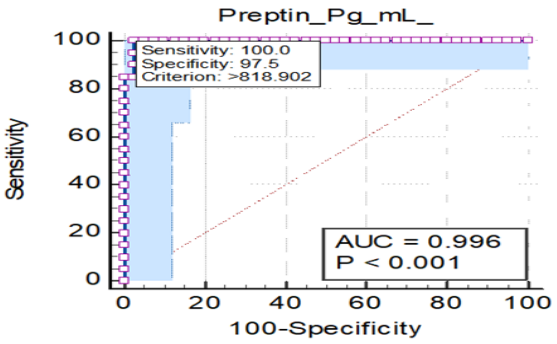

Figure (2): The ROC curve for Preptin distinguishing between CAD withT2DM and controls.

Figure 3 shows that Preptin shows a sensitivity of 97.5 and a specificity of 92.5 when distinguishing between (CAD without T2DM, and CAD with T2DM) and controls.

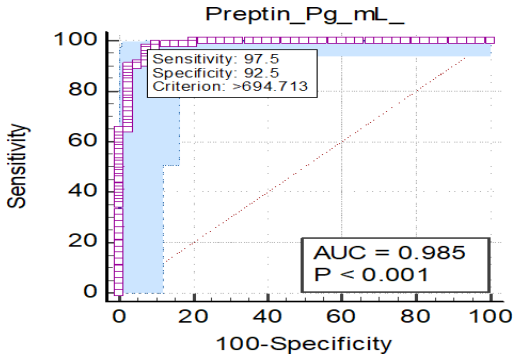

Figure (3): The ROC curve for Preptin distinguishing between all CAD cases and controls.

Discussion:

The finding of the current study that Preptin can be used as a diagnostic marker for CAD patients with T2DM is in line with the findings of Hussein et al who reported that an individual's CAD with T2DM had significantly higher Preptin levels (19). Tahir et al showed that Preptin is essential for regulating the metabolism of sugar. Hence, Preptin levels are elevated in diabetics and heart disease patients, which compromises the control over the metabolism and puts the patient at risk for several additional disease conditions (20). Hassan et al also demonstrated that Preptin levels can predict enhanced pathogenesis of CAD independently and have a significant impact on their advancement. It was linked to atherosclerosis, which is thought to be one of the primary causes of CAD (18). This may be due to Preptin functioning as a physiological amplifier of insulin secretion in response to glucose levels (15). The association between each research group and the following variables was addressed in this study: age, BMI, WHR, lipid profile, urea, creatinine, uric acid, and Preptin. A previous study reported no significant difference in BMI and WHR in Indian patients with CAD when compared to the control (21). The results of the current study that WHR was lower in CAD patients with DM than those without. The two parameters usually rise together with the rise of the prevalence of DM (22), (23). DM is frequently associated with dyslipidemia, which is defined by elevated plasma levels of (TG), (LDL), (TC), and (HDL). Dyslipidemia is a complex condition of lipoprotein metabolism that results from the interplay of hereditary and environmental variables. In individuals diagnosed with T2DM, atherosclerosis and the development of CAD are accelerated (24). Many studies have shown that serum creatinine across all study groups, significantly correlates with the severity of CAD (25).

Limitation: Number of pertinent.

Conclusions:

Preptin may serve as a predictive marker for the progression of declining heart function in people with T2DM. It also works well as a diagnostic tool to distinguish between patients with CAD and those without. In patients CAD with T2DM, the level of (FBG) has risen dramatically. On the other hand, patients' CAD with and without T2DM showed increased TC, TG, LDL, VLDL, Urea, Creatinine, and uric acid levels. HDL levels in both groups were significantly lower compared to the control group.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's Approval-Ethical Clearance: The project was approved by the local ethical committee in (Ibn Al-Bitar Center for Cardiac Surgery in Baghdad, Iraq. According to the code number (4846/22) on (31/ 8/2023)).

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تقييم البريبيتين في مرضى إعتلال الشريان التاجي الذين يعانون من مرض السكري وبدونه

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الخلاصة:
خلفية البحث: البريبيتين هو ببتيد الغدد الصماء مع 34 من الأحماض الأمينية. مقترنا بالإنسولين، يتم إنتاجه بواسطة خلايا β من عامل النمو الشبيه بالأنسولين E-peptide 2. ومع ذلك، بالإضافة إلى الإنسولين، يتم إفراز هرمون البنكرياس (البريبيتن) إستجابة لإرتفاع مستويات الجلوكوز في الدم. يُمثل التأثير الأيضي الأساسي للبريبيتن في زيادة تخليق الإنسولين، والذي يتحقق من خلال كل من آلية التضخيم وطريق التحفيز الذي يعتمد على إشارات الكالسيوم.
الأهداف: تحديد البريبيتين في المرضى الذين يعانون من إعتلال الشريان التاجي مع وبدون داء السكري من النوع الثاني (T2DM).
المنهجية: تم تقييم مستوى البريبيتن في المرضى الذين يعانون من إعتلال الشريان التاجي مع وبدون داء السكري من النوع الثاني (T2DM). شملت الدراسة 120 مشاركا تتراوح أعمارهم بين 40 و 60 عاما شاركوا بين آب وكانون الأول 2023. تمت المقارنة بأربعين شخصا يتمتعون بصحة جيدة (متطابقين في العمر والجنس) مع ثمانين مريضا عراقيا في مركز ابن البيطار لجراحة القلب في بغداد، العراق. لكل مجموعة بحثية، تم حساب مؤشر كتلة الجسم (BMI) ونسبة الخصر إلى الورك (WHR).
النتائج: وجدت الدراسة أن مجموعة المرضى (إعتلال الشريان التاجي مع وبدون داء السكري من النوع الثاني (T2DM)) لديها ارتفاع كبير للغاية في مصل بريبتين مقارنة بمجموعة السيطرة. كانت مستويات البريبتين والمتغيرين (البروتين الدهني عالي الكثافة (HDL) وحمض اليوريك) مرتبطة إرتباطا ذو دلالة إحصائية عالية. أظهر منحنى ROC للبريبيتين قيمة فاصلة واضحة (<601.71>818.096، و>694.713) مع المساحة الموجودة أسفل المنحنى (0.973، 0.996، و0.985) مع p<0.001 على التوالي عند حسابها في ثلاث مجموعات: إعتلال الشريان التاجي بدون داء السكري من النوع الثاني، إعتلال الشريان التاجي مع داء السكري من النوع الثاني، ومجموعة الاصحاء للمقارنة).
الإستنتاجات: قد يكون البريبتين بمثابة علامة تنبؤية لتطور إنخفاض وظائف القلب لدى الأشخاص المصابين بداء السكري من النوع الثاني. كما أنه يعمل بشكل جيد كأداة تشخيصية للتمييز بين الأشخاص الذين يعانون من إعتلال الشريان التاجي مع وبدون داء السكري من النوع الثاني والاصحاء.
مفتاح الكلمات: إعتلال الشريان التاجي، الدهون، البريبتن، السكري نوع الثاني، حمض اليوريك.

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Research Article

The Anti-Inflammatory Effect of *Chenopodium murale* in Comparison to *Salvia frigida* on Atopic Eczema

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Abstract:

Background: Atopic dermatitis (AD) is a prevalent chronic inflammatory skin condition with a familial tendency. It affects approximately 10%-20% of children and 1%-3% of adults worldwide. *Chenopodium murale* is clinically proven for treating many medical conditions, such as AD, due to its easy application and efficacy. *Salvia* plant has an anti-inflammatory effect on AD cases treated with phenolic compounds.

Objective: To determine the anti-inflammatory effect of the phytosterol fraction of *Chenopodium murale* (CM) in comparison to *Salvia frigida* (SF).

Methods: This study was conducted from December 2020 to June 2021 in the Department of Pharmacology, College of Medicine, Al Nahrain University. Fifty mice were included in the study, subdivided equally into five subgroups [control, induction, Tacrolimus-1%, Phytosterol-3%, and Phenolic-5%]. Biological and histological parameters were measured, and their means were compared using the independent t-test, and the one-way ANOVA was used to estimate the mean of differences.

Results: The Tacrolimus-1% group showed a significant decrease in white blood cells, Ig-E, and inflammation means than other groups; a significant decrease in mean epidermal thickness than the Phytosterol-3% groups; and a significant decrease in IL-13 and erosion than the Phenolic-5% groups. The phytosterol-3% group showed a significant decrease in the mean parakeratosis, erosion, and observational severity (OS) score than other groups. The phenolic-5% group showed a significant decrease in the mean epidermal thickness than other groups and a significant decrease in OS score than the Tacrolimus-1% groups.

Conclusion: The topical applications of the phytosterol fraction of *Chenopodium murale* or the phenolic compound of *Salvia frigida* were effective and promising in treating atopic dermatitis. While the phenolic compound of *Salvia frigida* is effective, it is somewhat less than that of the phytosterol fraction of *Chenopodium murale*.

Keywords: Atopic dermatitis; *Chenopodium murale;* Phenolic compound; *Salvia frigida*; Tacrolimus

Introduction

Atopic eczema dermatitis [AD] is a skin condition characterized by inflammation, itching, redness, drying, and scaling. Thirty percent of AD patients also have asthma. AD tends to be persistent with periods of relapse and remission. While some patients may improve during puberty, others may experience lifelong symptoms. Different microbial infection can occur as a result of AD. Restoring skin barriers by moistening is considered crucial in managing AD symptoms like itchiness and inflammatory response. (1-3)

A macrolide lactone, Tacrolimus, derived from fungi, is a widely utilized immune-suppressive medication. Its diminutive size grants it strong skin penetration capabilities. Despite the fact that it effectively treats severe AD and aids in controlling acute flare-ups and preventing new occurrences by its immune-regulating mechanism. However, it is associated with side effects such as skin burning and itchiness (4).

Natural plant extracts exhibit diverse pharmacological effects, including their ability to serve as antioxidants due to their redox property. This enables them to function like reducing agents, hydrogen donors, and quenchers of single oxygen. Phytosterol fraction (PF) shows promise as a nutritious factor in certain conditions like GIT disorders, harnessing both systemic metabolic and local anti-inflammatory effects. Previous studies have demonstrated the utility of plants across various ailments, including anthelmintic, gastric upset, anti-spasmodic, and excessive sweating, while providing relief from conditions such as dysmenorrhea, asthma, colds and migraines (5-6). Chenopodium murale (CM) exhibits similar pharmacological properties to PF; such as anti-

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oxidant, anti-bacterial, anti-inflammatory, alongside efficacy in treating skin diseases (6-10).

Salvia-frigida (SF) stands out as one of the extensively utilized medicinal plants in Turkey (11). Past research concentrated on analyzing acetone extract of SF's aerial parts, resulted in the discovery and characterization of two oleanane-type triterpenoids and two cycloartane-type triterpenoids, in addition to substances like α_amyrin and β_sitosterol (12-14). Antioxidant properties attributed to Phenolic Compounds (PC) of SF and flavonoids are believed to contribute to the upregulation or protection of the antioxidant defense system (15, 16).

The study aimed to assessment of *Chenopodium murale* anti-inflammatory effectiveness in comparison to that of *Salvia frigida* in treating atopic dermatitis**.**

Subject, Materials and Methods:

This study was randomized clinical trial, carried out on mice, for the period from January - July 2021 in the Department of Pharmacology, College of Medicine, Al-Nahrain University. Ethical and scientific procedures for the animal experiment protocols were rigorously reviewed and approved by the University Review Council (No. 857 on 28/9/2020).

Experimental Animal and Study Design: The study involved 50 apparently-healthy adult male Albino-mice weighing 25 to 30 grams. The mice acclimatized for seven days with well-ventilation and isolation in 24 Celsius. They were housed at the College of Veterinary, with a 12-hour light cycle. The study's practical component took place at the College of Veterinary Medicine, University of Baghdad, Iraq. Out of the 50 mice, 10 were apparently-healthy (Control Group), while 40 mice were induced with 1 – Chloro - 2, 4 - dinitrobenzene [DNCB][15] to develop AD. The induced mice were divided into four groups: Not treated (Induction group); managed by Tacrolimus ointment 0.1% (Tacrolimus-1% group), managed by 5% topically applied SF cream (Phenolic-5% group), managed by 3% topically applied CM cream (Phytosterol-3% group) (17). Topical treatments were administered once per day for three weeks. (18-20)

Inductions:

Mice Models of DNCB; AD-Induction: Atopic dermatitis was induced in mice by shaving hair from the dorsal skin, followed by the topical application of 150 µL of 1% Dinitrochlorobenzene (DNCB) in a 3:1 (v/v) acetone/olive oil solution. After four days, 0.2% DNCB dissolved in an acetone/olive oil mixture (3:1 vol/vol) was applied three times a week for three weeks. Once skin sensitization was visually confirmed, the mice were managed by the test sample (21). Figure 1 for details.

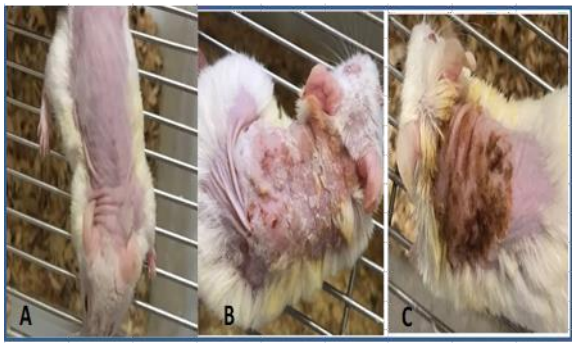

Figure 1: Skin condition: Healthy [A] and Lesions after induction [B and C]

Plant material: Identifying and authentication of the CM and SF plants was done by a specialist professor. Herbs were extracted and verified at the Pharmacognosy department and Medicinal Plants in the College of Pharmacy/ Al-Mustansiriyah University. The plant leaves were processed thorough washing, shade drying, and grinding into coarse powder using a mechanical grinder.

Extractions and fractionations of PF from *CM*: The shade-dried leaves, totaling 250 grams, were subjected to extraction with a 90% ethanol solution (600 milliliters) using a reflux apparatus until complete exhaustion and evaporation, yielding a crude fraction. The crude extracts were then acidified with 5% hydrochloric acid to reach a pH of 2, followed by partitioning with an equal volume of ethyl acetate to obtain 2 layers (aqueous and ethyl acetate). The ethyl acetate layer was collected, evaporated, and subsequently basified with 300 milliliters of NaOH-5%. The mixture was then separated with chloroform to produce two layers: A methanol-80% layer and a petroleum ether layer. The chloroform layer was further treated with petroleum ether to extract the phytosterol in the petroleum ether fraction. This fraction was then transferred to a Petri dish coated with tinfoil and stored at freezer temperature (-8 c^{o}). (18) High-performance liquid chromatography was conducted to examine the PF of CM, revealing significant presence of Beta-sitosterol as a primary component.

Preparations of PF-**3% cream:** Extraction of 3-grams from the PF of CM were weighed and dissolved with alcohol-3 milliliters. The mixture was shaken for 4 minutes until complete dissolution, resulting in a clear solution. One-hundred grams was subsequently weighted and adjusted with Aquasoft cream further shaking was done for five-minutes using a spatula (19).

***Salvia frigida* Extraction:** A total of 150 grams of shade dried and crushed leaves underwent defatting through soaking in hexane for 24-hours, followed by drying at 25^{o}c. Then, further extraction with Soxhlet apparatus with powder-packed into thimbles and 1.75 liters of 85% aqueous methanol was extracted as solvents after 24 hours. Then, the extracted material was filtered and evaporated through reduced pressure, yielding 12 grams of dry extract. Of the residue, 4 grams

were suspended in 100 ml of wate,, adding approximately four milliliters NaOH-5% to obtain basic-solution with a pH of 10. The mixture was then aliquoted with ethyl acetate (19). Finally, collection, evaporation, and dryness of the aqueous layer were done, representing the phenol-rich fraction was stored for further use.

Preparations of PC-5% Cream: Extraction of five grams PC from SF were weighed and dissolved with alcohol-3 milliliters. The solution was shaken for 4 minutes until complete dissolution and clarity were achieved. Subsequently, the weight was adjusted with Aquasoft cream and further shaking was done for five-minutes using a spatula (22).

Phytochemical tests: Two chemical tests [(I) Liebermann-Burchard test and (II) H2SO4 test)] were conducted on ethanol extraction through standard procedure to assess PF of CM (18).

Topical Treatment Application, parameter, and animals sacrificing: Topical administrations of Tacrolimus-0.1% ointment (20), Phenolic-5% cream (22), and Phytosterol-3% cream (18) was done to the AD areas of animals for 21 days, once/day (9-am), commencing from 5th day of induced AD. The comparison parameters included white blood cells, eosinophil%, s. Ig-E levels, IL-4 and IL-13 concentrations, and histopathological examination of AD skin lesions. These results were then compared with those of the control groups, and an observational severity score was determined.

Sample Collection and Histopathological Analysis
After 21 days of treatment, the mice were anesthetized using ether, and blood samples were collected in EDTA tubes for CBC and serum Ig-E analysis. Subsequently, the mice were euthanized using cervical dislocations, and the affected skin area of AD was removed for histological examination and homogenized skin preparation. Dorsal skin samples were fixed in 10% formaldehyde, paraffin-embedded, and cut into 6 µm sections. These sections were stained with hematoxylin and eosin (H & E) for histological evaluation of the inflammatory degree and AD-associated changes (23). Histopathological analysis was performed on skin for all groups in the 21st day of therapy. The sections were evaluated and scored by a pathologist using a semi-quantitative scoring system, assessing parameters such as epidermal thickness, erosions, inflammations, and oedema (0-3 scores, with 0 indicating no abnormalities, 1 indicating mild abnormalities, 2 indicating mild to moderate abnormalities, and 3 indicating moderate abnormalities) (21). These evaluations were performed in the department of histopathology/Ibn Sina Medical and Pharmaceutical Sciences University.

Preparations of Skin-Tissue Homogenates (STH): The 2nd part of the mice skin underwent a washing process using normal saline, with chilled phosphate buffered saline (1X PBS) being used. After weighing, homogenization; for every 100-grams of tissue with one-milliliters of 1X PBS was done using a tissue homogenizer (24). The homogenate was stored overnight at 20°C, and two freeze-thaw cycles were performed to break the cell membrane. After centrifugation, the supernatant was collected and preserved at -20°C to examine the level of IL-4 and IL-13.

Serum Ig-E, IL-4, and IL-13 Assays: Serum Ig-E, IL-4, and IL-13 levels were assessed using an ELISA Kit from CUSABIO/China Kit (23).

Assessing the Observational Severity Score (OS score): On day 21 of treatment, the severity of atopic dermatitis on the dorsal area was evaluated for each group. Erythema, dryness, erosion, and edema were scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The clinical skin score was defined as the sum of individual scores, ranging from 0-12 (25).

Statistical Analysis:
The data was entered into Microsoft excel 365 and loaded into the Statistical Package for Social Sciences (SPSS) version 26. Parametric data are presented as mean ± standard deviation. Categorical data are presented as numbers and percentages. The Independent t-test and one-way ANOVA test were used to measure the differences between groups parametric variables. A P value of <0.05 was considered statistically significant.

Results:
All biomarkers and histological parameters showed significantly higher levels in the induction-group than that of the control, $P<0.05$, Figure 2.

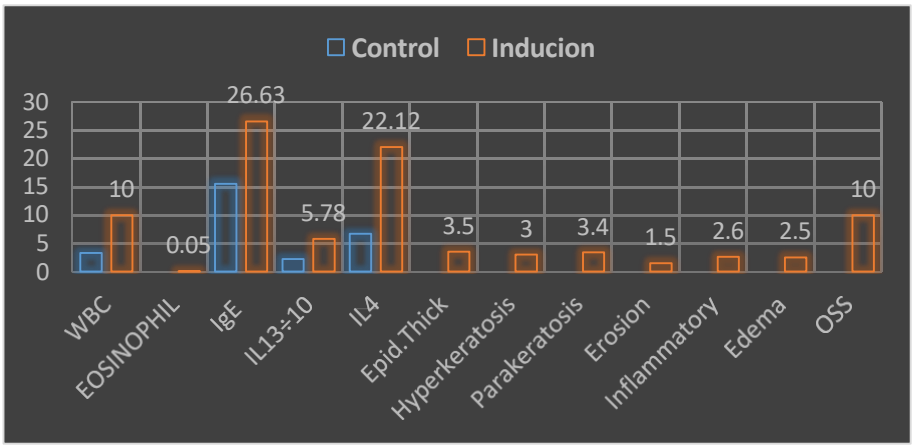

Figure 2: Biological and histological comparisons between control and induction groups

Biological comparisons between all studied groups showed significant decreases in the level of (WBC, Eosinophil, Ig-E, IL-13, and IL4) among Tacrolimus-1%, Phytosterol-3%, and Phenolic-5% groups compared to the induction group (P<0.001). Tacrolimus-1% group showed a significantly lower mean WBC count and Ig-E than other groups (<0.05) and a significantly lower mean IL-13 than Phenolic-5% groups (P<0.05). No significant differences were observed in biological parameters between Phytosterol-3% and Phenolic-5% groups (P>0.05), table 1.

Table 1: Biological comparisons between all study groups

Variables		Induction	Tacrolimus-1%	Phytosterol-3%	Phenolic-5%
WBC [x103 /µl]	Mean±SD	10.0±2.10	6.0± 2.02	7.1± 2.01	7.6± 3.03
	P-*a*		< 0.001	< 0.001	<0.001
	P-*b*			0.04	0.04
	P-c				0.56
Eosinophil [x103 /µl]	Mean±SD	0.05±0.02	0.02± 2.02	0.03±0.09	0.03± 0.03
	P-*a*		< 0.001	< 0.001	< 0.001
	P-*b*			0.11	0.12
	P-c				0.34
Ig-E [ng/ml]	Mean±SD	26.6±5.15	16.0±6.08	17.5±6.61	20.4±5.92
	P-*a*		< 0.001	< 0.001	< 0.001
	P-*b*			0.9	0.045
	P-c				0.91
IL-13 [pg/ml]	Mean±SD	57.8±10.52	31.8±21.29	31.6±12.31	37.2±18.00
	P-*a*		< 0.001	< 0.001	<0.001
	P-*b*			0.06	0.031
	P-c				0.42
IL4 [pg/ml]	Mean±SD	22.1±6.21	9.1±4.03	9.7±2.88	11.6±2.23
	P-*a*		< 0.001	< 0.001	<0.001
	P-*b*			.49	.07
	P-c				.57

a= Comparisons among Induction and others; b= Comparisons among tacrolimus-1% and Phytosterol-3%; Phenolic-5%; c= Comparisons among Phytosterol-3% and Phenolic-5%

Histological comparisons between all study groups showed a significantly lower levels of (Epidermal thickness, hyperkeratosis, parakeratosis, erosion, inflammatory cells, extracellular edema, and OSS) among Tacrolimus-1%, Phytosterol-3%, and Phenolic-5% groups from that of the induction group (P<0.001). The tacrolimus-1% group; showed a significantly lower mean inflammatory cells than other groups (P<0.05), a significantly lower mean epidermal thickness than Phytosterol-3% group (P<0.05), and a significantly lower mean erosion than Phenolic-5% group (P<0.05). The phytosterol-3% group showed a significantly lower parakeratosis, erosion and OS score than other groups (P<0.05). The phenolic-5% group showed a significantly lower mean epidermal thickness than the other groups (P<0.05), and a significantly lower OS score than Tacrolimus-1% groups (P<0.05), Table 2. Figure 3 (a, b and c) shows some of the above histological changes.

Table 2: Histological c ɔmparisons betv een all study groups

Variables		Induction	Tacrolimus-1%	Phytosterol-3%	Phenolic-5%
Epidermal Thickness	Mean±SD	3.5±0.52	1.2±1.22	2.2±0.78	1.0±0.66
	P-*a*		< 0.001	< 0.001	< 0.001
	P-*b*			0.025	0.04
	P-c				0.002
Hyperkeratosis	Mean±SD	3.0±0.81	1.6±0.51	1.6±0.51	1.6±0.51
	P-*a*		< 0.001	< 0.001	< 0.001
	P-*b*			0.99	0.99
	P-c				1
Parakeratosis	Mean±SD	3.4±0.69	1.2±0.78	1.0±0.003	1.2±0.78
	P-*a*		< 0.001	< 0.001	< 0.001
	P-*b*			<0.001	0.9
	P-c				0.43
Erosion	Mean±SD	1.5±0.52	0.2±0.42	0.2±0.35	0.4±0.51
	P-*a*		< 0.001	< 0.001	< 0.001
	P-*b*			0.17	.045
	P-c				.035
Inflammatory Cells	Mean±SD	2.6±0.51	1.7±0.42	1.8±0.42	1.8±0.78
	P-*a*		< 0.001	< 0.001	< 0.001
	P-*b*			.046	.81
	P-c				1
Extracellular Edema	Mean±SD	2.5±0.52	1.2±0.51	1.2±0.42	1.1±0.56
	P-*a*		< 0.001	< 0.001	< 0.001
	P-*b*			0.45	0.46
	P-c				0.66
OSS*	Mean±SD	10.0±.81	4.5±1.08	3.5±0.97	3.7±1.33
	P-*a*		< 0.001	< 0.001	< 0.001
	P-*b*			0.028	.03
	P-c				.7

a= Comparisons among Induction and others; b= Comparisons among tacrolimus-1% and Phytosterol-3% and Phenolic-5%; c= Comparisons among Phytosterol-3% and Phenolic-5%; *OSS= observation-severity-score.

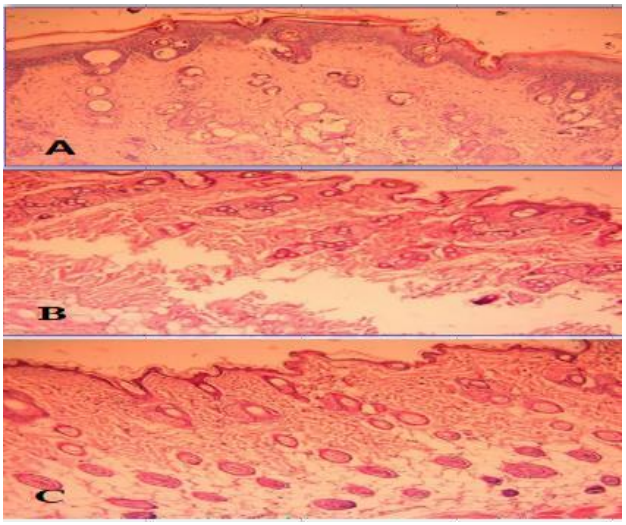

Figure 3: Histological changes [10x] among a. Induction group; b. Phytosterol-3% group; and c. Phenolic-5% group. H and E -stain

Discussion:

Atopic dermatitis (AD) is detrimentally impacting the quality of life and daily activities, with a comparable or even more severe impact than other chronic skin and systemic diseases (26).

In the current study, the comparison between the selected parameters in the untreated group with induced atopic dermatitis (AD) showed noticeable signs of inflammation and a significant increase in thickness, as well as elevated OS scores. This aligns with a previous study reporting a substantial increase in various WBC components in untreated AD-induced groups (27). Eosinophil counts were found to be significantly elevated in AD induced non-treated group in agreement with another study which showed increased eosinophil in patients having eczema with persistent lesions (28). In addition, a significant increase in skin tissue IL-13 and IL-4, and serum Ig-E were observed in the AD non-treated group, in congruency with other studies (29, 30). Another difference was found in OS score in the AD induced non-treated group, in agreement with a study reporting significantly more severe symptoms and high OS score, Gil, et al (31).

Upon the application of the topical 3% cream of phytosterol fraction of CM, a significant decrease in signs of inflammation, histopathological changes, and OS score were evident in comparison to the induction group, which may be explained by the β Sitosterol anti-atopic effects of CM. The anti-inflammatory action of β Sitosterol has been linked to the regulation of mediators of inflammation, demonstrating the therapeutic potential in inflammatory skin conditions like AD. Animal studies also support this, indicating that β Sitosterol decreases the release of inflammatory cytokines and oedema, He *et al.* (32)

Several studies supported our findings that the extracts of the Chenopodium murale significantly suppressed the test fungal growth (33) and exhibited mild to moderate inhibitory activities against different bacteria (34).

The results of the current study are in agreement with those of TrivellatoGrassi et al on the anti-inflammatory and anti-nociceptive effects of Chenopodium, identifying the mechanism of action as the inhibition of mediators and enzymes involved in the inflammatory and pain processes. (17) This confirms the validity of the common use of this plant for treating inflammation and pain and helping wound healing processes.

Han et al found that β-sitosterol reduced AD clinical symptoms such as dryness, eczematous, erythema and serum histamine and Ig-E levels in induced AD in mice. Additionally, β-sitosterol inhibited the IL-6 expression in AD like skin lesions, significantly reduced the levels of inflammation-related mRNA and protein in the AD skin lesions, significantly reduced the levels of histamine, Ig-E, and IL-4, and reduced the activation of mast cell when used in the treatment of AD skin lesions (20), which supports the current results.

In the current study, the application of the topical 3 phenolic compounds resulted in a significant decrease in the signs of inflammation, histopathological changes, and OS score in comparison to the induction group, supporting the role of the phenolic compounds of SF. Studies on Salvia plants, specifically those treated with phenolic compounds showed the anti-inflammatory effects in AD-treated groups, and highlighted the diverse properties of Salvia plants, including anti-inflammatory, anti-cancer, anti-cholinesterase, anti-microbial, anti-malarial, and antioxidant effects (35-41).

In the present study a significant decrease was found in the WBC count and IL-4 between 5% phenolic compound treatment group and AD induced non-treated group after three weeks of treatment, in consistence with Paulin et al study who found that salvia plant has anti-inflammatory effects (42). Paulin et al and Raal et al reported that Salvia species have also been used for a long time in folk medicine against fever, rheumatism, perspiration, sexual debility, chronic bronchitis, mental and neurological conditions. Essential oil of salvia and their preparations are externally used for inflammations and infections of the mucous membranes of throat and mouth (43, 44).

Histo-pathologically, a highly significant reduction in epidermal thickness, hyperkeratosis, parakeratosis, erosion, inflammatory cells, extracellular edema, and OS score was found after phenolic compound treatment. Many studies confirm these results, highlighting the properties of Salvia plant and its effects on AD and other skin lesions (45, 46). Dai et al reported that Phenolic compounds, especially flavonoids, have a great antioxidant effect that has been shown to be more effective than vitamins C and E and carotenoids (47). Upon comparing the effects of the phenolic compound of Salvia frigida and the phytosterol fraction of *Chenopodium murale*, it is noted that the phenolic compound reduces epidermal thickness significantly after three weeks of treatment. In contrast, the phytosterol fraction-treated group displays a more significant decrease in IL-13, parakeratosis, and OS score. The Tacrolimus-1% group exhibits a highly significant decrease in WBCs and inflammation but a comparable reduction of erosion to the phytosterol fraction-treated group.

The topical applications of various treatments in AD in the current study successfully mitigated responses of inflammation. This suppression led to a decrease in blood concentrations of histamine due to the reduction of IL-13 and the inactivation of mast cells, similar to the results reported (48).

Limitations of the Study:

This study did not include clinical data from human participants. Although animal models offer valuable insights into human diseases, they may not fully capture the complexity of atopic dermatitis as it occurs in humans. While the findings in the mouse model are encouraging, further research is required to evaluate the safety and effectiveness of *Chenopodium murale* and *Salvia frigida* on Atopic Eczema in human subjects.

Conclusions:

The topical applications of phytosterol fraction of *Chenopodium murale* or phenolic compound of *Salvia frigida* was effective and promising in treating atopic dermatitis. While the phenolic compound of *Salvia frigida* is effective, it is somewhat less than that of the phytosterol fraction of *Chenopodium murale*.

Authors' declaration:

The manuscript is an original work, not previously published or sent to other journals. We hereby confirm that all the figures and tables in the manuscript are ours. The project was approved by the local ethical and scientific care procedures for the animal by Al Nahrain University Review Council; code no. = 857 on 28/9/2020.

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Authors' contributions:

Study conception & design: (Zahraa Y. Hassan). Literature search: (Zahraa Y. Hassan). Data acquisition: (Tuka Y. Hassan). Data analysis & interpretation: (Tuka Y. Hassan). Manuscript preparation: (Ahmed Al-Kinany). Manuscript editing & review: (Ahmed Al-Kinany)

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التأثير المضاد للإلتهاب لنبات العفينة بالمقارنة مع نبات القصعين برودي في علاج الأكزيما التأتبية لدى الفئران

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الخلاصة

الخلفية: إلتهاب الجلد التأتبي هو حالة جلدية مزمنة شائعة ذات طبيعة إلتهابية ولها ميل وراثي. يصيب حوالي 10%-20% من الأطفال و1%-3% من البالغين في جميع أنحاء العالم. لقد ثبت سريريا أن نبات العفينة فعال في علاج العديد من الحالات الطبية مثل التهاب الجلد التأتبي بسبب إمكانية تطبيقه وفعاليته. كما أن لنبات القصعين برودي تأثيرا مضادا للإلتهابات بين مجموعة مرضى إلتهاب الجلد التأتبي الذين عولجوا بمركبات الفينول.

الهدف: تحديد التأثير المضاد للإلتهابات لنبات العفينة بالمقارنة مع نبات القصعين برودي في علاج الأكزيما التأتبية لدى الفئران.

المنهجية: أجريت هذه الدراسة في الفترة من كانون الأول 2020 إلى حزيران 2021 في قسم الصيدلة-كلية الطب-جامعة النهرين. تم تضمين خمسين عينة من الفئران في الدراسة، وتم تقسيمهم إلى خمس مجموعات فرعية بالتساوي]الضابطة، المحفزة بدون علاج، تاكروليموس-1%، فيتوستيرول-3%، وفينوليك-5%[. تم قياس المعايير البيولوجية والنسيجية باستخدام إختبار تي المستقل (أو تحليل التباين الأحادي ANOVA) لتقدير متوسط الفروقات.

النتائج: أظهرت مجموعة التاكروليموس-1% إنخفاضا ملحوظا في عدد خلايا الدم البيضاء والغلوبيولين المناعي-إي والخلايا الإلتهابية مقارنة بالمجموعات الأخرى، وإنخفاضا أكبر في متوسط سمك البشرة مقارنة بمجموعة فيتوستيرول-3%، وانخفاضا أكبر في الإنترلوكين-13 والتآكل مقارنة بمجموعة الفينول5 ٪. كما أظهرت مجموعة فيتوستيرول-3% إنخفاضا أكبر في متوسطات نظير التقرن والتآكل ودرجة شدة الملاحظة مقارنة بالمجموعات الأخرى. كما أظهرت مجموعة الفينول -5% إنخفاضا أكثر في متوسط سماكة البشرة مقارنة بالمجموعات الأخرى وإنخفاضا أكثر في درجة شدة الملاحظة من مجموعة التاكروليموس-1٪.

الإستنتاج: كانت التطبيقات الموضعية لجزيئات الفيتوستيرول من نبات العفينة أو مركبات الفينول من نبات القصعين برودي فعالة وواعدة في علاج إلتهاب الجلد التأتبي. في حين أن المركب الفينولي لنبات القصعين برودي فعال، إلا أن تأثيره أقل قليلا من تأثير جزيئات الفيتوستيرول من نبات العفينة.

الكلمات المفتاحية: جزيئات الفيتوستيرول، نبات العفينة، التهاب الجلد التأتبي، مركب الفينول، نبات القصعين برودي، تاكروليموس

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Evaluation of Human β-defensin-3 Diagnostic Role in a Group of Iraqi Patients with Osteoporosis

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Abstract

Background: Osteoporosis (OP) is a prevalent age-related condition that increases the risk of fracture and bone fragility, as a result of loss of bone mass as well as micro-architectural degradation of the bone, thereby reducing the mass and strength of bone. Human β-defensin (HBD-3) is an anti-inflammatory peptide and a crucial part of the human innate immune system. Giving early therapeutic intervention for OP requires an early diagnosis.

Objectives: To evaluate the serum HBD-3 accuracy of diagnosis in patients with osteoporosis.

Methods: The study was conducted in the National Joint Center at Yarmouk Teaching Hospital in Baghdad during September - October 2023.

Eighty participants were recruited, all of whom had clinical examinations and had their bone status measured by dual-energy X-ray absorptiometry (DXA). Levels of serum HBD-3 and vitamin D3 (Vit D3) were determined by the ELISA technique. Calcium level (Ca^{+2}) was measured using a spectrophotometer. A comparative study was conducted between forty patients with OP and 40 control. The study included females and males with an age range between (40-60) years.

Results: The serum level of HBD-3 in the OP group was significantly higher ($p < 0.001$) than that of the healthy controls. The area under the curve (AUC) was found to be (1.000) in the ROC curve analysis for serum HBD-3 level.

Conclusion: Serum HBD-3 can be a valuable indicator of OP in middle-aged individuals, and may be a helpful biological marker for OP diagnosis.

Keywords: Calcium; Human β-defensin-3; Osteoporosis; T-score; Vitamin D3.

Introduction:

Osteoporosis is a disease of the skeleton caused by the interaction of intricate and composite pathways in molecules that lead to the loss of bone mass as well as micro-architectural degradation of bone, thereby reducing the mass and strength of bone. Reduced bone mineral density (BMD) is a significant effect linked to weak, fragile, and broken bones (1-4) Both sexes are susceptible to OP and its worst effects (5-7) include fractures and chronic pain. However, women are more susceptible than men, due to accelerated bone mass loss caused by decreased estrogen levels, with the prevalence being higher in postmenopausal women (8-9). Increased bone resorption results in a phase of faster bone depletion and Ca^{+2} exhaustion from the skeleton into the extracellular fluid. These changes exacerbate bone loss by creating an imbalance of Ca^{+2} throughout the body (10-12). Vit D3 has a major impact on calcium-phosphate homeostasis and ideal bone growth. It is worth mentioning that an inadequate amount of Vit D3 raises the risk of OP fractures. Physiologically active Vit D3 enhances Ca^{+2} intestinal absorption while promoting osteoclastic maturation and bone growth (13-14) by modulating Ca^{+2} transport proteins in the small intestine. Adequate serum Ca^{+2} concentrations are necessary for the correct mineralization of bones (15-16). Vitamin D3, obtained from food or cutaneous synthesis, is first converted to the physiologically active version 25-hydroxyvitamin D [25(OH)D] in the liver and undergoes a hydroxylation process. The kidneys then use this form to create 1,25-dihydroxyvitamin D [1,25(OH)2D] which is named calcitriol. The latter is linked to calcium homeostasis and phosphate absorption in the intestine maintaining adequate levels of calcium and phosphate in the bloodstream (15,17). One important element of hydroxyapatite is calcium, the mineral compound that makes up bone tissue. For maintaining the strength and mineralization of bones, this process is essential (18). Vit D3 also binds to its receptor, known as the vitamin D receptor (VDR), which is found on the surface of bone cells. This binding stimulates the production of proteins involved in bone formation, such as osteocalcin and collagen. Ultimately, this leads to increased bone matrix formation and mineralization, which promotes bone growth and density and reduces osteoporosis (19) Defensins are a subclass of antimicrobial peptides (AMPs) which are 3.5–4.5 kDa tiny cationic proteins rich in cysteines that are present in immune system cells with a range between 33 - 47 amino acid residues in length have varying order (20-21), and composition that serve as

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the body natural defense system. Pathogenic microorganisms are killed by defensins by permeabilizing their cytoplasmic membranes (22-24). Mostly expressed in certain epithelial tissues, human β-defensin (HBD) is a crucial portion of the human innate immune system and it plays a vital function in tumor formation, metastasis, injury repair, and inflammatory diseases (25-27). HBD-3 is an antibacterial and immunomodulatory protein secreted by skin, salivary glands, and bone marrow cells (27). It is an essential component of the innate immune system, which serves as the first line of defense against microorganisms on mucosal surfaces like the skin, lungs, eyes, and airways (28,29). HBD-3 is typically expressed at a low concentration as part of the oral mucosa's natural immune barrier (27,30,31). Moreover, rheumatoid arthritis (RA) and other autoimmune diseases have also been connected to HBD-3. HBD-3 has a positive charge (+11) (32). HBD-3 is installed from three anti-parallel β-strands and an N-terminal α-helix that make up the 45 residues of HBD-3, which is stabilized via three disulfide bonds inside the molecule made up of six cysteine residues: C11-C40, C18-C33, and C23-C4 (28). According to certain findings HBD-3 can be utilized as a marker for treatment follow-up for patients with RA due to its association with proinflammatory cytokines (24,31). Correspondingly, HBD-3 is a physiological component that rises during term labor and is found in amniotic fluid, demonstrating that this defensin engages in host defense processes in the amniotic cavity to ward off pathogens or warning signs (33). A previous study recommended using HBD-3 as a therapeutic target to treat cutaneous conditions marked by impaired autophagy and skin barriers, such as atopic dermatitis (AD) (34). Furthermore, HBD-3 was able to support bone repair *in vivo* while also reducing the inflammatory destruction caused by periodontitis (35). In a different research, scientists examined how human-defensin-3-C15, a component of HBD-3, inhibits osteoclast activity to stop bone resorption. HBD-3 has prevented the rise in tartrate-resistant acid phosphate (TRAP+) multinucleated cell formation that was brought about by RANKL. Moreover, the establishment of the RANKL-induced podosome belt is prevented by HBD-3, a feature of osteoclasts that are mature and capable of resorbing bone (36-38). This research aims to measure the levels of HBD-3 in the serum of patients with OP and of healthy individuals to see if there is any relationship between them and disease features.

Patients and Methods:

Osteoporosis Patients:

It is a cross section study which was conducted on 80 participants between (40 -60) years of age, who were not suffering from any significant diseases and were recruited from the National Joint Center at Yarmouk Teaching Hospital in Baghdad for the period between September and October, 2023. The participants were divided into two groups: 40 participants suffering from OP (study group), and 40 healthy individuals (control group). All participants underwent an examination of OP activity by a joint physician using the dual energy X-ray absorptiometry (DXA scan) at the lumbar and femoral neck spine regions vertebrae (L1–L4). Patients were categorized according to the following: The T-score was used to identify OP (< -2.5 standard deviations) and healthy individuals (BMD within 1 SD) of a young normal adult (T-score ≥ -1). A blood sample was tested for (Vit D3, Ca^{+2}) as a routine biochemical blood procedure for all subjects, in addition to measuring the levels of serum HBD-3 in the blood samples for all patients. The weight (Kg) and height (m) of each participant was measured to calculate the body mass index (BMI) Kg/m^2. The waist and hip circumferences were also measured to calculate the waist/hip ratio (WHR). People suffering from diabetes mellitus, heart disease, rheumatoid arthritis, kidney diseases, cancer, hysterectomized women, Addison's disease and other diseases were excluded from this research.

This study was approved by the Iraqi Ministry of Health / Center for Education and Human Development, Yarmouk Iraqi Teaching Hospital Committee of Ethics, and the University of Baghdad Ethical Committee. The ethical standards of the Helsinki Declaration were adhered to in the procedures.

Blood Sample Collection and Laboratory Analysis:

Five milliliters of blood were collected from the participants without using a tourniquet, leaving them to coagulate in a clot activator tube for 15 minutes at room temperature. The serum was separated using a centrifuge for 5 minutes, was stored in 2ml Eppendorf containers and kept at -4◦C. Serum HBD-3 (Cloud-Clone Corp., USA, SEE132Hu), and Vit D3 (Cloud-Clone Corp., USA, CEA920Ge) concentrations were assessed using an ELISA plate reader from Germany's Human. Serum Ca^{+2} (Linear Chemicals, Spain) concentration was evaluated using A spectrophotometer.

Statistical Analysis:

Statistical analysis was conducted using version 26 of SPSS. The median, 25^{th} and 75^{th} percentiles were used. The Mann-Whitney test was used to identify numerical elements that weren't normally distributed. The ROC curve method was utilized to assess the serum HBD-3 level cut-off value. Additionally, calculations were made for the specificity, sensitivity, negative predictive value, and positive predictive value. *P*-values that are less than 0.05 were regarded as significant.

Results:

Table 1 shows the median (25th and 75th percentiles) values for age, BMI and WHR for the OP cases and their healthy controls. The differences between the means of the two groups were not statistically significant.

Table (1): The median and (25th and 75th percentiles) of age, BMI and WHR for the OP cases and their healthy controls

Variable	OP case(n = 40)	Control (n = 40)	*P* value
Age (year)	57.0 (52 - 59)	55.0 (45 - 58.75)	0.648
BMI (kg/m^2)	28.2 (24.5 - 33.2)	30.5 (26.7 - 34.7)	0.273
WHR	0.93 (0.91- 0.95)	0.92 (0.90 - 0.93)	0.407

-The median (25th and 75th percentiles) of the obtained data were evaluated using the Mann-Whitney test at the 0.05 threshold; The two independent group differed significantly from one another.

As it turns out, there was a clear significant difference between the concentration levels of each (Vit D3, Ca) for the OP patients [6.44 (4.05-9.44) ng/dl, 7.99 (7.81-8.15) mg/dl] with [67.0 (38.30-79.10) ng/dl, 9.3 (8.80-9.80) mg/dl] healthy individuals, p-value (<0.001), table 2. Serum HBD-3 levels were noticeably higher in OP patients (2.84 (2.52-3.36) ng/ml) than in the healthy individuals (0.990 (0.890-1.10) ng/ml) with p-value (<0.001). The statistical analysis demonstrated that the OP group and the healthy individuals differ significantly as displayed in (Table 2).

Table (2): The median (25th and 75th percentiles) of lab and radiological investigations for the OP cases and their controls

Variable	OP case (n = 40)	Control (n = 40)	*P*-value
Vit D3 (ng/dl)	6.44 (4.05-9.44)	67.00 (38.30-79.10)	<0.001
Ca (mg/dl)	7.99 (7.81-8.15)	9.30 (8.80-9.80)	<0.001
T/score%	-2.95 (-3.27- -2.60)	0.05 (-0.80-0.38)	<0.001
HBD-3 (ng/ml)	2.84 (2.52-3.36)	0.99 (0.89-1.10)	<0.001

The median (25th and 75th percentiles) of the obtained data were evaluated using the Mann-Whitney test at the 0.05 threshold; The two independent group differed significantly from one another.

Table 3 shows the specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) for studied biomarkers. For each biomarker in the study, the ideal cut-off value was obtained from the ROC curve using the Youden index to determine how well the serum HBD-3 concentration can distinguish between OP cases and healthy individuals using ROC curve analysis, (Figure 1) with enhanced validity high sensitivity and specificity (100.0, 100.0), respectively. The ROC curve, which has an AUC of 1.000 (P-Value <0.001) reached the ideal degree of accurate diagnosis of OP.

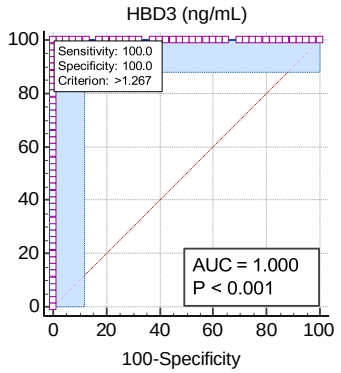

Figure (1): The ROC curve for the predictive value of HBD-3 serum concentration in OP patients (n = 40) compared to controls (n = 40) AUC is 1.000 (95%), *P*-Value 0.001.

Table (3): Validity criteria of test variables to distinguish between groups of people with and without OP

Variable	AUC	P-Value	cut off value	Sensitivity	Specificity	Accuracy	PPV	NPV
HBD-3	1.000	0.001	>1.267	100.0	100.0	1.000	10 0.0	10 0.0

*AUC and the ability to discriminate between osteoporosis sufferers and healthy individuals.

Discussion:

The study results showed no significant differences in (age, BMI and WHR) between the OP cases and healthy controls, indicating that age is not related to the severity of OP, and that it may be related to factors like race, lifestyle, diet, prescription drugs and concomitant diseases. This result is in line with the study of Ahmed *et al.* (22), while it disagrees with the study of Alfadhul (10) which showed that younger age groups were more knowledgeable about OP and therefore less susceptible to the disease. When comparing the OP patients to the healthy controls, there was significantly lower concentrations of vitamin D3, Ca+2 among those with OP. This is in line with the results of Jafer, et al. (39) and Farhan, *et al.* (40). The findings of the current study emphasized the role of vitamin D in preventing OP, as all the OP cases had low levels of vitamin D, which may be one of the main causes of the disease. The current study showed a highly significant difference in HBD-3 levels among OP cases compared to healthy controls. The T-score was significantly higher in OP cases. Park, et al. (36) pointed to the role of the human-defensin-3-C15, a component of HBD-3 in inhibiting osteoclast activity to stop bone resorption. The prohibited HBD-3 prevents the rise in tartrate-resistant acid phosphate (TRAP+) multinucleated cell formation that is brought about by RANKL, and inhibits the formation of the RANKL-induced podosome belt, a feature of osteoclasts that are mature and capable of resorbing bone. So HBD-3 was evaluated as an anti-bone resorption agent (36). This

is consistent with the results of the current research, which found high concentrations of anti-inflammatory protein HBD-3 in OP patients compared to controls, indicating its defensive activity against the causes of OP. Mohammed, *et al.* (32), studied rheumatoid arthritis patients and was in agreement with the results of the current study, as they found that higher HBD-3 levels were observed in rheumatoid arthritis cases, an indication that it can be used as a marker to monitor the treatment of the disease, as that disease leads to bone loss and OP over time (32). A high concentration of HBD-3 in OP cases in the current study compared to controls, indicating its defensive activity against the causes of osteoporosis. This confirms its diagnostic role for the disease, and probably suggests future research to explore the potential therapeutic possibilities of HBD-3 for OP patients.

Conclusions:

Serum HBD-3 can be a valuable indicator of OP in middle-aged individuals. The activity of the disease is reflected in the levels of this marker, with OP patients having higher levels of HBD-3 than healthy individuals. Serum HBD-3 levels may be a helpful biological marker for OP diagnosis.

Authors' Declaration:

We hereby confirm that all the Figures and Tables in the manuscript are ours. The Department of Chemistry, College of Science for Women, University of Baghdad approved the project according to the code number (4168 /22 on 26/7/2023).

Conflicts of interest: None.

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Authors' Contributions:

Study conception & design: (Layla Othman Farhan & Zahraa Salim Hassan). Literature search: (Zahraa Salim Hassan). Data acquisition: (Layla Othman Farhan & Zahraa Salim Hassan). Data analysis & interpretation: (Layla Othman Farhan & Zahraa Salim Hassan).Manuscript preparation: (Zahraa Salim Hassan). Manuscript editing & review: (Zahraa Salim Hassan).

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دراسة بيتا-ديفينسين-3 البشري في المرضى العراقيين المصابين بهشاشة العظام

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الخلاصة:
خلفية البحث: هشاشة العظام هي حالة منتشرة مرتبطة بالعمر وتزيد من خطر الإصابة بالكسور وهشاشة العظام، نتيجة فقدان كتلة العظام وكذلك التدهور المعماري الدقيق للعظام، وبالتالي تقليل كتلة العظام وقوتها. بيتا-ديفينسين-3 البشري هو الببتيد المضاد للالتهابات وجزء مهم من جهاز المناعة الفطري البشري.
الأهداف: تقييم دقة تشخيص بيتا-ديفينسين-3 البشري في مصل الدم لدى المرضى الذين يعانون من هشاشة العظام.
الحالات والمنهجية: تمت دراسة ثمانين مشاركا، خضعوا جميعا لفحوصات صحية وتم قياس حالة عظامهم بواسطة قياس امتصاص الأشعة السينية المزدوج الطاقة (DXA) وتحديد مستوى بيتا-ديفينسين-3 البشري في مصل الدم وفيتامين D3 (Vit D3) بواسطة تقنية ELISA. تم قياس الكالسيوم (Ca) باستخدام مقياس الطيف الضوئي. أجريت دراسة مقارنة بين أربعين مريضا يعانون من هشاشة العظام و 40 من الاصحاء. وشملت الدراسة الإناث والذكور الذين تتراوح أعمارهم بين (40-60) سنة. أجريت الدراسة في المركز الوطني للمفاصل في مستشفى اليرموك التعليمي في بغداد خلال الفترة من أيلول إلى تشرين الأول 2023.
النتائج: بالمقارنة مع مجموعات الأفراد الأصحاء، كان مستوى بيتا-ديفينسين-3 البشري في مصل مجموعة هشاشة العظام أعلى بكثير ($P < 0.001$). تم العثور على المنطقة تحت المنحنى (AUC) لتكون (1.000) في تحليل منحنى ROC لمستوى بيتا-ديفينسين-3 البشري في الدم.
الاستنتاجات: يمكن أن يكون مستوى بيتا-ديفينسين-3 البشري في مصل الدم مؤشرا قيما لهشاشة العظام لدى الأفراد في منتصف العمر، وقد يكون علامة بيولوجية مفيدة لتشخيص هشاشة العظام.
مفتاح الكلمات: الكالسيوم، بيتا-ديفينسين-3 البشري، هشاشة العظام، T-score، فيتامين د.

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Research Article

The Role of Microelements in Lumbar Disc Degeneration in Patients Undergoing Lumbar Spine Surgery

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Abstract

Background: Lumbar disc degeneration (LDD) is a common musculoskeletal disorder that frequently causes low back pain (LBP). In addition to the discomfort of lower back pain, it can accompany pain in one or both legs. The lumbar spine and sacrum, consisting of five vertebrae and one bone, determine the spine's balance. Microelements are essential in bone metabolism and are associated with preventing osteoporosis and alleviating musculoskeletal pain.

Objectives: To examine the correlation between lumbar spinal surgery and the concentrations of microelements, namely zinc and copper.

Methods: A case-control study was conducted in Ghazi Al-Hariri Hospital in Baghdad, Iraq, during the October 2023 to January 2024. The study included 120 participants ranging in age from 18 to 70 years. Sixty participants underwent lumbar spine surgery and were diagnosed using X-ray or MRI scans. The other 60 were healthy controls. The zinc (Zn) and copper (Cu) levels in the blood were determined using an atomic absorption spectrometer. The body mass index (BMI) was determined using the formula: BMI (kg/m^2) = weight/height^2.

Results: The patients had a lower mean zinc level (57.3 ± 14.56 *Mmol/L*) and a higher mean copper level (106.6 ± 39.41 *Mmol/L*) in comparison to healthy controls (96.4 ± 17.38 *Mmol/L*) and (61.0 ± 9.53 *Mmol/L*) respectively. There was a weak relationship and a significant correlation between copper and zinc (r=-0.2). A very strong relationship and a significant correlation between copper and Cu / Zn ratio (r = 0.85) while zinc had a significant very strong correlation relationship with Cu / Zn ratio (r =-0.7) in patients.

Conclusion: The present study underscores the noteworthy association between microelements (Cu, Zn) and degenerative lumbar discs underscoring the significance of pre-operative evaluation in achieving the best possible surgical results. The study has demonstrated the utility of measuring serum zinc level and copper level especially their link with lumbar disc degeneration (LDD) as markers of patients undergoing lumbar spinal surgery.

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Introduction:

The lumbar spine, composed of five big vertebrae, and the sacrum, are crucial for spinal equilibrium, with lumbar lordosis influencing sagittal balance, posture, and upright walking, supporting bipedalism (1). Degenerative disc disease (DDD) is a complicated condition that is still poorly understood. Many theories have been put forth to explain the disease such as aging and the interaction of genetic and environmental variables. It has been demonstrated that the disproportion of anabolic and catabolic activity of extracellular matrix (ECM) enzymes including cathepsins, aggrecans and matrix metalloproteinases (MMPs) influences a significant percentage of the degeneration process (2).

Since low back pain is a symptom rather than a diagnosis, it might be caused by a variety of disorders both recognized and undiscovered.

Specific or non-specific low back pain (LBP) is possible. Specific low back pain (LBP) refers to pain that originates in another area of the body or is brought on by a specific disease or anatomical issue in the spine (3).

A surgical method called lumbar interbody fusion (LIF) is used to treat degenerative lumber segments, their decompressed neural components and any facet joint problems that may be related (4).Trace elements (TE) were explored in several illnesses such as osteoarthritis (OA), a whole-joint disease characterized by pathological changes in all joint tissues including subchondral bone sclerosis, cartilage loss and synovial inflammation (5). Most of the investigations focus on bone tissue, which is thought to be the storehouse for trace elements and reflects their cycling across the entire organism (6). Aside from that, recent research links TE to bone metabolic failure (7). Additionally, abnormalities in serum TE (Zn and Cu) are linked to cardiac failure and coronary artery disease (8).

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The body contains very small amounts of trace elements, which are vital micronutrients. They are essential to the healthy operation of the immune system and have a significant impact on several physiological functions (9). Trace elements such as copper and zinc, play a part in numerous biological activities as micro sources (10). Zinc (Zn) is a crucial component of bone health and a cofactor in metalloenzymes (11). A significant amount of the body zinc is found in the skeleton, where it is involved in intracellular signaling, endocrine axis modulation and long-bone growth (12). It promotes osteoblast growth and bone production, prevents osteoclastic bone resorption and shields osteoblasts from apoptosis. A zinc deficit affects collagen production, collagenase activity and bone mineralization. It also compromises the integrity of bone tissue (13). Copper (Cu) decreases bone metabolism by inhibiting osteoblasts and osteoclasts. Lysyl oxidase is an enzyme that crosslinks collagen fibers, and Cu is a cofactor for this enzyme (14). Cu deficit raises the risk of osteoporosis and causes bone abnormalities. The element has a strong correlation with bone flexibility and tensile strength, and it is crucial in the lysyl oxidase enzyme production process, which is responsible for the crosslinking of elastin and collagen in the organic matrix of bone (15).

Copper influences the equilibrium of humoral and cellular immunity. For growth, development and strong bones, it is necessary (16). Additionally, serum Cu is high in type 2 diabetics (17). Among the three primary disorders in orthopedics are osteoporosis, fractures and arthritis and Cu is crucial to the management of these three conditions (18).

Supplementation with trace metals like copper can help prevent and minimize bone loss (19). A previous study reported that alkaline phosphatase (ALP) activity and osteocalcin concentration were significantly increased with zinc supplementation. This finding demonstrated that zinc positively influences osteoblastogenesis, resulting in increased osteoblast differentiation and proliferation (20).

The aim of this research is to examine the correlation between lumbar spinal surgery and the concentrations of microelements.

Patients and Methods:

The case-control study was conducted on 120 individuals at Ghazi Al-Hariri Hospital in Baghdad, Iraq, during the period from October 2023 to January 2024. The age range of participants was between 18 and 70 years old. the participants were divided into two groups: 60 were patients who underwent lumbar spine surgery, and 60 healthy individuals who served as a group of controls from the same areas of the patients and were randomly selected. Inclusion criteria for both cases and controls were that they did not suffer from kidney or liver disease without known zinc and copper supplementation.

The study questionnaire included a set of questions on demographic and clinical characteristics. X-rays or MRI scans was utilized for imaging the lumbar spines. Atomic absorption spectroscopy was used to measure the level of zinc and copper. Weight and height were measured to calculate the body mass index (BMI), and to classify the participants according to World Health Organization (WHO) (21).

Statistical Analysis:

The data was managed using SPSS version 25.0 software. Frequency, percentage, mean and standard deviation were used to describe the data. To investigate the association between the qualitative variables, the chi-square test was utilized. The independent t-test was used to evaluate the difference between means and the Pearson correlation coefficient was used to analyze the correlation between two quantitative variables. With values <0.3 signifying no correlation, 0.3 - <0.5 denoting weak correlation, 0.5 - <0.7 moderate strength and >0.7 strong correlation. P-values of < 0.05 were regarded as significant. Receiver operating characteristic (ROC) analysis was used to determine the ideal threshold for study cases, which had high specificity and sensitivity.

Results:

The mean age of the cases was (50.9 ± 13.76 years) compared to (44.5 ± 6.21 years) for controls *p*-value < 0.001.

The mean BMI was 26.2 ± 3.16 kg/m^2. Patients had a significantly higher BMI mean (28.1 ± 3.41 kg/m^2) in comparison to healthy controls (24.4 ± 1.14 kg/m^2), *p*-value <0.001. Regarding BMI groups, 45.4% had a normal BMI; the percentages for overweight were 41.2%, obese 12.6% and severely obese 0.8%, Table 1.

Table 1: Demographic characteristics of the study groups

Demographic		Patients	Controls	Total	*p*- value
Age (years)		50.9 ± 13.76 (1.77)	44.5 ± 6.21 (0.80)	40.2 ± 15.12	<0.001ǂ
BMI (kg/m^2)		28.1 ± 3.41 (0.44)	24.4 ± 1.14	26.2 ± 3.16	<0.001*ǂ
Sex	Male	28 (46.7%)	33 (55%)	61 (50.8%)	0.46
	Female	32 (53.3%)	27 (45%)	59 (49.2%)	
	Normal	10 (16.7%)	44 (74.6%)	54 (45.4%)	<0.001*ǂ
	Overweigh	34 (56.7%)	15 (25.4%)	49 (41.2%)	
	Obese	15 (25.0%)	0 (0.0%)	15 (12.6%)	
	Morbid ob	1 (1.7%)	0 (0.0%)	1 (0.8%)	

Mean ± SD (SE), N (%), **p*-value is significant, ǂ independent t-test, †chi-square test

The patients had a lower mean zinc value (57.3 ± 14.56 *Mmol/L*) compared to the controls (96.4 ± 17.38 *Mmol/L*), p-value = 0.001. The mean copper and Cu/Zn ratios were significantly higher (106.6 ± 39.41 *Mmol/L*) and (2.0 ± 1.06) in cases compared to the control (61.0 ± 9.53*Mmol/L*) (0.6 ± 0.12), *p*-value <0.001, Table 2.

Table 2: Mean ± SD of zinc, copper and Zn/Cu ratio in the study groups

Microelement	Patients	Controls	*p*-value
Zinc (*Mmol/L*)	57.3 ± 14.56 (1.89)	96.4 ± 17.38 (2.24)	0.001*
Copper (*Mmol/L*	106.6 ± 39.41 (5.08)	61.0 ± 9.53 (1.23)	<0.001*
Copper/Zinc	2.0 ± 1.06 (0.13)	0.6 ± 0.12 (0.01)	<0.001*

Mean ± SD (SE), **p*-value is significant

The correlation coefficient was used to determine linear relationships between copper and zinc in patients with lumbar spinal surgery. The results showed that there was a weak relationship and a significant correlation between copper and zinc ($p \leq 0.05$, r=-0.2), Figure 1.

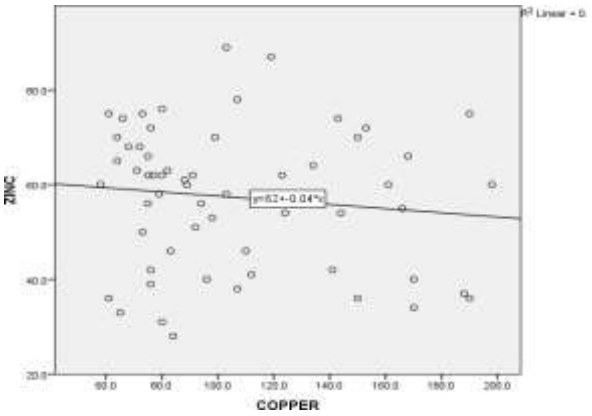

Figure 1: Simple linear regression of Copper and Zinc for lumbar spinal surgery cases.

The results showed a positive relationship and a significant correlation between copper and copper/zinc (p = <0.001, r = 0.85), Figure 2.

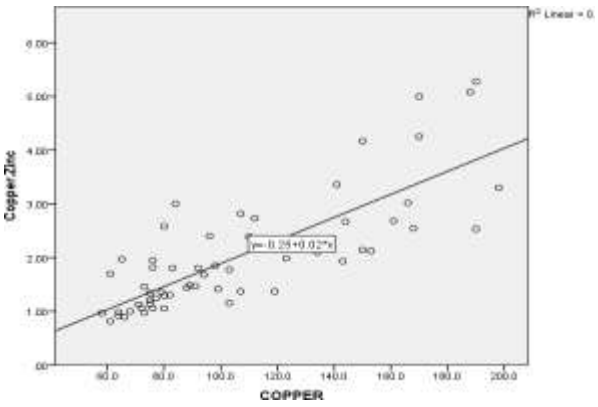

Figure 2: Simple linear regression of Copper and Cu/Zn for lumbar spinal surgery cases.

They also showed a positive relationship and a significant correlation between Cu/Zn and Zn ($p \leq 0.001$, r =0.7), Figure 3.

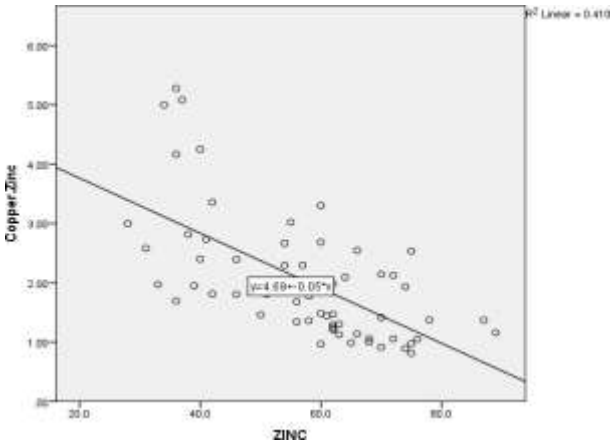

Figure 3: Simple linear regression of Copper/Zinc and Zinc for lumbar spinal surgery cases.

Table 3 shows the area under the curve and ROC for evaluating serum zinc, copper and their ratio concentrations as potential diagnostic indicators of lumbar disc degeneration in lumbar spine surgery. Serum zinc, copper and their ratio biomarkers exhibited high diagnostic accuracy for predicting lumbar spine surgery.

The copper/zinc ratio produced an AUC of 0.995 (0.988-1.000; P<0.001). The best cut-off value of copper/zinc for the detection of lumbar disc degeneration is 0.9111 with a sensitivity of 96.7%, a specificity of 96.7%, a PPV of 95.1%, an NPN of 96.6% and an accuracy of 95.8%.

The copper produced an AUC of 0.926 (0.883–0.969**;** P<0.001). The best cut-off value of copper for the early detection of lumbar disc degeneration is 72.5 *ug/dL* with a sensitivity of 83.3%, a specificity of 88.3%, a PPV 87.7%, an NPN 84.1% and an accuracy of 85.8%. The zinc has an AUC of 0.979 (0.958-1.000**;** *p*<0.001). The best cut-off value of zinc for the early detection of lumbar disc degeneration is 75 *ug/dL* with a sensitivity of 93.3%, a specificity of 95%, PPV of 94%, NPV of 93.4% and accuracy of 94.2%.

Table 3: The ROC curve for the optimal threshold that assesses serum zinc, copper and their ratio for diagnosing lumbar disc degeneration

Test Result Variable(s)	Copper	Zinc	Copper/Zinc
Cut-off points	72.5	75	0.9111
AUC	0.926	0.979	0.995
Sensitivity %	83.3 %	93.3%	96.7%
Specificity %	88.3%	95%	96.7%
PPV	87.7%	94%	95.1%
NPV	84.8%	93.4%	96.6%
Accuracy	85.8%	94.2%	95.8%
CI (95%)	(0.883-0.969)	(0.958-1.000)	(0.988-1.000)
P value	<0.001[S]	<0.001[S]	<0.001[S]

S= Significant, PPV= Positive protective value, NPV= Negative predictive value, AUC= Area under curve, CI= confidence interv

In evaluating the efficacy of copper, zinc and copper/zinc ratio in lumbar disc degeneration

detection, the findings showed that zinc (sensitivity 93.3%) was the most sensitive biomarker for lumbar disc degeneration, followed by copper (sensitivity 83.3%). However, the copper/ zinc ratio (sensitivity 96.7%) reflected a highly specific biomarker for lumbar disc degeneration, Figure 4.

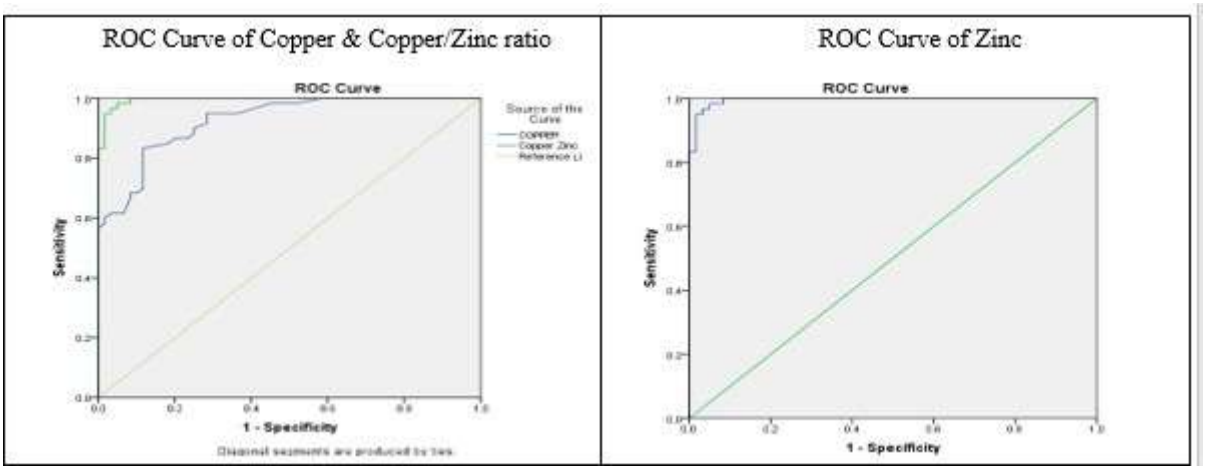

Figure 4: ROC curves for optimal diagnostic point analysis for predicting lumbar disc degeneration in lumbar spine surgery using copper, zinc and their ratio.

Discussion:

The significantly higher mean age in the patients than the controls found in the current study is consistent with the findings of Lee *et al.* who combined information from other studies that considered advanced age to be a risk factor for lumbar kyphosis and lumbar disc degeneration (22). Rajasekaran *et al*., also found that aging causes changes in the lumbar vertebrae and intervertebral discs (23).

The higher mean BMI in patients than controls is consistent with the findings of Flippin *et al.*, based on a community-based spine registry that covers a range of diseases. An increase in BMI was associated with a statistically significant increase in operating time (24), that due to a combination of intervertebral disc degeneration, fatty infiltration of paraspinal muscles and medical changes such as bone marrow lesions visible on magnetic resonance imaging and suggestive of low back pain. Obesity is also linked to degenerative spinal pathology in the lumbar spine (25).

The significantly lower zinc levels in patients than controls in the current study is consistent with the results of Akoniuk *et al* (26). According to Molenda *et al*., individuals with osteoporotic illness have lower zinc levels in their bones compared to healthy individuals, Zinc is an essential co-factor for alkaline phosphatase, a protein that is involved in the synthesis of many components of the bone matrix and is especially crucial for appropriate collagen synthesis and bone mineralization (27).

The finding of the current study that patients had significantly higher copper levels than controls supports that of Mahmood where patients' serum copper concentrations were much higher than controls (28). Elevated serum copper ions are an indicator of osteoporosis, fractures and joints. Copper ions are released from ceruloplasmin during the stage of an inflammatory reaction, which is a crucial component of the immune response and results in elevated serum copper (29).

Conversely, copper acts as a co-factor for lysyl oxidase an enzyme that initiates and controls the production of collagen and elastin. Copper deficiency results in a significant decrease in the activity of this enzyme in areas of bone, which is thought to lead to a decrease in collagen crosslinking. This can affect the stability and structure of collagen in bones, as well as cause abnormalities in skeletal growth and osteoporosis (30).

The finding of the current study of a significantly higher Cu/Zn ratio in patients compared to controls is consistent with those of Jakoneiuk *et al*. According to this evidence, a high serum Cu/Zn ratio could indicate an inflammation if it results from a drop in serum Zn or a rise in serum Cu (26). It is also consistent with another study which found that the Cu/Zn ratio was favorably correlated with mineral content and bone density, indicating that the ratio may be a significant predictor of bone health. The high serum Cu/Zn ratio may be connected with a decreased ability to maintain or reestablish homeostasis following a disruptive event (31).

The weak but significant correlation between copper and zinc found in the current study agrees with a the results of another study which found a positive correlation between Zn and lumbar vertebrae bone mineral density. According to this finding, osteoporosis is caused by zinc deficiency. In the same way, it has long been known that Cu deficit causes pathological alterations that are indicative of osteoporosis and limits bone formation (32). Gaier *et al*., discovered several significant associations between serum Cu and Zn and clinically important markers of bone and physical function (33). The degenerative process affects human intervertebral discs, affecting trace elements like copper and zinc. The concentration in bone tissue reflects body changes and periodic reactions, reflecting the accumulation of these elements (34).

The significant positive correlation between copper and the Cu/Zn ratio in the current study matches a previous study that found significant relationships between high Cu/Zn ratios and high serum Cu with lower bone mineral density (BMD), lean mass, strength and power and lower extremity function. The results support the use of the Cu/Zn ratio as a functional and predictive biomarker for overall health. Those with elevated serum Cu and a high Cu/Zn ratio had low BMD (33). It is also consistent with the number of studies that discovered positive relationships between Cu concentrations and the Cu/Zn ratio (35, 36). Our patient had a very strong relationship and a significant correlation between the copper/zinc ratio and zinc. Unfortunately, not all previous studies have measured markers of the correlation between copper/zinc ratio and zinc, which may be one reason for the observed difference in early healing response among patients who underwent lumbar spine surgery.

The current study revealed that measuring serum copper/zinc (at a cut-off value of > 0.9111 *ug/dL* and zinc (at a cut-off value of > 75 *ug/dL*, while copper (at a cut-off value of > 72.5 *ug/dL* were the best biomarkers for distinguishing lumbar disc degeneration with lumbar spine surgery from healthy groups. These biomarkers have a higher AUC for serum copper/zinc, followed by zinc and then copper. The results contribute to the theory of delayed lumbar disc healing as a major mechanism involved in lumbar disc degeneration in patients undergoing lumbar spine surgery.

Limitation: The current study's limitation was its limited sample size; a larger number is needed to generalize the existing findings on the Iraqi population.

Conclusion: The low serum zinc level is a predictor of lumbar disc degeneration in patients undergoing lumbar spine surgery, particularly its association with lumbar disc degeneration, and the patient had a weak correlation and a high correlation between copper and zinc. The serum level of copper in the patient has a very strong relationship and significant correlation between copper/zinc, and a very strong relationship and significant correlation between copper/zinc and zinc.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration Approval-Ethical Clearance: The project was approved by the local ethical committee in Ghazi AL-Hariri Hospital in Baghdad, Iraq. according to the code number (40681) on (15 /10/ 2023).

Conflict of Insert: None

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Authors' contributions:

The manuscript should mention the contribution of each author to the research done:

Study conception & design: (Manal K Rasheed &Ali T. Abdul Wahid). Literature search: (Sadiq R. Karkush). Data acquisition: (Sadiq Rahim Karkush). Data analysis & interpretation: (All Authors). Manuscript preparation: (Sadiq R. Karkush). Manuscript editing & review: (Sadiq Rahim Karkush).

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دور العناصر الدقيقة في إنحطاط القرص القطني في المرضى الذين يخضعون لجراحة العمود الفقري القطني

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الخلاصة

الخلفية: إنحطاط القرص القطني هو إضطراب عضلي هيكلي شائع يتسبب في كثير من الأحيان في آلم أسفل الظهر. بالإضافة إلى عدم الارتياح الناتج عن آلم أسفل الظهر قد يصاحبهما الم في إحدى الساقين أو كلتيهما. العمود الفقري القطني والعجز الذي يتكون من خمس فقرات وعظم واحد يحدد توازن العمود الفقري. العناصر الدقيقة ضرورية في إستقلاب العظام وترتبط مع الوقاية من هشاشة العظام وتخفيف من آلم العضلات والعظام. **الاهداف :** فحص العلاقة بين جراحة العمود الفقري القطني وتركيزات النحاس والزنك.

المواد والمنهجية: أجريت دراسة حالة وضبط في مستشفى غازي الحريري في بغداد، العراق، خلال الفترة من تشرين الاول ٢٠٢٣ إلى كانون الثاني ٢٠٢٤. شملت الدراسة ١٢٠ مشاركا تتراوح أعمارهم بين ٧٠-١٨ سنة. خضع ستون منهم لعملية جراحية في العمود الفقري القطني وتم تشخيصهم بالأشعة السينية أو التصوير بالرنين المغناطيسي وكان ستون منهم يتمتعون بصحة جيدة وكانوا بمثابة مجموعة مراقبة. تم قياس مستويات النحاس والزنك في المصل بواسطة جهاز قياس الامتصاص الذري. تم تحديد مؤشر كتلة الجسم (كجم / م)٢ = الوزن / الطول٢ .

النتائج: كشفت النتائج أن المرضى لديهم متوسط أقل للزنك ٣.٥٧(± ٥٦.١٤ مليمول / لتر) مقارنة مع الأصحاء ٤١.٩٦(± ٣٨.١٧ مليمول / لتر)، القيمة الاحتمالية (P <0.001). أظهرت النتائج وجود علاقة ضعيفة وارتباط معنوي بين النحاس والزنك (r=-0.2). ومع ذلك، هناك علاقة قوية جدا وارتباط معنوي بين النحاس ونسبة النحاس/الزنك (r = 0.85 ،p <0.001)، بينما كان للزنك علاقة ارتباط معنوية قوية جدا بين الزنك مع نسبة النحاس/الزنك (r = -0.7) في المرضى .

الإستنتاجات: تؤكد الدراسة الحالية على الارتباط الجدير بالملاحظة بين العناصر الدقيقة (النحاس والزنك) وإنحطاط الاقراص القطنية مما يؤكد أهمية التقييم قبل الجراحة في تحقيق أفضل النتائج الجراحية الممكنة. أظهرت الدراسة فائدة قياس مستوى الزنك ومستوى النحاس في الدم وخاصة ارتباطهما بتحطم القرص القطني كعلامات على إنحطاط القرص القطني في المرضى الذين يخضعون لجراحة العمود الفقري القطني.

مفتاح الكلمات: إنحطاط القرص القطني؛ النحاس؛ الزنك؛ جراحة العمود الفقري القطني؛ألم أسفل الظهر.

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Research Article

Frequency of 25-Hydroxyvitamin D Deficiency in Pediatric Patients with Immune Thrombocytopenia: Disease Phase and Therapy Options

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Abstract

Background: Hypovitaminosis D can cause immunological irregularities in the development of immune thrombocytopenia.

Objectives: To identify the frequency of low levels of 25-hydroxyvitamin D in children with Immune thrombocytopenia (ITP), and to assess the effect of the disease phase and type of treatment on vitamin D level.

Methods: This case-control study was carried out on 88 children (63 had been diagnosed with immune thrombocytopenia and 25 healthy children as controls) during November 2023 and April 2024. The patients were sub-grouped according to global classification of vitamin D level into three groups: less than 10 ng/ml (n = 47), 10–20 ng/ml (n = 16), and 20-30 ng/ml (none of the patients or controls fell in this group). The cases were sub-classified according to their disease phase: Acute (n = 21), persistent (n = 24), and chronic (n = 18)**.** The serum 25-hydroxyvitamin D level was measured using the enzyme-linked immunosorbent assay (ELISA) technique.

Results: Around 75% of ITP children had a serum 25-hydroxyvitamin D level of less than 10 ng/ml. The mean (± SEM) values of the serum 25-hydroxyvitamin D of the ITP children of acute (9.5±1.84 ng/ml) and chronic (8.0±1.13 ng/ml) phases were lower than those of controls (10.0±1.32 ng/ml, $p > 0.05$), but not significantly so. The mean values of 25 hydroxyvitamin D of ITP children were lower than those of the controls, irrespective of the type of treatment.

Conclusion: Vitamin D deficiency is prevalent among children with immune thrombocytopenia, particularly those in the chronic phase.

Keywords: Autoimmune; Immune Thrombocytopenic; Purpura; Thrombocytopenic; 25-hydroxy vitamin D.

Introduction:

Immune thrombocytopenia (ITP) is an autoimmune disorder defined by the immune system's destruction of platelets in the peripheral blood. The imbalance between the rates of platelet generation and elimination in the bone marrow leads to different levels of vulnerability to bleeding. A minority of patients experience severe hemorrhaging that poses a risk to their lives. ITP is diagnosed when the platelet count falls below 100,000 per cubic millimeters (1). Most patients present with skin bleeding including purpura and skin ecchymoses, and nasal or oral bleeding. Increased menstrual bleeding and urogenital hemorrhage were reported (2). ITP is classified as newly diagnosed, persistent, or chronic, based on the duration of the disease. Newly diagnosed ITP is defined as a disease diagnosed within three months of the onset of thrombocytopenia symptoms. Persistent ITP is the disease that lasts 3-12 months. A chronic form that lasts more than 12 months (3). ITP is the most prevalent cause of acquired thrombocytopenia in childhood, affecting 2 to 5 / 100 000 Children (4). Vitamin D insufficiency is prevalent in both developed and developing countries. In the United States, it has an equal impact on children and adults (5). Studies have demonstrated that vitamin D has a considerable impact on both innate and adaptive immune responses. It can improve phagocytosis and control the activity of the T helper and regulatory cells (6–8). Immunological abnormalities leading to chronic ITP can be caused by hypovitaminosis D, and treating with vitamin D can alter the immune system and lower the likelihood of chronic disease (9). Children with ITP (whether newly diagnosed, persistent, or chronic) typically have vitamin D insufficiency. Vitamin D has been suggested as a potential treatment for autoimmune illnesses due to the correlation between vitamin D and the occurrence or severity of these diseases(10). Many children with ITP, similar to those with other autoimmune disorders, frequently suffer from hypovitaminosis D. Children with hypovitaminosis D have more severe ITP, suggesting that vitamin D therapy could be a

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novel strategy to treat this disease (11). Patients with autoimmune diseases have a higher prevalence of vitamin D insufficiency compared to healthy individuals. Glucocorticoids, a type of drug used to treat autoimmune illnesses, are a risk factor for vitamin D insufficiency (12). The increased prevalence of hypovitaminosis D in autoimmune illnesses is not entirely understood. Excessive use of corticosteroids may partly explain this phenomenon. It leads to increased breakdown of vitamin D by increasing the expression of certain receptors and enzymes, such as steroids and xenobiotic nuclear receptors (SXR) and CYP3A4, respectively (13). The study conducted in Egypt on primary ITP patients who were less than 18 years old, both sexes, found that the Vitamin D values are significantly lower in chronic and persistent ITP children than those in controls (6). Another study conducted in Croatia on 2–18-year-old children found that Vitamin D deficiency is very common in children with both newly diagnosed and chronic ITP forms. Innate and adaptive immune responses are modulated by vitamin D, an immunomodulatory drug that targets a variety of immune cells, including monocytes, macrophages, dendritic cells (DCs), T lymphocytes, and B lymphocytes. Vitamin D also decreases the likelihood of developing autoimmune illnesses. Furthermore, there are indications that autoimmune illnesses are susceptible to vitamin D. Significant amounts of Vitamin D receptors (VDR) are present in T- lymphocyte and macrophage populations, and they play an important role in T cell-mediated immunity (14).

Increased platelet count, less bleeding, remission induction, and overall patient well-being are the goals of immune thrombocytopenia treatment. Corticosteroids serve as the first line of defense against platelet destruction by preventing the development of autoantibodies and excessive cytotoxic T-cell activity. Other possible treatment options include Intravenous immunoglobulin, mycophenolate, and thrombopoietin receptor agonists (TPO-RAs) in the event of bleeding affecting lifestyle, as well as platelets transfusion in cases of life-threatening bleeding in rare cases (15). Thus, the aim of the current study was to determine how often children with ITP have low levels of 25-hydroxyvitamin D and to evaluate how the disease stage and therapy type affect vitamin D levels.

Patients and Methods:

This study was conducted in the Children Welfare Teaching Hospital, Medical City, Baghdad, Iraq, by the Department of Biochemistry / College of Medicine / University of Baghdad, between November 2023 and April 2024. The study included 88 children, 63 of them had been diagnosed with ITP by a pediatric hematologist, and 25 healthy children as controls who were free from any acute and chronic illnesses, they were selected from the children of colleagues and relatives from Baghdad and other governments after details asking about their medical history. The ITP group and control group were sub-classified according to their serum vitamin D levels into three groups: Those with less than 10 ng/ml of vitamin D, those with vitamin D levels from 10 to 20 ng/ml, and those whose vitamin D levels ranged from 20 to 30 ng/ml (16).

The ITP children were also sub-classified according to the disease phase into The ‘newly diagnosed ITP group’ (ITP duration within three months), the ‘persistent ITP group’ (ITP duration between 3 - 12 months), and the ‘chronic ITP group’ (ITP duration that is more than 12 months) (3). The ITP children included in this study were also sub-classified according to their type of treatment into Group 1 (Romiplostim therapy group), which included 18 children, Group 2 (the steroid therapy group), which included 19 children treated with prednisolone only, and Group 3 (other modality including; prednisolone and IVIG, or prednisolone and mycophenolate) included 26 children treated with prednisolone and IVIG, or prednisolone and mycophenolate. The dose of each medicine was defined by a consultant pediatric hematologist according to the severity of the disease. It has to be mentioned that the platelets count reported in the study was the count at the time of the patient’s visit to the outpatient clinic in the hospital. Some of these counts are normal as a response to treatment while others were still low during treatment.

The cases were selected at the age range of 1 - 16 years, and they were all undergoing treatment. The first line of their treatment was prednisolone alone or in combination with intravascular immunoglobulin (IVIG). The second line was mycophenolate. The third line was Romiplostim.

This study was approved by the scientific and ethical committees of the Department of Biochemistry, College of Medicine, University of Baghdad. Ethical approval was also obtained from the Children Welfare Teaching Hospital, Medical City, and Ministry of Health / Iraq. Verbal consent was obtained from the children's guardians in this study.

The control group consisted of 25 healthy children selected from the children of colleagues and relatives who were healthy and not suffering from any acute or chronic illness.

The exclusion criteria included those patients who had a blood transfusion during the previous month, had active infections, and any case of suspected inherited platelet disorders based on history, physical examination, and laboratory results.

Five milliliters (ml) of blood was aspirated from the peripheral vein of each patient and control group and allowed to clot for 15 minutes, then centrifuged for 10 minutes at 2500 rpm. The separated serum was stored at -45º C till the day of lab testing, which included measurements of vitamin D, using a semiautomatic ELISA Reader (Huma Reader, by the Human Diagnostics German company, Washer (COMBIWASH)). The principle of the ELISA technique with the biotin double antibody sandwich method was used for the evaluation of human vitamin

D. The wells were coated with the vitamin D monoclonal antibody and allowed to incubate. The next step was to combine streptavidin-HRP with biotin-labeled anti-vitamin D antibodies, to create an immunological complex. After incubation and washing, the enzymes that remained unbound were removed. Substrates A and B were combined. In the presence of acid, the solution would undergo a color shift, first from blue to yellow. The human vitamin D content was positively correlated with the solution color. The platelet counts were measured using Huma Count 30 ^{TS} Human, Germany.

Statistical analysis was done using the SPSS version 25.0 software which described the data using percentages, means, and standard error of the mean (SEM). The ANOVA test was used to assess the differences between means of numerical data when more than two means were tested. The correlation between the numerical data was evaluated using the Pearson correlation regression. A P value of < 0.05 was considered significant.

Results:

Out of the 63 ITP children, there were 34 females (54%) and 29 males (46%). Table 1 shows the mean (±SEM) values of age, platelet counts, and 25-hydroxy vitamin D in the blood of ITP children and controls. The mean platelet count value was significantly lower in the ITP children compared to the controls (p = 0.0001). ITP children's serum 25-hydroxy vitamin D mean value was very low but not significantly different from the controls.

Table 1: Mean (± SEM) of age, platelet counts, and 25-hydroxy vitamin D in the ITP cases and controls

Parameter	ITP group (n=63)	Controls (n=25)	*P* Value
Age (years)	7.2±0.51	8.3±0.93	> 0.05
Platelet count (10^9/L)	117.5±18.15*	376.2±7.51	< 0.0001
25-hydroxy vitamin D (ng/ml)	7.9±0.50	10.0±1.32	> 0.05

Table 2 shows the percentage of cases and controls in each of the three 25-hydroxy vitamin D level subgroups. Three-quarters of the ITP cases had blood vitamin D levels of < 10 ng/mL compared to 64% of the controls. A quarter of the ITP cases had vitamin D levels of (10–20 ng/ml), compared to 36% of the controls. There were no cases or controls with Vitamin D levels of more than 20 ng/ml. The Chi-square test revealed no significant association between vitamin D level and the study group.

Table 2: Frequency and percentage of 25-hydroxy vitamin D based on a normal reference range

Group	25-hydroxy vitamin D level (ng/ml)	
	<10 – No. (%)	10–20 – No. (%)
Patients (n = 63)	47 (75%)	16 (25%)
Controls (n =25)	16 (64%)	9 (36%)

Table 3 shows the mean (± SEM) values of 25 hydroxy vitamin D in the serum according to the phase of disease (newly diagnosed, persistent, or chronic) of cases and controls. The mean values of serum 25-hydroxy vitamin D of the newly diagnosed and chronic phases were not statistically significant, but they were lower than those of the controls. The lowest level of 25- hydroxy vitamin D was found in the chronic phase of the disease. There were also non-significant differences among and between these groups of patients.

Table 3: Mean values (±SEM) of 25-hydroxy vitamin D concentrations of the ITP cases groups and controls

Parameter	ITP Groups			Control (n=25)
	Newly diagnosed (n=21)	Persistent (n=24)	Chronic (n=18)	
25-hydroxy vitamin D (ng/ml)	9.5±1.84	11.4±1.92	8.0±1.13	10.0±1.32

NS: Non- significant (p>0.05)

Table 4 shows the mean (± SEM) values of 25-hydroxy vitamin D concentrations in the serum according to the type of treatment. The mean values of the prednisolone group, prednisolone, and IVIG, prednisolone and mycophenolate group, and romiplostim group were lower than the controls, but they did not reach a significant level. No significant differences were found across the analyzed patient subgroups.

The results also revealed that receiver operating characteristic (ROC) and area under the curve (AUC) for 25-hydroxy vitamin D in differentiation between acute ITP children and controls was (AUC=0.67) at cutoff (5.8 ng/ml) with (sensitivity=52.4, specificity=92.0). Similarly, 25-hydroxy vitamin D has AUC=0.64, at cutoff (6.3 ng/ml) with (sensitivity=55.6, and specificity=80.0) in differentiation between chronic ITP children and controls. Serum 25-hydroxy vitamin D has a significant positive correlation with platelet counts in the Romiplostim group (r=0.51, p=0.032).

Table 4: Mean (±SEM) values of 25-hydroxyvitamin D concentration according to the type of treatment

Parameter	Romiplostim (n=18)	Prednisolone only (n=19)	Prednisolone + IVIG, or prednisolone + mycophenolate (n=26)	Control (n=25)
25-hydroxy vitamin D (ng/ml)	8.2±0.96	8.1±0.96	7.7±0.81	10.0±1.32

Discussion:

The female predominance in the ITP cases of the current study is consistent with that observed by Shaheen *et al.* and Čulić *et al.*, who reported that females were predominant among ITP children in their group (14,17). They found that the mean age of the ITP children was 6.69 years, which agrees with the present study. The blood 25-hydroxy vitamin D level is the most reliable biochemical indicator of vitamin D status, as it reveals the amount of vitamin D that the body makes from the diet, sunlight, and the conversion of vitamin D reserves by the liver (17). 1,25(OH)2D3 exerts its effects by attaching to the vitamin D receptors (VDR). Evidence suggests that VDR is not only found in the colon, bones, and kidneys. also the peripheral blood monocytes and activated lymphocytes. Thus, VDR is recognized to participate in several immunomodulatory functions (18). Matinkia et.al. reported that Vitamin D may be used as a novel immunomodulatory treatment for people with ITP. Thus, there is a supplementary benefit for Vitamin D in individuals with ITP (19). Petrovic et al. reported that most of the ITP children had low levels of vitamin D. which is consistent with the findings of the current study that most of the ITP children had serum 25-hydroxy vitamin D levels below 10 ng/ml (10). Hypovitaminosis D affects the severity of ITP in children at the time of diagnosis, and therapy with vitamin D might be a new possible alternative for ITP treatment (10).

The current study looked at 25-hydroxy vitamin D blood levels in children with ITP at various phases of the illness and found no statistically significant differences. Similarly, Shaheen *et al.* found no statistically significant differences in the mean blood vitamin D concentration between the control group, patients with persistent and chronic ITP, and those newly diagnosed with ITP, even after adjusting for age (17). Lui *et al.* provided an analogy (20) with no significant difference in blood vitamin D levels between healthy controls and ITP patients. In contrast, Čulić *et al.* found that chronic ITP patients had significantly lower blood vitamin D levels compared to acute ITP patients. This was explained by the fact that ITP therapy also reduced their 25(OH)D values (14).

The non-significant differences in 25-hydroxy vitamin D blood levels among types of treatment of ITP in the current study may be due to that all groups began with prednisolone use. No published reports were found about this issue. The osteoblastic and osteoclastic processes that result in the equilibrium of minerals and vitamin D may be impacted by the glucocorticoid treatment of ITP (13). Of note, the levels of platelets reported in the cases were taken at a single point of time during treatment, many of them were normal in terms of response to treatment.

Vitamin D is used as a new medicinal method when high amounts of IFNγ are implicated in the development of illnesses. Individuals just diagnosed with ITP, as well as those who have been living with the ailment for an extended period may find Vitamin D therapy advantageous (21).

Limitation: Inability to include newly diagnosed children with ITP because of limited cases encountered during the time of the study.

Conclusion:

In children who have immunological thrombocytopenia, vitamin D deficiency is prevalent and may be very severe, particularly in the chronic phase.

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Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in the place where the research was conducted or samples collected and treated) according to the code number (107) on (18/ 4/ 2024).

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Authors' contributions:

The manuscript should mention the contribution of each author to the research done:

Study conception & design: (Basil O Saleh, Hasanein H Ghali). Literature search: (Huda K Abbas). Data acquisition: (Huda K Abbas). Data analysis & interpretation: (Huda K Abbas & Basil O Saleh). Manuscript preparation: (Basil O Saleh, Hasanein H Ghali & Huda K Abbas). Manuscript editing & review: (Basil O Saleh, Hasanein H Ghali).

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مدى تكرار نقص 25 هيدروكسي فيتامين د لدى الأطفال المصابين بتكسر الصفيحات الدموية المناعي: مرحلة المرض وتاثير نوع العلاج

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خلفية البحث: يمكن لنقص فيتامين د أن يسبب إضطرابات مناعية في تطور نقص الصفيحات المناعي المزمن.
الأهداف: تحديد معدل تكرار 25 هيدروكسي فيتامين د ذو مستوى منخفض في الأطفال الذين لديهم تكسر الصفيحات الدموية المناعي وأيضا لتقييم تاثير المرض ومرحله ونوع العلاج على مستوى فيتامين د.
الحالات والمنهجية: تم إجراء دراسة الحالات والشواهد هذه في مستشفى حماية الأطفال التعليمي في مدينة الطب، قسم الكيمياء الحياتية السريرية في كلية الطب / جامعة بغداد بين شهري نوفمبر 2023 وأبريل 2024. شملت الدراسة 88 طفلا، 63 منهم تم تشخيص إصابتهم سابقا بنقص الصفيحات المناعي و25 طفلا يتمتعون بصحة جيدة كمجموعة تحكم. تم تقسيم الأطفال الذين يعانون من نقص الصفيحات المناعي إلى مجموعات فرعية، وفقا لمستويات فيتامين د في الدم إلى ثلاث مجموعات أقل من 10 نانوغرام/مل، 10 -20 نانوغرام/مل، 30- 20 نانوغرام/مل. كانت هناك تصنيفات فرعية للأطفال الذين يعانون من نقص الصفيحات المناعي وفقا لمراحل المرض لديهم: حاد، ومستمر، ومزمن. تم قياس مستوى -25هيدروكسي فيتامين د في الدم باستخدام تقنية الاليزا.
النتائج: كشفت النتائج أن غالبية الأطفال ITP (75٪) لديهم مستوى 25 هيدروكسي فيتامين د في الدم أقل من 10 نانوجرام / مل. كانت القيم المتوسطة (± SEM) لمصل 25 هيدروكسي فيتامين د لدى أطفال ITP في المراحل الحادة والمزمنة أقل من تلك الخاصة بالضوابط، لكنها لم تصل إلى مستوى مهم. علاوة على ذلك، كانت قيم 25 هيدروكسي فيتامين د في المصل لدى الأطفال ITP أقل من تلك الخاصة بالضوابط، بغض النظر عن نوع العلاج.
الإستنتاجات: في الأطفال الذين يعانون من نقص الصفيحات المناعي، يكون نقص فيتامين د شائعا وقد يكون شديدا جدا، وخاصة في المرحلة المزمنة. قد يلقي الارتباط الإيجابي الكبير بين 25 هيدروكسي فيتامين د وعدد الصفائح الدموية في مجموعة روميبلوستيم الضوء على نهج العلاج الجديد لهذا المرض.
مفتاح الكلمات: فرفرية نقص الصفيحات الدموية المناعي، 25 هيدروكسي فيتامين د، نقص الصفيحات المناعي، المناعة الذاتية.

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Research Article

Association between Alpha- Klotho Protein, Calcium, and Phosphate concentrations in Adult Iraqi Patients with Beta-Thalassemia Major

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Abstract

Background: Beta-thalassemia major is a prevalent global condition characterized by a rapid breakdown of red blood cells. Regular blood transfusions can give rise to problems such as cardiovascular disease, diabetes, osteoporosis, and renal disorders. Alpha-Klotho protein is a protein that has anti-aging properties and is involved in several functions, including reducing oxidative stress, regulating energy metabolism through several routes, and managing calcium and phosphate metabolism.

Objective: This study aimed to assess changes in calcium and phosphate levels, Alpha-Klotho protein concentration, and their associations with cardiac dysfunction in patients with Beta-thalassemia major.

Methods: The study was conducted at Al-Sadr General Hospital and Ibn Albaladi Center of Blood Diseases, Baghdad, and involved 90 participants who were grouped into three groups: Group A: 30 patients with Beta-thalassemia major and heart dysfunction; Group B:30 patients with Beta-thalassemia major without any signs of heart dysfunction; and Group C:30 healthy individuals as a control group. The indicators examined were serum levels of Alpha-klotho protein, calcium, phosphate, and Ferritin. ELISA method was used to assess serum Alpha-klotho protein, whereas serum Ca, serum phosphate, and serum Ferritin were analyzed using the Beckman Coulter AU clinical chemistry analyzers.

Results: The mean values of Serum Alpha-Klotho protein, phosphate, and Ferritin in the patients with beta-thalassemia were greater than those in the control group with P value<0.05. Patients with thalassemia had decreased levels of serum calcium compared to the control group. Additionally, a strong negative association was observed between serum calcium and phosphate levels.

Conclusion: Patients with beta-thalassemia major have significant alterations in calcium and phosphate levels under the control of Klotho protein levels.

Keywords: Calcium; Ferritin, Klotho, Phosphate, Thalassemia.

Introduction:

Thalassemia is a hereditary autosomal recessive blood condition defined by the improper production of hemoglobin (1). Thalassemia is mainly classified into: alpha-thalassemia and beta-thalassemia depending upon the reduced or absent-minded synthesis of the alpha-globin chain or Beta-globin chain of hemoglobin. Beta-thalassemia is categorized into major, moderate, and mild forms according to clinical criteria (2). Beta-thalassemia major is a severe illness recognized as a global issue (3). Ferritin is a widely distributed protein that stores and detoxifies iron. It is crucial in regulating iron balance by keeping it soluble and non-harmful (4). Iron overload occurs in β-TM patients due to numerous blood transfusions, inadequate erythropoiesis, and increased iron absorption via the gastrointestinal tract. The presence of secondary hemosiderosis adversely affects several organs in the body, such as the heart, liver, and endocrine system. Serum ferritin is a frequently employed indicator of Iron levels in individuals with β-TM (5). Calcium and phosphate are crucial elements necessary for maintaining bone strength and stiffness. Furthermore, they play a vital role in muscle activity, the transmissionof nerve impulses, intracellular signaling, and the secretion of different hormones (6). Klotho is a co-receptor for the hormone fibroblast growth factor 23 (FGF23) and has anti-aging properties. Klotho is enzymatically broken and then released into circulation as a substance derived mainly from the kidney. It exerts a wide range of actions in virtually all organs (7). It controls the reabsorption of calcium and phosphate in the kidney and regulates vitamin D metabolism (8). Klotho is mainly produced in the kidney and binds to FGF receptors (FGFRs), enhancing their attraction to FGF-23 and facilitating the excretion of phosphate in urine. The expression of Klotho decreases as renal function declines. Klotho is a substance in the body that has multiple roles and acts as a protective factor for the heart by regulating ion channels. This action is independent of FGF-23 and phosphate (9). High ferritin levels due to iron overload might affect calcium and phosphate metabolism, which may also be linked to Klotho levels. Evaluate the klotho levels in beta-thalassemia patients and correlate them with

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calcium, phosphate, and ferritin levels could provide insights into the complex interactions among klotho, calcium, phosphate, and ferritin, which may lead to better management strategies for beta-thalassemia.
This study aimed to assess changes in calcium and phosphate levels, Alpha-Klotho protein concentration, and their associations with cardiac dysfunction in patients with Beta-thalassemia major.

Patients, Materials, and Methods
Patients and control:
In the present study, 90 subjects were recruited. Their age ranged from 18 to 30 years. Each participant completed a questionnaire that included the following information: code number, age, sex, date, address, ethnicity, family history of thalassemia, weight, height, and medical history. The study was conducted at Al-Sadr General Hospital and Ibn-AL-Baladi Center of Blood Diseases in Baghdad from 1st, March 2023 to 31st, August 2023. The individuals were categorized into three groups based on clinical and physiological examinations of heart function and the physician's diagnosis using ECG and ECHO tests conducted at Al-Sadr General Hospital and Ibn-AL-Baladi Center of Blood Diseases. Group A consists of 30 patients with β-TM who have heart dysfunction. Group B consists of 30 patients with β-TM who do not exhibit any signs of heart dysfunction. Group C consists of 30 healthy individuals who serve as the control group. This study excluded patients with comorbidities such as Diabetes mellitus, liver disease, brain disease, and kidney disease. Additionally, patients with cancer, obesity, and active infection were also excluded. It is important to note that the medication administered to the patients may have influenced the study's outcomes. All patients and healthy subjects, or their parents, were asked to agree to participate in this study, and their consent was publicly recognized. Blood sampling. Subjects' blood samples were withdrawn during morning hours from 8:00 a.m. to 11:00 a.m. by venesection using a 10 ml disposable syringe. The blood was collected into gel tubes that aid blood clotting and separation of serum. The blood in gel tubes was allowed to clot at 37°C for roughly ten to fifteen minutes. It was then centrifuged at 2000rpm the acceleration due to gravity for ten to fifteen minutes. The resulting serum was separated in sterile Eppendorf tubes and stored at -20°C. For analysis, 0.5 ml of serum was utilized. The following biomarkers were measured in the blood: serum α-Klotho protein, serum calcium, serum phosphate, and serum ferritin. The serum α-Klotho protein is determined using an enzyme-linked immunosorbent assay (ELISA) kit. This kit is a sandwich enzyme immunoassay designed for in vitro quantitative measurement. The ELISA kit is the Klotho-(KL)-SEH757Hu Cloud-Clone Corp (USA). In addition to measuring the Alpha-Klotho protein, the Clinical Automation system by Beckman Coulter is used to measure serum calcium, serum phosphate, and serum ferritin.
Calculation of Body Mass Index
The body mass index (BMI) calculated as weight (Kilograms) divided by the square of height (in meters) was the only anthropometric parameter specified. All subjects were weighted on the same scale, barefoot. Height was measured using a measuring tape.

Statistical Analysis:
The statistical analysis was conducted using the MedCalc software, specifically version 19.6.1. Continuous data were summarized using the median and interquartile ranges and the mean± Standard Deviation (SD). The comparison results were expressed as mean ± SD based on analysis of variance (ANOVA) for each study. A Pearson correlation analysis was performed to see if there was a significant association between the parameters. The alpha level for statistical significance was established at a threshold of $P < 0.05$.

Results:
The demographic characteristics of the participants in the present study are displayed in Table (1). The frequency distribution of people according to sex did not show any significant difference between the beta thalassemia major groups (A and B) and the control group, there were in each group 17 (57.0%) males and 13 (43.0%) females. In Table (1) the Mean±SD for age across the three groups (A, B, and C) are statistically similar, as indicated by the P-value of 0.65 suggesting that age is not a differentiating factor among these groups. There is a difference in BMI across the groups. The group A has a Mean±SD BMI of 20.92± 0.90 Kg/m², group B has 21.12± 0.95 Kg/m², and the group C has 23.72± 1.41 Kg/m². The p-value <0.01 suggests a significant variance, with the control group(C) differing notably from the other two groups (A and B).

Table 1 Descriptive analysis of Age and BMI across the three study groups

Parameter	Group	Mean± SD	Group	P value
Age (Years)	Group A	23.60± 4.75	C	0.753
	Group B	22.67± 4.78	A	0.658
	Group C	22.83± 2.03	B	0.987
BMI Kg/m²	Group A	20.92± 0.90	C	< 0.01
	Group B	21.12± 0.95	A	0.752
	Group C	23.72± 1.41	B	< 0.01

Note: Each parameter's mean and standard deviation (Mean± SD) are provided, along with the P-value indicating the significance of the differences between the groups.

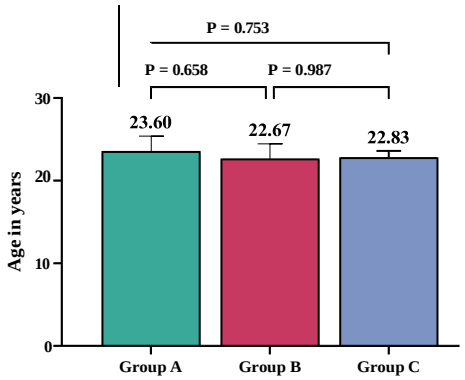

Figure 1 Mean values of age by Groups with 95.00% CI (Confidence intervals) Error Bars.

Table 2 A comparison of serum Klotho, serum Ca, serum phosphate, and Ferritin across the three study groups

Parameter	Group	Mean± SD	Groups	P-value
Serum Klotho (ng/mL),	Group A	4.46± 1.03	C	<0.001
	Group B	5.34± 0.57	A	<0.001
	Group C	1.48± 0.51	B	<0.001
Serum Ca (mmol/L),	Group A	7.94± 1.56	C	<0.001
	Group B	8.71± 0.66	A	0.012
	Group C	8.93± 0.30	B	0.684
Serum PO4 (mmol/L)	Group A	6.57± 1.64	C	<0.001
	Group B	5.89± 1.15	A	0.085
	Group C	3.82± 0.49	B	<0.001
Serum Ferritin (ng/Ml)	Group A	4276.73± 2401.39	C	<0.001
	Group B	4703.17± 3390.18	A	0.777
	Group C	53.20± 17.01	B	<0.001

The results are presented as mean ± standard deviation (SD), and the statistical significance is denoted by the P-value.

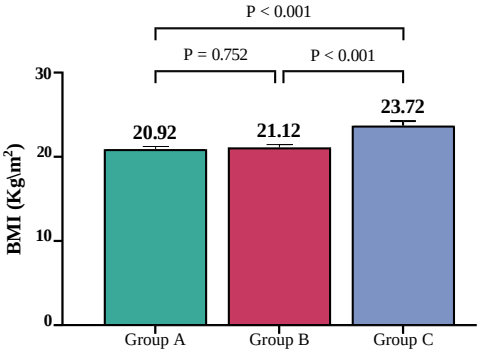

Figure 2 Mean values of BMI by Groups with 95.00% CI Error Bars.

Table 2 compared mean±SD serum levels of Klotho, Ca, phosphate, and Ferritin across groups A, B, and C which were 4.46±1.03 (ng/mL), 5.34±0.57 (ng/mL), and 1.48±0.51 (ng/mL) respectively. group A and B exhibited significantly higher mean values than group C, with a P-value< 0.001, indicating a statistically significant difference, as shown in Table (1). The calcium mean±SD levels in groups A, B, and group C are 7.94 ± 1.56, 8.71 ± 0.66, and 8.93 ± 0.30, respectively. The P-value for this parameter is <0.001, indicating significant statistical differences between the groups. Notably, group A showed a lower mean calcium level than the other two groups, which were statistically similar.

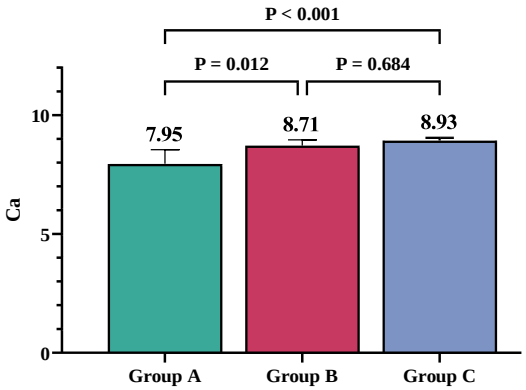

Figure 3 Mean serum levels of Ca by Groups with 95.00% CI Error Bars.

The mean±SD phosphate level for group A was 6.57 ± 1.64 mmol/L, whereas group B had an average level of 5.89 ± 1.15 mmol/L. On the other hand, the group C group has a noticeably lower average level of 3.82 ± 0.49 mmol/L. With a P-value of less than 0.001, this parameter exhibits substantial statistical disparities

across the groups. Group A and Group B had greater phosphate levels than Group C.

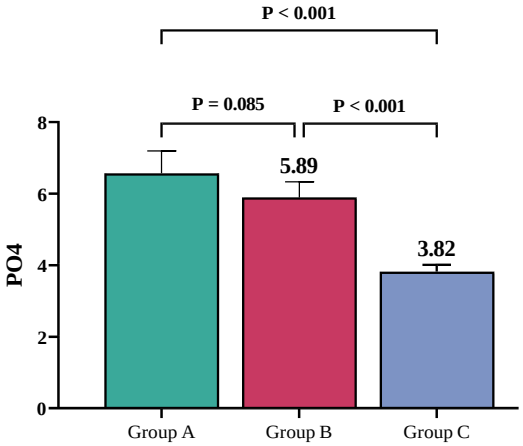

Figure 4 Mean serum levels of PO_4 by Groups with 95.00% CI Error Bars.

Finally, group A had a mean±SD serum Ferritin level of 4276.73 ± 445.93 (ng/mL), which was similar to group B which had a mean±SD serum level of 4703.17 ± 629.54(ng/mL), group C with a significantly lower mean of 53.20 ± 3.16(ng/mL). The *P*-value <0.001 strongly suggested significant differences between the groups, with both group A and group B showing markedly higher Ferritin levels compared to the control group (group C).

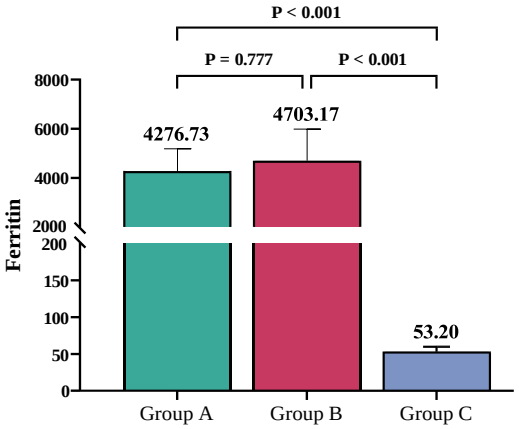

Figure 5: Mean serum levels of Ferritin by Groups with 95.00% CI Error Bars**.**

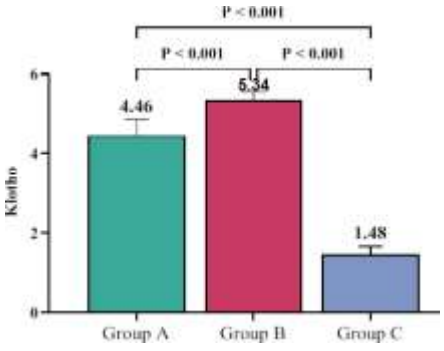

Figure 6: Mean serum levels of Klotho by Groups with 95.00% CI Error Bars.

The matrix for group A shows substantial positive correlations (r=0.41, *P* value<0.05) between phosphate and Ferritin, as well as negative correlations (r= -0.41, *P* value<0.05) between phosphate and Ca.

Table 3 Correlation matrix (Pearson) for group A

Variable	Klotho	Ca	Ferritin	PO4	BMI
Klotho	1	-0.22	-0.18	0.03	-0.22
Ca	-0.22	1	-0.07	-0.41	0.24
Ferritin	-0.18	-0.07	1	0.41	0.10
PO4	0.03	-0.41	0.41	1	-0.25
BMI	-0.22	0.24	0.10	-0.25	1

Values in bold are different from 0 with a significance of level alpha=0.05

In the matrix for group B, there is a significant negative correlation between phosphate and Ca (r= -54, *P* value<0.05). It is important to note that although these relationships are statistically significant, they did not indicate causality. Several causes may alter the connections between these variables, and the varied patterns identified between the two groups may indicate disparities in their demographic or clinical features. These differences emphasize the significance of considering group-specific dynamics when analyzing biomarker correlations in clinical or research environments.

Table 4 Correlation matrix (Pearson) for group B

Variable	Klotho	Ca	Ferritin	PO4	BMI
Klotho	1	-0.20	0.32	-0.12	0.26
Ca	-0.20	1	-0.12	-0.54	-0.08
Ferritin	0.32	-0.12	1	-0.10	0.21
PO4	-0.12	-0.54	-0.10	1	-0.10
BMI	0.26	-0.08	0.21	-0.10	1

Discussion:

This study found no statistically significant sex difference in the distribution of patients and controls between males (57.0%) and females (43.0%). β-Thalassemia major patients' groups showed a significant increase in serum ferritin levels compared to the control group. This finding was consistent with previous Iraqi studies by Talib et.al, Ali EA et.al, and Maki Al-Hindyet.al., which showed a significant increase in serum ferritin levels compared to healthy subjects (10-12). Iron excess often arises from two mechanisms: blood transfusion and insufficient erythropoiesis. In individuals with thalassemia, mutations lead to increased production of GDF15 protein which acts as an inhibitor of the peptide-

hepcidin hormone and transmits a signal to the liver, causing a drop in the amount of Hepcidin and improved iron absorption from the diet. Consequently, erythrocytes that are not functioning correctly are captured in the spleen, causing iron release, ultimately leading to an elevation in ferritin levels (13) There was no significant difference in serum ferritin in β-TM patients group B against group A (*P*-value>0.05) with a slight increase in group B. Which can be explained due to the treatment protocol for heart disease by increased dosage of iron chelators (14) and the influence of cardiovascular medications (15). Thalassemia patients in group A had a significant difference in phosphate levels as compared to groups B and C and A significant reduction in serum calcium levels compared to groups B and C, this result agreed with Sultana MA(6). The elevation in serum phosphate and associated reduction of serum calcium in thalassemia patients with heart dysfunction are attributed to several factors, such as iron accumulation in different tissues, including osteoblasts, frequent blood transfusions, or the use of desferrioxamine as a chelation treatment for iron overload (10). These findings were consistent with prior research showing elevated levels of serum phosphate in individuals with beta-thalassemia major due to chronic hemolysis and transfusions (16), hypoparathyroidism, and reduced kidney function. In this study, patients with renal insufficiency were not included. Therefore, the elevated phosphate levels observed may be attributed to chronic hemolysis or hypoparathyroidism (17). Hyperphosphatemia plays a role in the onset and progression of various cardiovascular diseases and is a significant risk factor for elevated cardiovascular mortality. Previous research has demonstrated that elevated phosphate levels can lead to left ventricular hypertrophy (LVH), myocardial fibrosis, and a higher risk of cardiovascular mortality (18). Iron excess in thalassemia can also impact calcium absorption in the intestines, and there is a mutual relationship between the transportation of iron and calcium in thalassemia (19). Calcium entering cardiac fibers triggers the release of calcium from the sarcoplasmic reticulum, leading to an increase in intracellular calcium concentration. This calcium then binds to troponin C, which controls the interaction between actin and myosin, resulting in muscle contraction (20). Studies have demonstrated that hypocalcemia directly affects heart function, leading to reduced cardiac contractility. A drop is seen in the left ventricular work, stroke, and cardiac indexes. It is a possible factor leading to heart failure (21). The current study demonstrated that the serum Klotho levels in patients with β-TM in groups (A and B) were considerably elevated compared to those in group C. Thalassemia patients experience inflammation and oxidative stress damage due to the direct effects of iron poisoning. Su and Yang determined that α-Klotho may function as an acute phase response, as demonstrated by the elevation of serum α-Klotho protein in response to restraint stress. Crucially, α-Klotho functions as an anti-inflammatory regulator by controlling the synthesis of nuclear factor-κB-associated inflammatory proteins. This leads to a decrease in the production of various pro-inflammatory cytokines and the harmful effects of oxidative stress. α-Klotho provides defense against oxidative stress at both the cellular and organismal levels (22). Thalassemia patients experience impaired calcium absorption, resulting in reduced calcium levels (19). This condition is triggered by certain stimuli that cause the secretion of αKl (23). Soluble Klotho protects against cardiac hypertrophy by suppressing aberrant calcium signaling in the heart, regardless of FGF23 and phosphate levels (24). When comparing the results of thalassemia patients in groups A and B, we observed that the thalassemia group without heart disease has phosphate and calcium concentrations near the expected levels with a higher concentration of klotho than thalassemia patients with heart problems which had low calcium concentration and a significant increase in phosphate levels counteracted by a decrease in klotho concentration. The reduction of Klotho can enhance the action of prooxidative, proinflammatory, and proapoptotic factors, leading to damage of cardiomyocytes in individuals at risk of cardiovascular disease (25).

Limitation of study:

The study was based on only one Hospital and one center. Hence, the findings don't represent the whole population.

Conclusion:

Klotho protein plays a crucial role in regulating phosphate and calcium metabolism in the body. Patients with thalassemia major have significant alterations in calcium and phosphate under the control of Klotho protein levels. These changes in klotho protein can potentially lead to cardiovascular complications in the future. Estimating klotho protein in beta-thalassemia patients might be helpful for the early detection of calcium and phosphate dysregulation and prevent its complications.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current research, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in (Biochemistry Department) according to the code number (138) on (16/ 5/ 2024).

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Author contributions:

Study conception & design: (Ahmed J. Kadhim, Hedef D. El-Yaseen, Ali M. Jawad). Literature search: (Ahmed J. Kadhim, Hedef D. El-Yaseen, Ali M. Jawad). Data acquisition: (Ahmed J. Kadhim,

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العلاقة بين بروتين ألفا كلوثو وتركيزات الكالسيوم والفوسفات لدى مرضى بيتا ثلاسيميا الكبرى البالغين العراقيين

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الخلاصة:

خلفية البحث: بيتا ثلاسيميا الكبرى (β-TM) هي حالة عالمية منتشرة تتميز بالانهيار السريع لخلايا الدم الحمراء. يمكن أن تؤدي عمليات نقل الدم المنتظمة إلى مشاكل مثل أمراض القلب والأوعية الدموية والسكري وهشاشة العظام واضطرابات الكلى. بروتين كلوثو هو بروتين له خصائص مضادة للشيخوخة ويشارك في عدة وظائف، بما في ذلك تقليل الإجهاد التأكسدي، وتنظيم استقلاب الطاقة من خلال عدة طرق، وإدارة استقلاب الكالسيوم والفوسفات.

الاهداف: تشير فرضيتنا إلى أن التغيرات في مستويات بروتين كلوثو لدى الأفراد الذين يعانون من بيتا ثلاسيميا الكبرى قد تؤدي إلى تغييرات في استقلاب الكالسيوم والفوسفات. هدفت هذه الدراسة إلى تقييم التغيرات في مستويات الكالسيوم والفوسفات، وكذلك تركيز بروتين كلوثو، وارتباطها بخلل وظائف القلب لدى مرضى بيتا ثلاسيميا الكبرى.

المرضى وطرق العمل :المواد وطرق العمل: أجريت الدراسة في مستشفى ابن البلدي/بغداد وتكونت من 90 مشاركا تم تقسيمهم إلى ثلاث مجموعات: المجموعة (أ): 30 مريضا يعانون من مرض بيتا ثلاسيميا الكبرى وخلل في القلب. المجموعة ب: 30 مريضا مصابين بـ بيتا ثلاسيميا الكبرى دون أي علامات لخلل في القلب. والمجموعة ج: 30 فردا أصحاء كمجموعة ضابطة. وكانت المؤشرات التي تم فحصها هي بروتين الفا كلوثو والكالسيوم والفوسفات والفيريتين في مصل الدم. تم استخدام طريقة ELISA لتقييم الفا كلوثوبروتين في مصل الدم، في حين تم قياس تركيز الكالسيوم والفوسفات والفيريتين في مصل الدم باستخدام جهاز التحليل الذاتي للكيمياء السريرية بيكمان كولتر.

النتائج: كانت القيم المتوسطة مصل الدم كلوثو والفوسفات والفيريتين في المرضى الذين يعانون من مجموعات بيتا ثلاسيميا أكبر من تلك الموجودة في المجموعة الضابطة. كان لدى مرضى الثلاسيميا انخفاض في مستويات الكالسيوم في مصل الدم مقارنة بالمجموعة الضابطة. بالإضافة إلى ذلك، لوحظ وجود ارتباط سلبي قوي بين مستويات الكالسيوم والفوسفات.

الاستنتاج: كانت مستويات الكلوثو والفوسفات والفيريتين في مصل الدم أكبرفي مجموعات بيتا ثلاسيميا مقارنة بالمجموعة الضابطة. بالإضافة إلى ذلك، لوحظ وجود علاقة سلبية قوية بين الكالسيوم والفوسفات.

الكلمات المفتاحية: الثلاسيميا، كلوثو، الفوسفات، الكالسيوم، الفريتين.

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Research Article

Prevalence of Cholelithiasis and Associated Factors of Gallstone Formation after Laparoscopic Sleeve Gastrectomy in the Gastroenterology and Hepatology Teaching Hospital-Baghdad

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Abstract:

Background: Bariatric surgery (BS) is the most effective method for long-term weight loss. Rapid weight reduction after BS may contribute to the formation of gallstones.

Objectives: To assess the rate, and to identify the associated factors, of gallstone formation after laparoscopic sleeve gastrectomy.

Methods: This was a cross-sectional study among 128 patients with morbid obesity, who were treated with laparoscopic sleeve gastrectomy and followed up at two weeks, one month, six months, and one year after surgery between October 2018 and July 2020, at the Gastroenterology and Hepatology Teaching Hospital in Baghdad and the Dowaly Private Hospital. Data was collected using a structured questionnaire.

Results: The mean age of patients was 36.5±5.21 (17–54) for females and 41.6 ±3.04 (24–58) for males. Gallstone formation happened in 49 cases (38.3%). Of all males, four (36.4%) developed gallstones after (LSG), compared to 45 (38.5%) females. Sixty (46.9%) patients had a Body Mass Index (BMI) ≥40 kg/m2, of whom 29 (48.3%) developed gallstones after LSG ($p = 0.031$). The *p*-values for weight loss regarding timing and degree of loss show a significant relationship with gallstone formation after LSG ($p < 0.05$). Comorbid conditions were not significantly associated with the incidence of gallstone formation after LSG ($p > 0.05$).

Conclusions: More than a third of the cases developed gallstones after Laparoscopic Sleeve Gastrectomy. This was associated with Gross obesity prior to surgery (BMI ≥ 40 kg/m²), losing ≥25% of the original weight, and rapid weight loss during the first six months after LSG.

Keywords: Bariatric Surgery; BMI**;** Gallstone; Obesity; Laparoscopic Sleeve Gastrectomy.

Introduction:

Obesity is associated with an increased risk of hypertension (HT), diabetes (DM), pulmonary disease, hyperlipidemia, cardiomyopathy, malignancy, arthritis, infertility, sleep apnea, gallstone formation, and psychosocial impairments (1). Weight loss has been shown to reduce many conditions associated with obesity. Bariatric surgery (BS) is the most effective method for long-term weight loss (2). In addition to restricting and reducing the surface area for absorption, hormonal changes after bariatric surgery are the primary mechanism of action (3, 4). The specific criteria established are that bariatric surgery is appropriate for patients with a body mass index (BMI kg/m²) of more than 40 and patients with a BMI of 35–40, with associated medical conditions. (2). Gallstones develop in patients who experience rapid weight reduction after dietary restriction and BS (5). Patients with rapid weight reduction after BS may be exposed to a high level of anxiety, depression (6),

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increased bile cholesterol saturation, decreased bile acid secretion, increased mucin secretion by 10–20 times, and finally decreased gallbladder emptying, causing bile stasis, all of which mainly contribute to the formation of gallstones (7). Cholelithiasis is common after BS, with a high incidence during the first 12–14 months after the procedure (8). In Saudi Arabia, a study found that the overall incidence rate of gallbladder stones after BS was 61.4%. (9) It was found that the risk of gallstones was as high as 51.2%, in patients who underwent Roux-en-Y gastric bypass (R-YGB) and were followed for a year afterward (10). The rate of developing cholelithiasis after laparoscopic sleeve gastrectomy (LSG) ranged from 29%–48%, as reported in the literature (11, 12). Other studies have reported that R-YGB surgery has a greater risk of gallstone formation than SG (13, 14). The role of prophylactic cholecystectomy at the time of BS remains controversial. In asymptomatic patients, who require cholecystectomy after BS, concomitant

cholecystectomy during BS prevents them from being exposed to a second surgery (15). The present study assessed the rate and investigated the possible factors associated with cholelithiasis development after a sleeve gastrectomy. It also evaluated the association between weight loss parameters and gallstone development in these patients.

Patients and Methods:

This was a cross-sectional study on 128 patients with morbid obesity who were treated with LSG between October 2018 and July 2020, at the Gastroenterology Hepatology Teaching Hospital of Baghdad and the Dowaly Private Hospital. Data collection began preoperatively and continued until the final postoperative visit. The patients were selected according to BS guidelines, and the suitable candidates were operated on by a single senior surgeon using the same surgical procedure. Patients who had a complete follow-up (at two weeks, one month, six months, and one year) after BS, at an outpatient clinic, were included in this study. We excluded from this study patients who had gallbladder disease and those with a history of cholecystectomy. The demographic data of patients were collected together with their medical history, clinical examination, and hormonal study preoperatively. The complete blood count, biochemical parameters, anthropometric measurements; and weight before surgery and at six and 12 months after surgery were recorded. Positive findings according to the abdominal ultrasound or MRI reports were recorded. Ethical approval was obtained from the committee of the Iraqi Board for Medical Specializations. Verbal informed consent was obtained from all patients.

Statistical Analysis:

All data were collected using Excel for Windows and an analysis with the Statistical Package for Social Sciences (SPSS) version 25 was performed. The Chi-squared test or Fisher's exact test was used for nominal variables, as appropriate. The *t*-test was used to compare continuous variables. A *P*-value less than 0.05 was considered significant.

Results:

The mean age of patients included in this study was 36.5±5.21 (17–54) years for females and 41.6±3.04 (24–58) years for males. Gallstone formation happened in 49 (38.3%) patients, Figure 1.

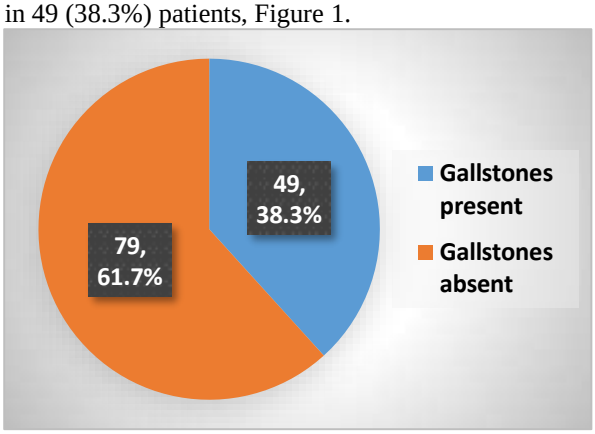

Figure 1: Gallstones formation after bariatric surgery.

Age and gender did not appear to be associated with gallstone formation after LSG, P value >0.05 (Table 1). Four out of 11 males (36.4%) developed gallstones after LSG compared to 45 (38.5%) out of 117 females. Age was also not associated with gallstone formation, *p*-value > 0.05. Parity did not appear to be associated with gallstone formation (P=0.97).

Table 1: Distribution of the cases by gender, age, parity, and gallstone formation after LSG

Variable	Category	Gallstone		Chi-squared	*P*-value
		Present	Absent		
Gender	Male (n-11) (8.6%)	4 (36.4%)	7 (63.6%)	0.018	0.914
	Female (n-117) (91.4%)	45 (38.5%)	72 (61.5%)		
Age (years)	≤ 45 (N = 82)	28 (34.1%)	54 (65.9%)	0.165	0.617
	> 45 (N = 46)	21 (45.7%)	25 (54.3%)		
Parity	Null (N = 29)	11 (37.9%)	18 (62.1%)	0.0012	0.97
	Parous (N = 88)	34 (38.6%)	54 (61.4%)		

There were 19 (14.8%) diabetics among the cases, of whom only five (26.3%) developed gallstones, the *p*-value was (0.527) indicating no increase in the risk of gallstone formation after LSG, among diabetics. The same was true for cases with hypertension. The mean BMI preoperatively was 41 ± 8.4 (range from 35.4–55.7). out of 128 patients, 60 (46.9%) had a BMI ≥40 kg/m². Of those 29 (48.3%) developed gallstones after LSG, the *p*-value is (0.031), able 2.

Table 2: Distribution of the cases by diabetes, hypertension, BMI, and gallstone formation after LSG

Variable	Category	Gallstone		Chi-squared	*P*-value
		Present N = 49 (38.3%)	Absent N = 79 (61.7%)		
DM	Diabetic N = 19	5 (26.3%)	14 (73.7%)	0.135	0.527
	Non-diabetic N = 109	44 (40.4%)	65 (59.6%)		
HT	Hypertensive N = 21	6 (28.6%)	15 (71.4%)	0.1	0.595
	Non-hypertensive N = 107	43 (40.2%)	64 (59.8%)		
BMI	≥ 40 Kg / m2, N = 60	29 (48.3%)	31 (51.7%)	4.83	0.031
	< 40 kg/m2, N = 68	20 (29.4%)	48 (70.6%)		

The period and magnitude of weight loss are significantly associated with gallstone formation after LSG, *p*-values (0.032) and (0.047), table 3.

Table 3: Distribution of the cases by weight-loss period and magnitude and gallstone formation after LSG

Weight loss	Category	Gallstone N = 49 (38.3%)	No gallstone N = 79 (61.7%)	*P*-value
Period of weight loss	First six months (n=40)	23 (57.5%)	17 (42.5%)	0.032
	Second six months (n=88)	26 (29.5%)	62 (70.5%)	
Magnitude of weight loss	≥25%	31 (63.3%)	21 (26.6%)	0.047
	< 25%	18 (36.7%)	58 (73.4%)	

Table 4 shows that co-morbidities were not significantly associated with the development of gallstones after LSG.

Table 4: Distribution of the study group by co-morbidities and the development of gallstone after LSG

Co-morbidity	Gallstone	No gallstone	*P*-value
Sleep apnea	6 (35.3%)	11 (64.7%)	0.862
Hypothyroidism	1 (25%)	3 (75%)	0.527
Dyslipidemia	7 (35%)	13 (65%)	0.846

Discussion:

Laparoscopic sleeve gastrectomy is fast becoming one of the most effective surgeries in the management of obesity. It significantly reverses some metabolic abnormalities, such as diabetes mellitus, hypertension, sleep apnea, and dyslipidemia (16). Rapid weight loss is the only risk factor that contributes to the development of post-LSG gallbladder disease (17). Mishra et al. in 2016 reported that the incidence of development of gallstones post-bariatric surgery was 8.42% in the LSG group (18), while it was 38.3% in the current study, which is comparable to the results of the studies of Coupaye *et al* in Colombia in 2015 and Manatsathit *et al* in the USA in 2016 (29% and 48% respectively) (11, 12). The difference can be explained by the difference in sample size. The current study found a non-significant slightly more females than males developing gallstone disease after LSG, in disagreement with the results of Mishra T *et al.,* 2016 where cholelithiasis was much more common in females (18). Parous females showed more gallstones after BS, but not significantly so. Female sex hormones appear to be the underlying factor for the differences observed with cholelithiasis. Gender is one of the most prominent risk factors for developing gallstone disease. At all ages, women are generally more likely to develop cholelithiasis than men, due to naturally high estrogen levels in women as suggested by Cirillo *et al* in 2005 (19), multiparity as suggested by Galyani in 2013 (20), or taking estrogen-based oral contraceptives (19). Females are more likely to undergo cholecystectomy than men at all ages as reported by Racine et al. (2013) (21), gallstone formation in the current study was highest during the first six months after surgery, during the time of weight loss compared to the next six months, indicating that the follow-up period is important in identifying complications after LSG; similar to the findings of Elshaer *et al* (22). Kielani *et al.* (23), reported the incidence of gallstone formation to be highest during the first six months after surgery (33.8% in the first six months versus 21.6% in the next six months), postoperatively, which supports our findings, whereas, Elshaer *et al*. reported that gallstone formation after surgery was 33.3% in the first six months and 10.3% in next six months (22). Our finding that patients with BMI ≥40 kg/m² are at higher risk factor for gallstone formation after surgery agrees with that of Grover et al, that a BMI ≥40 kg/m², risk of developing gallstones is eight-fold higher than those with a normal BMI (24). Risk factors for gallstone development in comorbid diseases, such as, diabetes, hypertension, sleep apnea, hypothyroidism, and dyslipidemia were not found to be significant in our study as in other studies (22-26). The importance of our study lays in identifying the associated factors for gallstone formation after LSG that may be necessary for selecting patients for specific prophylactic interventions, such as regular ultrasound surveillance for gallstones.

Conclusions:

More than a third of cases developed gallstones after LSG. This was associated with Gross obesity prior to surgery (BMI ≥ 40 kg/m²), losing ≥25% of the original weight, and rapid weight loss during the first six months after LSG.

Authors' declaration:
The manuscript is an original work, not previously published or sent to other journals. We hereby confirm that all the figures and tables in the manuscript are ours. The project was approved by the local ethical committee of the Iraqi board for medical specialization, code no. 64.
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Study conception & design: (Dr. Abdulrahman Mahmood Mohammed). Literature search: (Dr. Abdulrahman Mahmood Mohammed). Data acquisition: (Dr. Tuka Younis Hassan). Data analysis & interpretation: (Dr. Tuka Younis Hassan). Manuscript preparation: (Dr. Abdulnaser Mahmood Mohammed). Manuscript editing & review: (Dr. Abdulnaser Mahmood Mohammed).

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انتشار حصوات المرارة والعوامل المساعدة في تكوينها بعد تكميم المعدة بالمنظار في مستشفى امراض الجهاز الهضمي التعليمي والكبد

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الخلاصة:

الخلفية: جراحة السمنة هي الطريقة الأكثر فعالية لفقدان الوزن على المدى الطويل .إن تخفيض الوزن السريع بعد جراحة السمنة قد يساهم في تكوين حصوات المرارة.

الهدف من الدراسة: احتساب معدل وتحديد العوامل المرتبطة بتكوين حصوات المرارة بعد عملية تكميم المعدة بالمنظار.

المرضى والمنهجية: كانت هذه دراسة مقطعية على 128 مريضا يعانون من السمنة المفرطة والذين تم علاجهم عن طريق تكميم المعدة بالمنظار وتمت متابعتهم بعد أسبوعين، وشهر واحد، و 6أشهر، وسنة واحدة بعد الجراحة في الفترة ما بين تشرين الأول 2018 وتموز 2020في مستشفى الجهازالهضمي والكبد التعليمي في بغداد ومستشفى الدولي الخاص .تم جمع البيانات باستخدام استبيان منظم .وتم الحصول على الموافقة الأخلاقية من لجنة البورد العراقي للاختصاصات الطبية ومن المشاركين .وتم استخدام البرنامج الإحصائي للعلوم الاجتماعيةلتحليل البيانات .

النتائج: كان متوسط عمر المرضى 36.5 ± 5.21 (17-54) للإناث و 41.6 ± 3.04 (24-58) للذكور. حدث تكوين حصوات المرارة في 49 حالة (38.3%). من بين جميع الذكور، أصيب أربعة (36.4%) بحصوات في المرارة بعد إجراء عملية تكميم المعدة بالمنظار، مقارنة بـ 45 (38.5%) من الإناث. كان لدى 60 مريضا (46.9%) مؤشر كتلة الجسم ≥40 كجم/م2، منهم 29 (48.3%) أصيبوا بحصوات في المرارة بعد تكميم المعدة بالمنظار (ع= 0.031). تظهر النتائج لفقدان الوزن فيما يتعلق بالتوقيت ودرجة الخسارة وجود علاقة كبيرة بتكوين حصوات المرارة بعد تكميم المعدة بالمنظار P) (0.05>. لم ترتبط الحالات المرضية المصاحبة بشكل كبير بحدوث تكوين حصوات المرارة بعد تكميم المعدة بالمنظار (P > 0.05).

الإستنتاجات: أصيب أكثر من ثلث الحالات بحصوات المرارة بعد عملية استبدال مفصل الركبة. وكان هذا مرتبطا بما يلي: السمنة المفرطة قبل الجراحة (مؤشر كتلة الجسم ≥ 40 كجم/م²)، وفقدان ≥ 25% من الوزن الأصلي، وفقدان الوزن السريع خلال الأشهر الستة الأولى بعد عملية استبدال مفصل الركبة.

الكلمات المفتاحية: السمنة، تكميم المعدة بالمنظار، جراحة السمنة، حصوة المرارة.

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Research Article

Correlation between MDA Level and Chitotriosidase-1 Activity in Seminal Fluid of Iraqi Infertile Males

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Abstract

Background: Male infertility is a multifactorial condition influenced by various physiological and biochemical factors. Seminal fluid composition plays a crucial role in sperm function and fertilization potential. Chitotriosidase is a chitinase enzyme released by activated macrophages and is highly conserved and controlled. The notable chitinase in humans plays a significant role in the body's immunological response and is linked to inflammation, infection, tissue damage, and remodeling processes. On the other hand, malondialdehyde is a marker of lipid peroxidation, reflecting oxidative stress levels.

Objective: This study aimed to explore the correlation between malondialdehyde levels and Chitotriosidase-1in seminal fluid in Iraqi infertile males.

Methods: Ninety males aged between twenty and forty-five were included in this cross-sectional study, all diagnosed with infertility by specialists at the infertility unit of Al-Batool Teaching Hospital between February 2022 and February 2023. The participants were categorized into three groups: the Normozoospermic Group (G1), the Asthenospermia Group (G2), and the Oligozoospermic Group (G3). Seminal malondialdehyde and Chitotriosidase-1 levels were measured by competitive Enzyme-linked immunosorbent assay.

Results: The study findings showed significantly higher levels of seminal fluid Chitotriosidase-1 found in the G2 group compared to the G3 and G1 groups. The seminal fluid malondialdehyde level for G1 was significantly lower than those for G2 and G3, which revealed a significant positive correlation between seminal fluid Chitotriosidase-1 activity and malondialdehyde levels ($r = 0.37$, $P < 0.05$) in the Asthenospermia Group.

Conclusion: There is a correlation between seminal fluid Chitotriosidase-1 activity and malondialdehyde level in the Asthenospermia Group. Novel diagnostic and therapeutic approaches for the treatment of male infertility may result from our growing understanding of the roles played by Chitotriosidase-1 and malondialdehyde in male reproductive health.

Keywords: Chitotriosidase-1; Male infertility; Malondialdehyde; Seminal plasma; Sperm quality.

Introduction

Fertility is the ability of the individual to reproduce through normal sexual acts. Normal fertility requires the production of enough healthy sperm, a problem with this step causes infertility (1). Several studies were carried out on the association of infection and inflammation with male infertility, which revealed great variations in the prevalence of genital infection in different parts of the world (2). Involvement of chitotriosidase-1 (CHIT1) in macrophage activation and differentiation has consequences for other immune cell types. Although this may point to a role for cHit1 in triggering an inflammatory response, data on the enzyme's involvement in the inflammation that contributes to male infertility is still lacking (3). Chitotriosidase-1 is an enzyme found in various tissues and bodily fluids, including blood and seminal fluid. In humans, CHIT1 is primarily produced and secreted by macrophages, where it serves as part of the innate immune response against chitin-containing pathogens. Elevated levels of CHIT1 activity have been associated with various conditions, including lysosomal storage disorders and certain inflammatory diseases (4). Malondialdehyde (MDA) is the most well-studied by-product of polyunsaturated fatty acid peroxidation caused by oxidative damage (5). Seminal levels of reactive oxygen species and MDA also increase in tandem with these findings, during infection and tissue

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damage, semen contains high amounts of the same cytokines that play an important role in immune regulation for the male gonad (6). This study aimed to explore the correlation between malondialdehyde levels and Chitotriosidase-1in seminal fluid in Iraqi infertile males.

Patients and Methods:

Study Population

Ninety males aged between twenty and forty-five were included in this cross-sectional study all were diagnosed with infertility by specialists at the infertility unit of Al-Batool Teaching Hospital / diyala governorate between February 2022 and February 2023. Subjects were divided according to seminal fluid analysis into three groups: the Normozoospermia Group (G1), the Asthenospermia Group (G2), and the Oligozoospermia Group (G3).

Inclusion criteria (according to Seminal Fluid Analysis (SFA) (WHO,1999)

❖ Normozoospermic Group (G1): must have a normal sperm count, Sperm morphology Particiy (shape and structure) should meet standard criteria for normal sperm, Sperm motility (ability to move) should meet standard criteria for normal sperm.

❖ Asthenospermia Group (G2): must have reduced sperm motility, other parameters such as sperm count and morphology may still fall within the normal range.

❖ Oligozoospermic Group (G3): must have a low sperm count, Sperm morphology and motility may be normal.

Exclusion criteria

Systemic disease such as DM.

1- Auto immune disease such as SLE, RA, Hashimoto's thyroiditis.
2- Severe oligospermia 5 million / ejaculation.
3- Azoospermia
4- Patients with varicocele.
5- Patients with disorders in his wife reproductive system.
6- Patients with undiscerning testis and with testicular torsion.
7- Hypogonadism
8- Patients previously taken Antimicrobials, corticosteroid, and Antioxidants.

Seminal fluid samples

All patients' semen samples were taken in sterile, clean cups, and placed in an incubator for 15-20 minutes to cause the semen to liquefy, throughout the course of three to four days of abstinence. The samples were then examined under a light microscope. Seminal plasma was obtained by centrifugation at 4000 rpm for 15 minutes were divided into two portions and kept until assay.

Measurements of MDA and Chitinase1 (CHIT1) by enzyme-linked immunosorbent assay (ELISA)

Seminal MDA and CHIT1 levels were measured by competitive Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer (Cloud-Clone Corp/USA/Cat No. CEA597Ge, SEJ374Hu, SEA181Hu).

Statistical Analysis:

The statistical analysis was conducted using Microsoft Excel for data input and preparation, which included organizing and cleaning the data for analysis, One-way ANOVA followed by multiple comparisons test was performed using GraphPad Prism version 19.5.1 for Windows, GraphPad Software, San Diego, California USA, and MedCalc® Statistical Software version 20.215was used to calculate and examine the strength and direction of relationships between variables, particularly between seminal plasma Malondialdehyde (SF-MDA) and CHIT1 levels. The statistical significance level was set at 0.05 for most tests, indicating that results with a [P-value less than 0.05 were considered statistically significant.

Results:

Age appeared to be uniformly distributed across the three groups with mean ages of 29. 67±1.043, 29.86±0.896, and 29.96±0.752 years, for G1, G2, and G3, respectively. The *P*= 0.972, strongly suggests that the differences between these groups were not statistically significant, demonstrated more discernible differences among the groups as shown in figure (1).

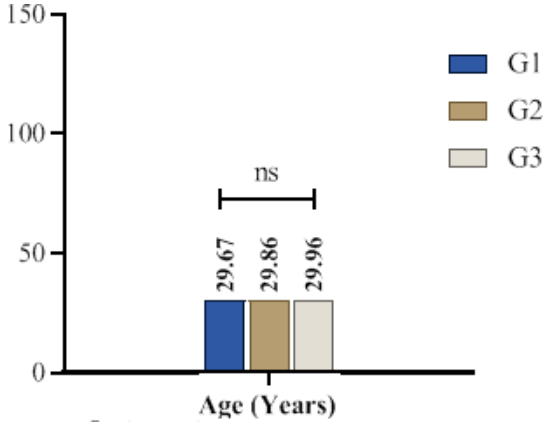

Figure 1: Age of males in the study groups.

In table 1 and figure (2), the results showed, that the Seminal Fluid Volume (SF Vol) revealed mean values of 2.550±0.1701, 2.083±0.1337, and 2.033±0.1809 ml for study groups, respectively. Although differences were observed, the effect size indicated by the R^2 value of 0.07 and a borderline *P*= 0.05 suggested that these differences are not substantial.

For Sperm Count, however, a striking disparity among the groups was evident. The means were 71.833±1.8489, 39.333±2.3700, and 11.000±0.7350 for

study groups, respectively. The R^2 value of 0.87 and a *P*= 0.001 indicated not only a statistically significant difference but also a large effect size, denoting a considerable variation in sperm count among these groups.

Motility categories, designated as A%, B%, C%, and D%, also showed substantive differences among the groups. For category A%, the mean values were 21.833±0.9123, 6.500±1.0491, and 2.000±0.9160 for study groups, respectively. Category B% displayed similar disparities with means of 33.833±0.5715, 18.000±1.0057, and 8.833±1.6380. Both A% and B% had R^2 values of 0.73 and *P*-values less than 0.001, signifying statistical significance and substantial effect sizes. Category C%, while also statistically significant with *P*= 0.01, showed a modest R^2 value of 0.10, implying a smaller effect size. Category D% exhibited an R^2 value of 0.77 and a *P*-value less than 0.001, indicating significant differences with a large effect size. Finally, the percentages of morphologically normal and abnormal sperms were 70.000±0.8970 and 30.333±0.8949 for G1, 40.833±2.5380 and 59.167±2.5380 for G2, and 23.833±3.5934 and 76.167±3.5934 for G3, respectively. These differences were statistically significant with *P*= 0.001 and R^2 values of 0.65, highlighting a sizable effect size.

Table 1 Semen profile for study groups

SFA	Study groups				
	G1	G2	G3	ANOVA	
	Mean± SE	Mean± SE	Mean± SE	R^2	P value
SF Vol ml	2.550± 0.1701	2.083± 0.1337	2.033± 0.1809	0.07	0.05
Sperm count	71.833± 1.8489	39.333± 2.3700	11.000± 0.7350	0.87	< 0.001
A%	21.833± 0.9123	6.500± 1.0491	2.000± 0.9160	0.73	< 0.001
B%	33.833± 0.5715	18.000± 1.0057	8.833± 1.6380	0.73	< 0.001
C%	10.000± 0.0000	10.833± 0.3460	9.333± 0.4632	0.10	0.01
D%	34.333± 1.0095	64.667± 1.9613	79.833± 2.5312	0.77	< 0.001
Normal%	70.000± 0.8970	40.833± 2.5380	23.833± 3.5934	0.65	< 0.001
Abnormal%	30.333± 0.8949	59.167± 2.5380	76.167± 3.5934	0.65	< 0.001

SFA=Seminal fluid analysis, SF vol=Seminal fluid volume, SE= Standard error of mean, and Motility categories=A%, B%, C%, and D%

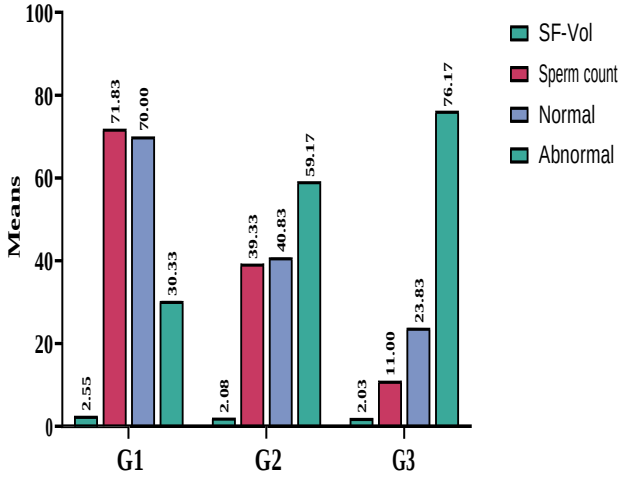

Figure 2: Means of Semen profile by the study groups.

Seminal fluid plasma Ch1t1 levels also displayed pronounced disparities among the groups with mean concentrations of 5.62±0.19, 15.96±0.29, and 12.46±0.18 (ng/ml) for study groups, respectively. As seen Table 2 and Figure 3

Table 2: The SF- Ch1t1 for study groups

Parameter	Mean ± SE			P value
	G1	G2	G3	
SF_Ch1t1 (ng/ml)	5.62± 0.19	15.96± 0.29	12.46± 0.18	< .001

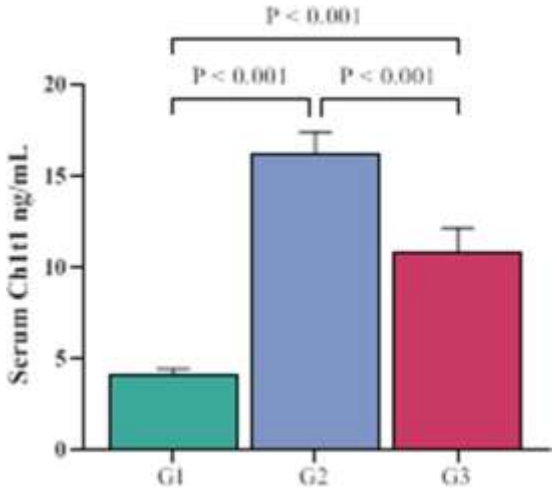

Figure3: Means of Ch1t1 by study groups.

The mean of SF-MDA for G1 236.59 ± 105.30(ng/ml), was significantly lower than for G2 1904.08 ± 877.85, similarly, compared to G3 1,042.78 ± 339.03 with *P*= 0.001. As seen Table 3, and Figure 4.

Table 3: The SF-MDA for study groups

Parameter	Group	n	Mean± SD	G1	G2	G3	Pr > F(Model)	Significant
SF-MDA ng/ml	G1	30	236.59± 105.30	236.59 a	1904.08 c	1042.78 b	< 0.001	Yes
	G2	30	1904.08± 877.85					
	G3	30	1042.78± 339.03					

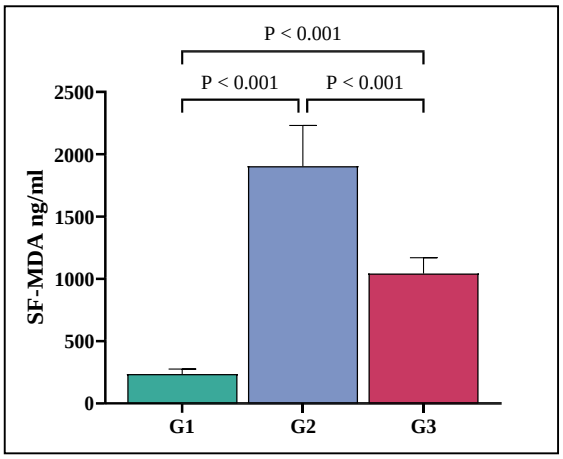

Figure 4: Comparison of SF-MDA between study groups.

The statement highlights a significant and substantial difference in sperm count among the Normozoospermic Group (G1), Asthenospermic Group (G2), and Oligozoospermic Group (G3). This indicated that the observed variation in sperm count is unlikely to have occurred by chance alone.

The correlation between SF MDA and SF Ch1t1 is modestly positive (r=0.37) and statistically significant (*P* value = 0.042) in G2 group. This suggested that there is a significant, albeit weak, positive association between SF MDA and SF Ch1t1 levels in the studied population. The modestly positive correlation between SF MDA and SF Ch1t1 may suggest a potential relationship between oxidative stress (MDA) and Ch1t1activity in the seminal fluid.

Table 4: Correlation between SF Ch1t1 and SF_MDA **in G2 group and G3**

Parameter	SF_Ch1t1 in G2		
	R	95.00% CI	*p*
SF_MDA	.37	[.01, .65]	.042
Parameter	SF_Ch1t1 in G3		
	R	95.00% CI	*p*
SF MDA	-.12	[-.46, .25]	.536

Discussion:

The observed variation is substantial and not merely a result of the sample size, a considerable difference in sperm count among the groups has important clinical implications, sperm count is a crucial factor in male fertility, and significant variations may affect the likelihood of successful conception, the study may explore potential causes or factors contributing to the observed differences in sperm count (7).

Factors such as lifestyle, environmental exposures, and genetic predispositions could be the cause of such variations among the different sperm health conditions, there are several reasons for a significant difference in sperm count among the groups included in the study (8). These factors may result from complex interactions between genetic, environmental, and health-related elements, variations in levels of hormones involved in sperm production, such as testosterone, may contribute to this difference, differences in genetic makeup could affect sperm count, genetics play a crucial role in determining reproductive characteristics(9).

For seminal fluid volume, although there are differences, the *P*-value (Table 1; $P \leq 0.05$) suggested that , there can be several reasons for differences in semen volume among different groups, including disruptions in hormone levels, particularly those involved in sperm production, can affect semen volume, imbalances in hormones like testosterone may influence semen production, variations in genes among individuals can contribute to differences in semen volume, genetic factors play a role in the physical characteristics of semen (10).

Exposure to specific environmental factors may influence natural sperm production and, consequently, semen volume. This could include exposure to harmful chemicals or elevated temperatures, as shown by (11).

The provided data revealed significant differences in motility categories (A%, B%, C%, and D%) among the study groups, namely the Normozoospermic Group (G1), Asthenospermic Group (G2), and Oligozoospermic Group (G3).

The statistically significant differences in Category A% suggested that there is a significant variation in the percentage of sperms showing progressive motility among the different groups ($P < 0.001$). This is a crucial parameter for male fertility, as sperm with progressive motility have a higher chance of reaching and fertilizing the egg (12).

Similar to Category A%, the significant differences ($P<$ 0.001) with a substantial effect size in Category B% indicated notable disparities in the percentage of sperms showing non-progressive motility. Non-progressive motility may still allow sperm to move but with less efficiency compared to progressive motility (13). Although statistically significant ($P<0.01$), the smaller effect size in Category C% suggested a less substantial impact compared to Categories A% and B%. This indicated that the percentage of sperms with local motility varies among the groups but to a lesser degree. Local motility may have limited functional relevance for fertilization compared to progressive motility (14). The significant differences ($P<$ 0.001) with a large effect size in Category D% highlighted considerable variations in the percentage of immotile sperm among the study groups. Immotile sperms have reduced or no movement, which can significantly impact fertility (15). The findings have clinical relevance, as sperm motility is a critical factor in male fertility, understanding the specific patterns of motility among different sperm health conditions can guide clinicians in diagnosing and addressing fertility issues in couples (16), as found by (17). With advancing age, there can be an effect on sperm motility, aging is often associated with a decline in overall body functions.

The provided information indicated significant differences in the percentages of morphologically normal and abnormal sperms among the three groups (G1, G2, and G3), these morphological differences may have implications for fertility and reproductive health in each group, this agreed with (18).

This finding can be important for the study's validity and interpretation, it suggested that the differences in sperm characteristics observed among the groups are more likely to be related to the specific condition (G1, G2, and G3) rather than being confounded by age or BMI differences (19).

As shown in Table (1) and Figure (4), seminal plasma MDA levels for G1 were significantly lower ($P<0.001$) than those for both G2 and G3.

Malondialdehyde (MDA) is a naturally occurring compound that serves as a marker for oxidative stress and lipid peroxidation in cells, it is a byproduct of the degradation of polyunsaturated fatty acids in cell membranes when cells are exposed to oxidative stress, such as from reactive oxygen species (ROS) (20), lipid peroxidation can occur, leading to the formation of MDA, this agrees with (21). Lower MDA levels in G1 might suggest reduced oxidative stress in the Normozoospermic Group (G1) compared to the Asthenospermic Group (G2) and Oligozoospermic Group (G3), oxidative stress is known to negatively impact sperm quality and function, high levels of ROS can lead to DNA damage, lipid peroxidation, and impaired sperm function. The observed difference in MDA levels among the groups may be associated with the differences in sperm parameters, especially considering that G2 is associated with asthenospermia (reduced sperm motility) (22).

Elevated MDA levels are associated with various pathological conditions, including inflammation, cardiovascular diseases, neurodegenerative disorders, and reproductive health issues (23).

Studies often investigate MDA levels concerning male fertility. Elevated MDA levels in seminal plasma or sperm cells may be linked to reduced sperm motility, viability, and overall sperm function, this agreed with (24, 25).

The study demonstrated significant differences in CHIT1 concentrations in seminal plasma between the normozoospermic and oligozoospermic groups, which aligned with the findings of this study. Inflammation in the male reproductive system can cause oligozoospermia (26).

This suggests a potential link between chitin metabolism and oxidative stress in the male reproductive system. Furthermore, subgroup analysis based on semen parameters revealed varying correlations between CHIT1 and MDA levels among different fertility profiles.

Malondialdehyde is a marker of oxidative stress, and elevated levels of MDA in seminal fluid have been associated with decreased sperm quality and male infertility. Asthenospermia, a condition characterized by reduced sperm motility, can be influenced by oxidative stress (20)

Ch1t1, on the other hand, is a protein involved in sperm maturation and function. Studies have shown that alterations in Ch1t1 levels in seminal fluid may be associated with male infertility and impaired sperm function (3).

In the context of asthenospermia, it is possible that elevated levels of MDA and alterations in Ch1t1 levels in seminal fluid could contribute to the condition. Oxidative stress induced by high MDA levels may affect sperm motility, while changes in Ch1t1 levels could impact sperm maturation and function.

Limitations:

There were some factors and reasons behind the limitations of the study: The small number of sample size that we were able to collect in the study, sample contamination was an obstacle to collecting a larger number of samples, Community customs in collecting semen samples restricted obtaining other samples, Some patients not providing their personal information to obtain the sample prevented obtaining more samples and information and the limited geographical area affected the generalization of the study to all patients in Iraq.

Conclusion:
The observed correlation between seminal fluid CHIT1 activity and MDA levels underscores the interplay between chitin metabolism and oxidative stress in male fertility. Understanding the role of CHIT1 and MDA in male reproductive health could lead to novel diagnostic and therapeutic strategies for male infertility management.

Authors' declaration:
We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in (Department of Biochemistry) according to the code number (19634) on (20/ 5/ 2024).
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Authors' contributions:
Study conception & design: (Ali S. Abdul Aziz, Hedef Dhafir El-Yassin, HK Kadhem). Literature search: (Ali S. Abdul Aziz, Hedef Dhafir El-Yassin, HK Kadhem). Data acquisition: (Ali S. Abdul Aziz, Hedef Dhafir El-Yassin, HK Kadhem). Data analysis & interpretation: (Ali S. Abdul Aziz, Hedef Dhafir El-Yassin, HK Kadhem). Manuscript preparation: (Ali S. Abdul Aziz, Hedef Dhafir El-Yassin, HK Kadhem). Manuscript editing & review: (Ali S. Abdul Aziz, Hedef Dhafir El-Yassin, HK Kadhem).

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العلاقة بين مستوى كيتوتريوسايديز-1 والمالونديالدهيد في السائل المنوي للذكور العراقيين العقيمين

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الخلاصة
خلفية البحث: العقم عند الرجال هو حالة متعددة العوامل تتأثر بعوامل فسيولوجية وكيميائية حيوية مختلفة .يلعب تكوين السائل المنوي دورا حاسما في وظيفة الحيوانات المنوية وإمكانية الإخصاب .كيتوتريوسايديز -1 هو إنزيم يشارك في استقلاب الكيتين، في حين أن المالونديالدهيد (هو علامة على بيروكسيد الدهون، مما يعكس مستويات الإجهاد التأكسدي . تهدف هذه الدراسة إلى استكشاف العلاقة بين مستويات المالونديالديهايد و كيتوتريوسايديز (في السائل المنوي عند الذكور العراقيين المصابين بالعقم والذين يعانون من التهاب صامت .
طرق العمل : تم تضمين تسعين رجلا تتراوح أعمارهم بين عشرين وخمسة وأربعين عاما في هذه الدراسة المقطعية، وتم تشخيص جميعهم بالعقم من قبل متخصصين في وحدة العقم في مستشفى البتول التعليمي بين فبراير 2022 وفبراير 2023. وتم تصنيف المشاركين إلى ثلاث مجموعات :مجموعة طبيعية النطاف(G1) ، ومجموعة وهن النطاف(G2) ، ومجموعة قليلة النطاف.(G3)
النتائج: النتائج التي توصلنا إليها، كانت هناك مستويات أعلى بكثير من كيتوتريوسايديز في السائل المنوي الموجودة في المجموعة G2 مقارنة بمجموعتي G3 و .G1كانت مستويات المالونديالديهايد للسائل المنوي لـ G1 أقل بكثير من تلك الخاصة بـ G2 و .G3كشفت عن وجود علاقة إيجابية مهمة بين نشاط السائل المنوي كيتوتريوسايديز ومستويات المالونديالديهايد($p < 0.05$، $r = 0.37$)في مجموعة. قليلة النطاف
الاستنتاجات: وجود علاقة إيجابية معنوية بين نشاط السائل المنوي بمستويات المالونديالديهايد و مستويات كيتوتريوسايديز في مجموعة وهن النطاف .
الكلمات المفتاحية :السائل المنوي البشري، كيتوتريوسايديز -1، الالتهاب الصامت، المالونديالدهايد. بيروكسيد الدهون

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Research Article

Analysis of MicroRNA -155-5p Expression in Patients with Primary Myelofibrosis

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Abstract

Background: Primary myelofibrosis is a chronic myeloproliferative neoplasm characterized by abnormal megakaryocyte proliferation and fibrosis that destroys healthy bone marrow. This results in extramedullary hematopoiesis, variable blood cell deficiencies, hepatosplenomegaly, general symptoms, progression to leukemia, and a reduced lifespan. Myelofibrosis can occur as a de novo myeloproliferative neoplastic disorder or evolve from other myeloproliferative neoplasms, including Polycythemia Vera or Essential Thrombocytosis. MicroRNAs (miRNAs) are short, non-protein-coding RNA molecules, typically 18–24 nucleotides in length. Dysregulation of miRNAs may contribute to the disease phenotype.

Objective: To investigate the expression level of MicroRNA-155-5p in patients with Primary Myelofibrosis compared to healthy controls and its correlation with common clinic-pathological factors.

Methods: twenty-eight patients with Primary Myelofibrosis and twenty healthy subjects were examined as controls. Expression analysis of MicroRNA-155-5p was performed using reverse transcription-quantitative polymerase chain reaction (qRT-PCR) on plasma isolated from peripheral blood.

Results: MicroRNA-155-5p expression was significantly upregulated in patients with Primary Myelofibrosis (P = 0.0001). However, no significant correlations were found between MicroRNA-155-5p and age, sex, Janus kinase 2 mutation status, or hematological parameters, including hemoglobin, white blood cell count, and platelet count.

Conclusion: MicroRNA-155-5p expression is not influenced by age, sex, Janus kinase 2 mutation status, or hematological parameters. Aberrant expression of MicroRNA-155-5p may contribute to the pathogenesis of Primary Myelofibrosis, warranting further research to understand the disease mechanisms better.

Keywords: Essential thrombocytosis; MicroRNA; Myeloproliferative neoplasms; Polycythemia Vera; Primary Myelofibrosis.

Introduction:

The classical myeloproliferative neoplasms (MPNs) are characterized by the proliferation of terminally differentiated myeloid cells. Primary myelofibrosis (PMF) is the most aggressive of the classic MPNs, marked by extensive heterogeneity in clinical manifestations and molecular markers (1). A significant proportion of patients harbor activated mutations, including the Janus kinase 2 (JAK2) mutation which drives cytokine-independent proliferation of hematopoietic progenitor cells by constitutively activating both canonical and non-canonical downstream pathways. Other driver mutations, such as calreticulin (CALR) and myeloproliferative leukemia virus oncogene (MPL), mediate persistent JAK-STAT signaling, a key process underlying the disease's pathophysiology (2, 3). Numerous Pathological mechanisms, including defective myeloid cell proliferation, aberrant stem cell trafficking, and increased production of inflammatory cytokines, contribute to the development of PMF (4). These processes lead to progressive changes in marrow histology, where all hematopoietic elements are initially preserved, followed by the accumulation of coarse reticulin fibers arranged in parallel bundles within the increased fibrous tissue. Ultimately, this progression culminates in the osteo-myelosclerotic stage (5,6). These mechanisms disrupt the normal medullary erythropoietic environment, resulting in anemia, bone marrow failure, splenomegaly, infections, bleeding, and constitutional symptoms (7,8). Patients with PMF have a median survival of 5.7 years, with a range of 4 to 7 years post-diagnosis (9). Currently, autologous hematopoietic stem cell transplantation remains the only treatment option capable of potentially prolonging survival or cure PMF (10). MicroRNAs (miRNAs) are a class of single-stranded, non-protein-coding RNA molecules, typically averaging 22 nucleotides in length (11). They bind to target messenger RNA (mRNA) and function as gene repressors, regulating gene and protein expression (12). MiRNAs play a

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critical role in controlling cellular processes such as apoptosis, proliferation, and differentiation (13).
Extracellular miRNAs have been extensively studied as potential biomarkers for various conditions and serve as signaling molecules facilitating intercellular communication (14). Among these, miRNA-155-5p is one of the most well-researched miRNAs. It plays a significant role in regulating immune cell differentiation and cytokine secretion, leading to cytokine hypersensitivity in bone marrow progenitors. Altered expression of miRNA-155-5p has been linked to hematological malignancies, making it a promising biological marker for these diseases (15).
The study aimed to analyze the expression level of miRNA-155-5p in patients with Primary Myelofibrosis (PMF) compared to healthy controls and evaluate its correlation with common clinicopathological factors.

Patients, Materials, and Methods:
This study employed a cross-sectional design and included patients diagnosed with Primary Myelofibrosis (PMF) according to the 2016 World Health Organization (WHO) criteria for myeloproliferative neoplasms (MPNs)(17). A control group of healthy individuals was also included.
With approval from the local Ethics Committee of the College of Medicine, University of Baghdad, 48 participants (28 patients with PMF and 20 healthy volunteers) were recruited. The study adhered to the Declaration of Helsinki ethical standards, and all participants provided written informed consent. Clinical data obtained from patients' records included:
Demographics: Age and sex
Disease Characteristics: Time and history of presentation, JAK2 mutation status
Hematological Parameters: Hemoglobin (Hb), total white blood cell count (WBC), and platelet count (PLT).
Inclusion Criteria
JAK2V617F mutation and confirmed negative for the **BCR-ABL1 fusion gene**. Patients were tested for the
None of the patients exhibited blastic transformation**.**
Sample Collection and Processing
For each participant, 2 mL of peripheral blood was collected in an EDTA tube. Plasma was separated by centrifugation within 3 hours of collection and then transferred into a 1.5 mL Eppendorf tube containing 300 µl of DNA/RNA Shield for preservation and stored at a temperature below -20 °C. The RNA was extracted within a period of two weeks and stored at a temperature below -20°C till the time of assessing the expression of MiRNA-155-5p using a qRT-PCR method.

RNA isolation and Reverse Transcriptase PCR procedures (RT-PCR)
Direct-zol™ RNA MiniPrep method (Cat. # R2051, ZYMO research, USA) was used to extract RNA from peripheral whole blood. Reverse transcriptase reactions contained 3µl isolated total RNA, 0.5µl stem-loop RT primer, 10 µl RNase Free water, and 2 µl Prime Script TM Reverse Transcriptase (Cat. # RR037A, Takara Bio, USA). For quantitative PCR (qPCR), a reagent system was used, this system was composed of (a fluorescent DNA-binding dye, GoTaq® Hot Start Polymerase, MgCl2, dNTPs, and a proprietary reaction buffer). The process was carried out using an automated Thermal Cycler (Sacace, Italy). The PCR conditions were as follows: denaturation at 95°C for 20 seconds, followed by 40 cycles of 20 seconds at 95°C, 20 seconds at 60°C, and the final extension step of 20 seconds at 72°C followed by the analysis of relative gene expression data using real-time quantitative PCR and the $2^{\wedge\text{-delta deltaCT}}$ method (18).

Statistical analysis: The method of inputting data was performed with Microsoft Excel 2019. The analysis was conducted using the statistical package for social sciences (SPSS version 26). A randomly selected sample (*t*-test) was employed to compare continuous parameters with categorical parameters. Chi-squared tests have been used to measure the association between categorical parameters while (ANOVA) tests were used to compare between categorical variables. ROC curve (receiver operating characteristic curve) was used to measure the area under the curve to measure the cutoff value. The linear regression test was used to measure the association between two continuous variables.

Results:
Out of 28 patients with PMF, 19 of them were males comprised 67.8% while 9 were females and comprised 30% (9). The mean age±SD of patients was (53.6 ± 12.2) years, whereas in the control group was (52.1 ± 15 years). The mean±SD MiRNA 155-5p was notably elevated in the patient group (1.04 ± 0.82) with PMF compared to the control group (0.32 ± 0.28), and this difference was statistically significant (*P*=0.0001; Table 1, and Figure 1)

Table1: MiRNA 155-5p level across studied groups

	Control	PMF	*P* value
Mean ± SD	0.32 ± 0.28	1.04 ± 0.82	0.0001

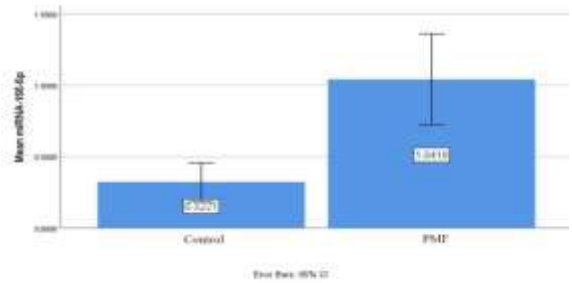


Figure. 1: The mean MiRNA 155-5p levels across the studied groups.

Age and Sex association with MiRNA 155-5p level

The mean MiRNA 155-5p levels among males and females within the PMF group showed no significant difference (*P*=0.81). A significant difference was observed among males and females of the PMF and control groups (*P*=0.001).

Regarding the age of participants, there was no significant correlation between age and MiRNA 155-5p across both patients and control groups (*P*>0.05) (Figures 2A and B).

JAK-2 mutation status, splenomegaly, and hematological parameters association with MiRNA 155-5p level

Regarding splenomegaly and JAK-2 mutation, there was no significant difference observed between patients with and without splenomegaly or JAK-2 mutation (*P*>0.05; Table 2).

Table 2: Association of splenomegaly and JAK2 mutation with MiRNA 155-5p mean level in PMF patients

Variable		Mean± SD	*P* value
Splenomegaly*	No	0.7±0.2	0.21
	Yes	1±0.8	
JAK-2 mutation**	Mutated	1.1±0.8	0.36
	Unmutated	0.8±0.5	

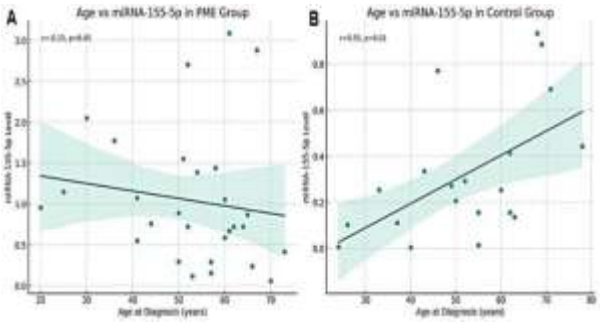
* The mean level of MiRNA-155-5p ± standard deviation in Primary Myelofibrosis patients with splenomegaly compared to those without splenomegaly.

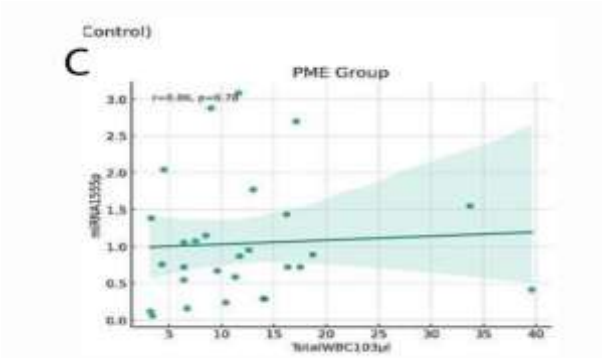
** The mean level of MiRNA-155-5p ± standard deviation in Primary Myelofibrosis patients with JAK-2 mutation compared to those Unmutated

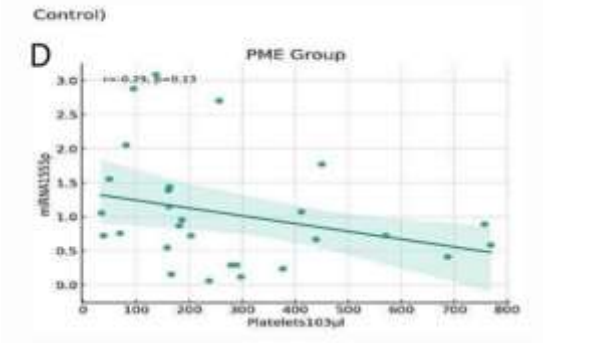
Regarding hematological parameters, there was no significant correlation between hematological parameters and MiRNA 155-5p level in PMF patients (*P*>0.05; Table 3, and Figures 2C, D, and E).

Table 3: Hematological parameters correlation with MiRNA 155-5p level

		PME
Hemoglobin (gm/dL)	No.	28
	r value	0.048
	P value	0.810
Total WBC (10^3/µl)	N	28
	r value	0.056
	P value	0.777
Platelets (10^3/µl)	N	28
	r value	-0.294
	P value	0.129







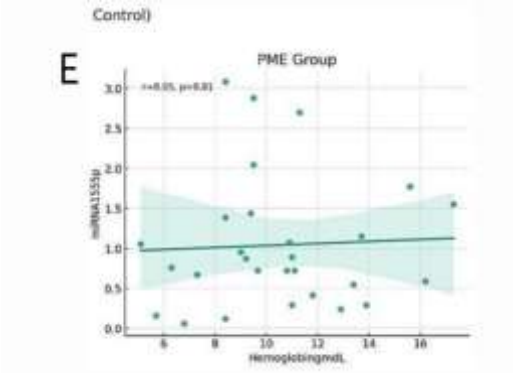


Figure 2 The association of miRNA 155-5p level with age of studied groups (A and B), total WBC count (C), platelets count (D), and hemoglobin level (E),

Discussion:

In this study and depending on qRT-PCR results, plasma mir155-5p expression was substantially elevated in patients with PMF in contrast with the control group. These results agreed with the study of Tombak *et al.* (22), and the study of Norfo *et al.* (23) where both demonstrated upregulation in mir155 expression leading to increased proinflammatory cytokine production which has a significant role in the pathophysiology of MF. The male-to-female ratio was (2:1) while the study of Alwan AF (24) showed (1.1:1), different from the study of Tombak *et al.* (22) which showed (0.6:1). These differences in ratios may be due to variable sample size in these studies. The level of miRNA 155-5p is not significantly associated with the sex of patients with PMF (no significant difference between males and females within PMF groups). The average age in the patient category was (53.6 ± 12.3) years while in the research of Tombak et al (22), was (54.8 ± 16,5) years, there is no significant correlation between the age of patients within PMF and the amount of miRNA 155-5p which was in agreement with the study of Tombak, *et al.* (22).

There were no significant differences between JAK-2 mutational status and level of miRNA 155-5p of patients with PMF groups included in this study was in agreement with Stolyar *et al.* (25) study and Tombak *et al.* (22), probably dysregulated miRNA 155-5p operates autonomously in the development of MPN, separately from JAK2 signaling.

The assessment of the correlation between the expression of miRNA155-5p and hematological parameters (HB level, WBC, and PLT counts) did not show any significant correlation between miRNA 155-5p level and any of the hematological parameters which were in agreement with Stolyar *et al.* (25) study.

It should be taken into account in future miRNA research that cells within the bone marrow environment, in addition to the mutated cell clone, contribute to the pathogenesis of MPNs and the expression of miRNAs.

Limitations: one of the limitations of the current study is the sample size**.**

Conclusion:

The aberrant expression of miRNA155-5p may contribute to PMF pathogenesis. Expression levels of miRNA 155-5p are not affected by age, sex, JAK2V617F status, and hematological parameters.

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Authors' Declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current research, have been given permission for re-publication attached to the manuscript. The project was approved by the Research Ethics Committee in the College of Medicine, University of Baghdad (issue number 26B, 25 Jan 2023)

Conflict of interest: None.

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Authors' contributions: Both authors (Dr. Jaffar Nori Sarah I. Khaleel) worked together to conduct a literature search, Data analysis & interpretation, Manuscript preparation, editing, and review.

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تحليل تعبير MicroRNA -155-5p في المرضى المصابين بالتليف النقوي الأولي

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الخلفية: تليف النقوي للعظم هو ورم نقوي تكاثري مزمن يتميز بتكاثر غير نمطي للخلايا العملاقة الليفية، مما يؤدي إلى تدمير نخاع العظام الصحي وبالتالي حدوث تكون الدم خارج نخاع العظام، وتقلبات في مستويات نقص خلايا الدم، وتضخم الكبد والطحال، وأعراض عامة، وتطور نحو اللوكيميا، وانخفاض متوسط العمر المتوقع. يمكن أن يكون التليف النقوي إما اضطراب تكاثري نقوي بدئي يسمى التليف النقوي الأولي (PMF) أو يمكن أن يتطور من الأورام النقوية التكاثرية الأخرى، بما في ذلك كثرة الحمر الحقيقية (PV) أو كثرة الصفيحات الأساسية .(ET) الميكرو RNAs، أو miRNAs اختصارا، هي نوع من RNAs أحادية الشريط وغير مشفرة للبروتينات بطول متوسط يبلغ 22 نيوكليوتيد. قد يعتبر اضطراب تنظيم الميكرو RNA (miRNA) كعوامل إضافية تؤثر على نمط المرض
أهداف هذه الدراسة: التحقيق في مستوى تعبير miRNA-155-5P في مرضى التليف النقوي الأولي (PMF) مقارنة بالأشخاص الأصحاء ومقارنة ارتباطه بالعوامل السريرية المرضية الشائعة.
المرضى والمواد وطرائق العمل: تم فحص ثمانية وعشرين مريضا بالتليف النقوي الأولي .(PMF) وتم استخدام عشرين شخصا صحيا كمجموعة ضابطة. تم إجراء تحليل التعبير عن miRNA-155-5p بواسطة تفاعل البلمرة المتسلسل الكمي في الوقت الحقيقي (RT-RCR) باستخدام البلازما المعزولة من الدم المحيطي للمرضى .
النتائج: كان تعبير miRNA-155-5p مرتفعا في مرضى التليف النقوي الأولي .(p=0.0001) الارتباطات بين miRNA-155-5p والعمر، الجنس، حالة JACK2 ومعايير الدم(الهيموغلوبين عدد الخلايا البيضاءWBC ، عدد الصفيحات الدموية PLT) لم تكن ذات دلالة إحصائية .
الاستنتاجات: تشير نتائجنا إلى أن المتغيرات الإضافية، بما في ذلك التعبير غير الطبيعي عنmiRNA-155-5p ، قد تساهم في مرض التليف النقوي، لذا هناك حاجة لمزيد من الأبحاث لفهم التسبب في هذه الاضطرابات في العصر الحالي.
الكلمات المفتاحية: كثرة الصفيحات الأساسية، الميكروRNA ، الأورام النقوية التكاثرية، كثرة الحمر الحقيقية، التليف النقوي الأولي.

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Research Article

Role of Inhibin B and Ratio of Luteinizing: Follicle-Stimulating Hormones in Phenotyping Polycystic Ovarian Syndrome

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Abstract:

Background: Polycystic ovary syndrome is among the leading causes of fertility-related problems and menstrual irregularities in women of reproductive age. The granulosa cells of the developing pre-antral and antral follicles produce inhibin B, which triggers chemical responses in the ovaries. Inhibin B is most often observed in the follicular phase when levels peak early and then decline over time
Objectives: This study was designed to investigate the role of serum inhibin B and the Luteinizing Hormone / Follicle Stimulating Hormone ratio in differentiation between the different phenotypes of polycystic ovary syndrome as well as to define the predominant PCOS phenotype**.**
Methods: This cross-sectional research was conducted in the Department of Biochemistry, College of Medicine, University of Baghdad from November 2023 to March 2024. The study included 111 women, ranging in age from18-40 years. Among these, 91 women were diagnosed with polycystic ovary syndrome (PCOS) based on the 2003 Criteria for the Rotterdam Consensus, and the other 20 were healthy women. Investigations included serum levels of inhibin B using the Enzyme Linked Immunosorbent Assay technique, follicle Stimulating Hormone, luteinizing Hormone and prolactin using Tosoh AIA-2000 Automated Immunoassay, to calculate the LH/FSH ratio.
Results: phenotype A was observed to be the predominant PCOS phenotype, while phenotype B was the rare form. The mean ±SEM values of the inhibin B levels for the phenotypes B (26.07±0.23 pg/ml, p=<0.001), C (25.96±1.68 pg/ml, p=<0.0001), and D (37.51±2.31 pg/ml, p=<0.0001), respectively, were significantly lower than those of the control women (57.68±2.07 pg/ml). However, the mean ±SEM value of inhibin B of phenotype A (50.46±7.12 pg/ml) was comparable to that of controls. The mean value of the LH levels of phenotype A (7.12±0.76 µIU/ml) showed significantly higher numbers than those of the control women (4.59±0.38 µIU/ml, p=0.03). Furthermore, the mean values of the LH/FSH ratio were significantly elevated in phenotypes A (p=0.001) and B (p=0.04) as compared to the controls.
Conclusion: Serum Inhibin B level and LH/FSH ratio can be used to distinguish between the different phenotypes of PCOS.
Keywords: Follicle stimulating hormone; Luteinizing hormone ratio; Inhibin B; Prolactin; Phenotypes; Polycystic ovary syndrome.

Introduction:

Polycystic ovary syndrome (PCOS) is one of the leading causes of fertility problems and menstrual irregularities in women of reproductive age (1–4). The World Health Organization (WHO) estimates that 4-8% of the global population has PCOS (5). Until recent times, the most commonly used diagnostic tool for PCOS is the Rotterdam criteria, which include polycystic ovarian morphology (PCOM), hyperandrogenism (HA), and oligomenorrhea and anovulation (OM) (6,7). If the patient meets two of the three criteria cited above hyperandrogenism can be diagnosed or oligo-amenorrhea when alternative etiologies have been ruled out (6,8). The Rotterdam criteria specify four main phenotypes of PCOS based on the clinical signs and symptoms (9). To get better outcomes, the phenotypes in PCOS patients can be identified using the appropriate methodology (10). Phenotype A was defined as oligomenorrhea -anovulation, HÀ and PCOM on ultrasound; phenotype B was identified as oligomenorrhea -anovulation and HA; phenotype C was described as HA and PCOM on ultrasound; and phenotype D was diagnosed as oligomenorrhea -anovulation and PCOM on ultrasound (6). In the Rotterdam consensus conference, it was agreed that subsequent sonographic definitions of the morphology of polycystic ovarian morphology

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(PCOM): expanded the ovarian volume (≥ 10cm^3) or more than 12 follicles per ovary, each measuring between 2 and 9 mm (6). The symptoms and signs showed definite and wide variations among women with PCOS (9). Inhibins, which belong to the transforming growth factor-β superfamily, comprise two constituents held together by disulfide bonds (11). These two constituents consist of an α-subunit and a βA-subunit or a βB-subunit, which together generate inhibin A or inhibin B, respectively, (12,13). The quantity of inhibin released into the ovaries is linked to the menstrual cycle (11). Subsequently, this hormone triggers chemical responses in the ovaries and testes of both sexes, in the granulosa and Sertoli cells, respectively. Inhibin B is most often observed in the follicular phase when levels peak early and then decline over time (11). Furthermore, patients with PCOS have lower FSH levels, which increase the LH/FSH ratio, boost the androgen synthesis from theca cells in the ovarium and, ultimately, create excess androgen (14). This disorder will halt new follicular growth and persistent anovulation (14). The aggregation of the tiny antral follicles results in the development of polycystic ovarian morphology (15–17). The differentiation between the four different phenotypes of PCOS is dependent on history of a woman regarding her cycle regularity, clinical examination considering hirsutism and acne, and ultrasonic study of ovaries. Hormones changes play important role in pathogenesis of PCOS. The aim of this study was to investigate the role of serum inhibin B and the Luteinizing Hormone (LH) / Follicle Stimulating Hormone (FSH) ratio in differentiation between the different phenotypes of polycystic ovary syndrome and to define the predominant PCOS phenotype**.**

Patients and Methods:

This case-control study was performed at the Department of Biochemistry, University of Baghdad College of Medicine, the Medical City of Baghdad Teaching Hospital, Baghdad, Iraq from November 2023 to March 2024. The study included 111 women in the age range of 18 to 40 years, of whom 91 had been previously diagnosed with polycystic ovarian syndrome (PCOS) by a consultant gynecologist and 20 healthy women as the control group. The PCOS women were sub-classified into four groups; (A, B, C, and D, respectively) based on their phenotypic characteristics which include polycystic ovarian morphology (PCOM), hyperandrogenism (HA), and oligomenorrhea and anovulation (OM) (6,10). The number of patient groups was limited because they were sub-classified into four groups as well as due to the limited time scheduled for study. This study was approved by the scientific and ethical committees of the Department of Biochemistry, College of Medicine, University of Baghdad (1500/ 26-11-2023); Ethical approval was also obtained from Baghdad Teaching Hospital, Medical City, Ministry of Health. Verbal consent was obtained from each of the women included as participants in this study. The inclusion criteria for women with PCOS involves the patient satisfying at least two criteria of the 2003 Rotterdam Consensus and they fall in the age of range 18-40 years. Polycystic ovarian morphology (PCOM) and hyperandrogenism (HA) are two characteristics of polycystic ovarian syndrome (8). According to this agreement, the patient must satisfy a minimum of two of the three main criteria listed below to be diagnosed with PCOS: (1) Anovulatory oligomenorrhea (2) hyperandrogenism (clinical or biochemical results), (3) polycystic ovaries (identified by ultrasound); also, other illnesses related to excess androgen, such as congenital adrenal hyperplasia, should be ruled out. After ruling out Cushing's disease, congenital adrenal hyperplasia, hyperprolactinemia, and androgen-secreting tumors, the patient is diagnosed with PCOS if at least two of these three criteria are met. Also, based on the Rotterdam criteria, four distinct phenotypes are associated with this syndrome: Hyperandrogenism and PCO and oligomenorrhea (A), oligomenorrhea and hyperandrogenism(B), PCO and hyperandrogenism(C), and oligomenorrhea and PCO (D) (6). The Exclusion criteria include those women on oral contraceptives at the time of blood draw and who have other diagnoses that mimic PCOS (i.e. prolactinoma, premature ovarian failure, congenital adrenal hyperplasia), thyroid gland dysfunctions, liver disease, kidney disease, and cancers. From each of the PCOS and control women, 5 ml of blood was drawn from a peripheral vein. This blood sample was left undisturbed to clot for 15 minutes and then centrifuged for 10 minutes at 2500 rpm. The separated serum was stored at -20 C until the time for measurements. Serum investigation included inhibin B level assessments using the semiautomatic ELISA Reader Huma, Reader by Human Diagnostics, a German company, and Washer (COMBIWASH) by HUMAN, Germany.

Statistical analysis:

The Statistical Package for Social Sciences (SPSS, version 25) was used for data analysis. The mean and standard error of the mean (SEM) were used to present the data obtained, and every statistical analysis was based on the data (18,19). The ANOVA test was used to evaluate the differences in the mean levels of the numeric data between more than two variables. The Area Under the Curve (AUC) and Receiver Operator Characteristic (ROC) were calculated and the cutoff value, sensitivity, and specificity of the parameters were obtained to differentiate between the four PCOS phenotypes and the control women as well as among the phenotypes themselves. Utilizing the Pearson correlation regression (r) the relationship between the numerical data was evaluated. *P*-value of less than 0.05 was considered statistically significant.

Results:

Table 1 presented the distribution of the four phenotypes of PCOS with phenotype A as the predominant one. Table 2 presented the mean ± SEM

values of age and body mass index (BMI) of the studied groups. The mean values of age of Phenotypes A (25.76±0.65 year, p=0.01), C (24.80±1.46 year, p=0.02), and D (25.94±0.97 year, p=0.02) were significantly higher than that of controls. However, there were insignificant differences in the mean value of age among the four phenotypes of PCOS women. The mean values of BMI of Phenotypes A (31.15±1.04 Kg/m2, p=0.001), C (28.91±1.24 Kg/m^2, p=0.01), and D (32.02±0.98 Kg/m2, p=0.001) were significantly higher than that of the control group along with non-significant differences among the phenotype groups. Table 3 shows the mean (±SEM) values of serum inhibin B, LH, FSH, prolactin, and the LH/FSH ratio of the studied phenotypes of PCOS and control women. The mean values of inhibin B levels of phenotypes B (26.07±0.23 pg/ml, p=0.001), C (25.96±1.68 pg/ml p=0.0001), and D (37.51±2.31pg/ml, p=0.0001) were significantly lower than those of control women. In addition, the mean value of Inhibin B levels of phenotype C was significantly lower than that of phenotype D (p=0.001) and phenotype A (p=0.049). The mean value of LH levels of phenotype A was significantly higher than that of control women (p=0.03). In addition, the mean values of LH of phenotypes B, C, and D were higher than those of controls but did not reach a significant level. There was a non-significant difference in mean values of LH among the four phenotypes of PCOS. The mean values of serum FSH levels were significantly lower in phenotypes B (p=0.02) and C (p=0.001) when compared with control women. There were non-significant differences among the four phenotypes of PCOS regarding mean serum value of FSH. The mean values of LH/FSH ratio were significantly elevated in phenotypes A (p=0.001) and B (p=0.04) when compared to controls. The mean value of serum prolactin was significantly increased in phenotype D in comparison with phenotype C (p=0.001) and control women (p=0.001), without any other significant differences. The study also found a significant positive correlation between serum LH and LH/FSH ratio in phenotype A (r=0.64, p=0.01), phenotype C (r=0.94, p=0.0001), and phenotype D (r=0.92, p=0.0001). However, there was no other significant correlation among the studied parameters in other groups. Also, the ROC and AUC study revealed that inhibin B at (cutoff ≤37.85 pq/ml) was the best measure for differentiation of phenotype C from controls with AUC value 0.997 (sensitivity=100 and specificity=95). The LH/FSH ratio was the best measure for differentiation of phenotype A from controls with an AUC value of 0.739 (sensitivity =69.05 and specificity=75.00). In differentiation between phenotypes C and D, inhibin B has AUC=0.80 at cutoff (< 37.851 ng/ml) with (sensitivity =100.0 % and specificity =48.39 %). Inhibin B also has AUC=0.805 at cutoff (< 26.502 ng/ml) with (sensitivity= 100.0% and specificity =77.42 %) in differentiation of phenotypes B and D. In addition, inhibin B has AUC=0.713 at cutoff (< 28.591 ng/ml) with (sensitivity= 64.28 % and specificity = 73.33 %) in differentiation of phenotypes A and C.

Table 1: Frequency and percentage of phenotypes distribution of the entire polycystic ovary syndrome women (Total number 91 women)

Phenotype A No. (%)	Phenotype B No. (%)	Phenotype C No. (%)	Phenotype D No. (%)
42 (46)	3 (3.2)	15 (16)	31 (34)

Table2: Mean ±SEM values of age and body mass index of polycystic ovarian syndrome Phenotypes and controls

Parameter	Phenotype A (n=42)	Phenotype B (n=3)	Phenotype C (n=15)	Phenotype D (n=31)	Control (n=20)
Age (year)	25.76±0.65●	29.33±1.86	24.80±1.46●	25.94±0.97●	29.95±1.42
BMI (Kg/m^2)	31.15±1.04●	28.35±1.91	28.91±1.24●	32.02±0.98●	25.05±0.64

ANOVA and *t*-test: ●: Significant decrease in mean values of age of phenotypes A (*P*=0.01), C (*P*=0.02) and D (*P*=0.02) and a significant increase in BMI in phenotypes A (*P*=0.001), C (*P*=0.01) and D (*P*=0.001) than in controls.

Table 3: Mean ±SEM values of inhibin B, luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin, and LH/FSH Ratio of polycystic ovarian syndrome groups and controls

Parameter	Phenotype A (n=42)	Phenotype B (n=3)	Phenotype C (n=15)	Phenotype D (n=31)	Control (n=20)
Inhibin B (Pg/ml)	50.46±7.12	26.07±0.23●	25.96±1.68●	37.51±2.31●	57.68±2.07
LH (µIU/ml)	7.12±0.76▪	4.30±1.22	4.69±0.84	5.52±0.72	4.59±0.38
FSH (µIU/ml)	7.71± 0.65	4.90± 1.10♦	6.37±0.42♦	7.09± 0.45	8.44± 0.49
Prolactin (ng/ml)	13.30±1.29	11.60± 1.81	10.14±1.16	16.56±1.57◘	9.90± 0.69
LH/FSH ratio	1.06±0.11*	0.95± 0.24*	0.80± 0.16	0.85± 0.14	0.58± 0.05

ANOVA and *t*-test: ●: Significant decrease in mean values of inhibin B of phenotypes B (*P*=0.001), C (p=0.0001) and D (p=0.0001) than in controls, a significant decrease in inhibin B in phenotype C than in phenotype D (p=0.001) & phenotype A(p=0.049) ▪ significant increase in LH in phenotypes A (p=0.03) than in controls. ♦: Significant decrease in FSH in phenotypes B (p=0.02) and C (p=0.001) compared to control. ◘: Significant increase in prolactin in phenotype D in comparison with phenotype C (p=0.01) and control women (p=0.001). *: LH/FSH ratio significantly elevated in phenotypes A (p=0.001) and B (p=0.04) when compared to controls.

Discussion:

The mean age value of the whole group of PCOS women in the present study was found to be 25.5 years that of the BMI was 30.69 Kg/m^2, which concurred with the findings reported by Carmina and Lobo (2022) They reported that the mean age of their PCOS women was 24.2 years (20), which differed from the findings of another study, where the mean age of their PCOS patients was found to be 28.2 years and BMI was 26.33 Kg/m^2 (21). The current study identified that phenotype A was the commonest one, after which was phenotype D of the women with PCOS, this finding corresponded with the previous study conducted by Malhotra *et al.* (2023), who found that phenotypes A and D were the commonest of the PCOS phenotypes (21). Moreover, Si *et al.* (2023) observed that phenotype B was the rarest subgroup (4%) among the women with PCOS (22). The current research found that the mean values of serum Inhibin B of the phenotypes (B, C, and D) were significantly lower than those of the controls, which was in agreement with the findings of Hussein *et al.* (2023) and Obaid *et al.* (2022) who recorded significantly lower levels of serum inhibin B in the women with PCOS women than in the controls (11, 23). However, Fazil *et al.* (2023) did not find any significant variation in the levels of serum inhibin B between the PCOS women and controls (24). In contrast, Farman *et al.* (2021) reported that the serum inhibin B levels of PCOS women were significantly higher than those of the controls (25). An essential function of inhibin B is to regulate ovarian function (26). Additionally, it is a crucial candidate gene for research on human ovarian function (26). Furthermore, Fawzy *et al.* (27) proposed that inhibin B could prevent the pituitary gland from producing FSH. The ovarian response diminishes and the FSH level rises while the inhibin B level is unable to maintain the FSH level within the normal range (26). As such, the baseline level of serum inhibin B may more accurately and immediately reveal the function of the ovarian reserve than the FSH level can do (26). The findings of the present study revealed that phenotype A had the highest level of inhibin B, while phenotypes B, C, and D had significantly lower levels of inhibin B, compared to the controls, This may be attributed to the fact that phenotype A possesses the triad characteristics of PCOS (hyperandrogenemia, oligomenorrhea-anovulation, polycystic ovarian morphology). To the best of our understanding, no previously published report or study dealt with inhibin B and the PCOS phenotypes. The current study also found that serum LH level and LH/FSH ratio were highest for phenotype A, which was in concurrence with the observation of Gürsu *et al.* (8). In the present study the significant decrease in FSH level in the B and C phenotypes, compared to that of the controls, concurred with the findings reported by Önal and Öztürk (2023) who recorded lower levels of serum FSH in PCOS phenotypes when compared to controls (28). These variations in hormone levels across the phenotypes could cast more light on the pathophysiology of women with PCOS, thus helping broaden the understanding of gynecologists regarding the heterogeneity of this disease and the creation of tailored treatment plans for each phenotype (28). Sharmin *et al.* (2023) indicated that phenotype A was the most common phenotypic and severe form of PCOS. These authors concluded that, compared with patients having the other phenotypes, those with phenotype A had significant biochemical hyperandrogenism, abnormal LH levels, and an altered LH / FSH ratio. The mildest phenotype was the normo-androgenic one (phenotype D). Ovulatory patients (phenotype C) were less common, most likely due to the less severe signs and hormonal imbalances. The phenotypic division facilitates the prediction of unfavorable consequences as well as enhances knowledge of the pathogenesis and severity of PCOS. Besides, the correct recognition of the distinct phenotypes has diagnostic consequences and ensures that patients receive the right care (29). Jamil *et al.* (2016) found that genotypes A and B had significantly higher total testosterone levels and LH/FSH ratio (30). Besides, Yilmaz *et al.* (2011) showed that phenotypes A, B, and C have higher LH/FSH ratios than those with phenotype D (31). In contrast, Duz *et al.* (2020) observed that phenotype D had significantly higher levels of LH and LH/FSH ratios, than did the other PCOS phenotypes (32). The results of the present study revealed that phenotype D had the highest serum prolactin level (Table 3). However, Gürsu *et al.* (2022) and Önal and Öztürk (2023) found non-significant differences in serum prolactin among and between the PCOS phenotypes and controls (8, 26).

Limitation:

The inability to include women who had received a recent diagnosis of polycystic ovarian syndrome because of the limited number of instances observed during the research period**.** Furthermore, the sample size was rather small because the sampling period was short as well as the sub-classification of PCOS patients.

Conclusion:

Phenotype A is predominant among the PCOS phenotypes in Iraqi women. Serum inhibin B level and LH/FSH ratio could be used in differentiation of the different phenotypes of PCOS.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local

ethical committee in (Baghdad Teaching Hospital) according to the code number (111) on (6/5/2024).

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Authors' Contributions:
Study conception, study design, and critical revision: (Zainab Gihad Falh, Dr Basil O Saleh and Dr Afraa M AL Naddawi) Acquisition of data analysis, drafting of manuscript, and interpretation of data: (Zainab Gihad Falh, Dr Basil O Saleh and Dr Afraa M AL Naddawi)

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الظاهرية لمتلازمة المبيض المتعدد دور انهيبين ب والهرمون الملوتن: نسبة الهرمون المنبه للجريب في تمايز الأنماط الكيسات

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الخلاصة:

خلفية البحث: تعد متلازمة المبيض المتعدد الكيسات (PCOS) سببا رئيسيا لمشاكل الخصوبة وعدم انتظام الدورة الشهرية لدى النساء في سن الإنجاب. تنتج الخلايا الحبيبية في الجريبات أمام الغدة والغربية النامية مادة الإنهيبين ب.

الأهداف: تم تصميم هذه الدراسة لمعرفة دور نسبة انهيبين B في الدم والهرمون اللوتيني (LH) / الهرمون المنبه للجريب (FSH) في التمييز بين الأنماط الظاهرية المختلفة لمتلازمة المبيض المتعدد الكيسات.

طرق العمل: تم إجراء هذا البحث المقطعي في قسم الكيمياء الحيوية، كلية الطب، جامعة بغداد في الفترة من نوفمبر 2023 إلى مارس 2024. وشمل 111 امرأة، الفئة العمرية (18-40 سنة)، تم تشخيص 91 من هؤلاء النساء بتكيس المبيض المتعدد. متلازمة تكيس المبايض (PCOS) وفقا لمعايير إجماع روتردام لعام 2003، وكانت 20 امرأة من النساء الأصحاء على ما يبدو. تم تقسيم النساء المصابات بمتلازمة تكيس المبايض إلى أربع مجموعات من النمط الظاهري (A، B، C، D). شملت التحقيقات قياسات مصل إنهيبين ب باستخدام تقنية مقايسة الامتصاص المناعي المرتبط بالإنزيم ((ELISA، FSH، LH، البرولاكتين باستخدام المقايسة المناعية الآلية AIA-2000 Tosoh وتم حساب نسبة LH/FSH.

النتائج: أظهرت النتائج أن النمط الظاهري A هو النمط السائد بين أنماط متلازمة تكيس المبايض، في حين أن النمط الظاهري B هو النمط النادر. كانت القيم المتوسطة (± SEM) لمستويات inhibin B للأنماط الظاهرية (p = 0.001) B، (p = <0.0001) C، (p = D <0.0001) أقل بكثير من تلك الخاصة بالنساء الضابطات. كانت القيمة المتوسطة لمستويات LH للنمط الظاهري A أعلى بكثير من تلك الخاصة بنساء السيطرة (P = 0.03). كانت القيم المتوسطة لنسبة LH / FSH مرتفعة بشكل ملحوظ في الأنماط الظاهرية (p = A 0.001) و (p = B 0.04) بالمقارنة مع الضوابط.

الاستنتاج: النمط الظاهري A هو النمط السائد لمتلازمة تكيس المبايض لدى النساء العراقيات. يمكن استخدام مستوى Inhibin B في المصل ونسبة LH/FSH في التمييز بين الأنماط الظاهرية المختلفة لمتلازمة تكيس المبايض.

الكلمات المفتاحية: إنهيبين B، نسبة LH/FSH، الأنماط الظاهرية، متلازمة تكيس المبايض , بروﻻكتين.

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Research Article

The Prevalence of Swarming Genes in *Escherichia coli* Isolated from UTI and Catheter-Associated UTI

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Abstract:

Background: Urinary tract infections (UTIs) are one of the most common bacterial illnesses among the public and in hospital settings. A prevalent nosocomial disease is catheter-associated urinary tract infection (CAUTI). The risk of infection increases with urinary catheterization, making it easier for Escherichia coli to colonize the urinary system. Uropathogenic *E. coli* (UPEC) specifically adapts to survive in challenging urinary tract conditions. Treating CAUTI accurately and effectively can be difficult. An important health concern nowadays is drug-resistant bacteria.

Objectives: To assess the prevalence of swarming genes in *E. coli* responsible for UTIs and catheter-associated UTIs, and determine their antibiotic resistance.

Methods: A total of 143 clinical specimens of urine and catheter samples were collected from two teaching hospitals in Baghdad city between October and December 2023. The bacteria were identified, and their antimicrobial susceptibility was tested. Conventional PCR methods were used to determine the bacteria and detect swarming genes (*flhC*, *flhD*, and *recA*).

Results: Out of 143 samples, 44 isolates were identified as *E. coli* (35 isolates from UTIs and 9 isolates from catheters). These isolates exhibited varying sensitivities to antibiotics, most being multi-drug resistant (MDR). They were highly resistant to tetracycline (72.7%) and highly susceptible to imipenem (93.2%). Among these isolates, 16 were identified (12 from UTIs and 4 from catheters). All the highly swarming and multidrug-resistant *E. coli* isolates were found to possess the three tested swarming genes (*flhC*, *flhD*, and *recA*), as determined by conventional PCR.

Conclusion: *Escherichia coli* is more prevalent in UTIs than in catheters. The number of isolates demonstrating the ability to swarm was found to be higher in UTIs, and these isolates also exhibited the capability to swim. Most *E. coli* isolates are multidrug-resistant and can swarm.

Key Words: *Escherichia coli*; *flhC; flhD*; Swarming motility; *recA*.

Introduction:

Urinary tract infection (UTI) is one of the most common bacterial diseases in humans. While E. coli is often associated with UTIs, it has also been found in the bladders of individuals who do not show any symptoms of lower urinary tract infection. This condition is known as asymptomatic bacteriuria (1). *E. coli* is also related to several extra-intestinal illnesses (2). Most *E. coli* strains found in the colon are harmless. However, certain pathogenic strains can cause contamination inside or outside the colon, depending on their virulence-associated traits (3). *E. coli* is the main and most common cause of UTI, cholangitis, cholecystitis, traveler's diarrhea**,** bacteremia, septicemia, neonatal meningitis, and others (4). One of the most common hospital-acquired infections is catheter-associated urinary tract infection (CAUTI). Urinary catheterization increases the risk of infection and promotes *E. coli* colonization of the urinary tract (5). *Escherichia coli* and other bacteria with peritrichous flagella can swim in a liquid environment by using a random walk pattern. This smooth movement, known as a run, happens when all flagella rotate counterclockwise to form a helical bundle. *E.coli* displays two types of flagella-driven motility: Swimming and swarming. Single-cell motility takes place when cells move in a liquid medium or soft semisolid agar while swarming happens when cells collectively move over semisolid surfaces (6). Flagellar-driven motility enables bacteria to colonize favorable sites in response to environmental cues, potentially increasing the risk of some species. Flagellated bacteria can move freely in bulk liquid or swarm on a semi-solid surface (7).

Escherichia coli uses several genes to control swarming, including *flhD*, *flhC*, and *recA*. The *flhD* operon, found at the beginning of the flagellar regulon, is the primary focus of control. It consists of two genes, *flhD*, and *flhC*, whose products combine to form the FlhD/FlhC heterotetrameric transcriptional regulatory

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complex (8)**.** *E. coli recA* has been discovered to have a new physiological function in promoting *E.coli*'s movement during swarming, but not during swimming. The exact molecular mechanism through which *recA* controls *E. coli*'s swarming movement is still unknown, however, it appears that *recA* does not affect swarming movement through the formation of large classical *recA* nucleofilaments (9)**.**

The first and crucial step in a UTI is when UPEC invades and colonizes the periurethral and vaginal areas. Bacterial motility is often associated with the severity of bacterial infections. In UPEC UTIs, the flagella enable motility and contribute to the bacterial virulence. A transcriptional regulator called PapX was found at the 3′ end of the P fimbrial operon in UPEC. When PapX is overexpressed, it reduces the synthesis of flagella, the flagellin protein (FlaA), and bacterial motility. On the other hand, the downregulation of PapX stimulates the transcription of the *flhDC* gene which is a transcriptional repressor that acts as a master regulator of flagellar motility. It plays crucial roles in adhesion, maturation, and proliferation. This is supported by the regulation of flagella-related gene expression during the growth phase of *E. coli* (10).

The study aimed to explore the most prevalent bacteria in UTIs and catheters, to determine which antibiotics are most resisted by the isolated bacteria, and identify the most prevalent genes associated with the swarming phenomena.

Materials and method:

Isolation and identification of bacteria: One hundred and forty-three samples were taken from urine (91 samples) and catheter surgery (52 samples) by transport swabs, from Al-Karama and Al-Yarmouk Teaching Hospitals between October and December 2023. The samples were taken and streaked onto MacConkey, EMB, and blood agar in a laboratory setting under aseptic conditions. The samples were then incubated at 37°C (11). The isolates underwent traditional biochemical testing to verify their identity, all tests were conducted following the standard procedures (12) (13).

Antibiotic susceptibility assay: The selection of antibiotics followed the Clinical & Laboratory Standards Institute (CLSI) 2023 recommendations, with disks for imipenem, amikacin, gentamicin, ciprofloxacin, azithromycin, tetracycline, cefotaxime, and ceftriaxone. By following the Kirby-Bauer protocol, the antibiotic sensitivity test was prepared (14). One or two colonies from an overnight nutrient agar plate culture were transferred into 3 mL of normal saline. A turbidity adjustment of 0.5 McFarland was made. Muller Hinton agar plates were inoculated using a sterile cotton swab dipped in the bacterial solution. The disks of various antibiotics were placed on the plate′s medium, for 18 to 24 hours. The plates were incubated at 37°C. The inhibition zones that resulted were quantified and contrasted with CLSI 2023 breakpoints. The isolate was categorized as susceptible, intermediate or resistant to a particular antibiotic by comparing it to the standard inhibition zones (15)**.**

Swarming and swimming assay: 1g% tryptone, 0.5g% glucose, 0.5g% NaCl, 0.5g% yeast extract, 0.5g% agar and 1.5g% Eiken agar was autoclaved at 121°C for 15 minutes, then cooled to 50°C and poured into Petri dishes (16). In this study, the media were modified by changing the agar percentage to 0.4 g% and the peptone percentage to 1 g%. Single colonies of *E.coli* were cultured in 5 ml of Brain-Heart infusion broth overnight. A 0.5 McFarland turbidity correction was used. On the two swarming agar plates, 5 µl of bacterial cultures was spotted. The plates were incubated at 37°C for 18 to 24 hours (17).

Swimming test media was prepared according to Kinoshita et al. (18), with modification, which include 1 g% peptone water, 0.5 g% NaCl, and 0.3 g% agar. Single colonies of *E.coli* were cultured in 5 ml of Brain-Heart infusion broth overnight. A 0.5 McFarland turbidity correction was used. 5 µl of bacterial cultures was spotted on a modified swimming medium.

Genotypic identification and detection of swarming genes: All steps are done according to Promega manufacturers (Part 9PIM712).

DNA extraction of bacteria: Nine *E.coli* isolates, six from urine and three from catheter surgery, were selected to detect the housekeeping gene (GAPDH) and swarming genes (flhC, flhD, and recA). Using a commercial Wizard genomic DNA purification kit (Promega, USA), genomic DNA was extracted from these isolates. The Quantus Fluorometer was then used to measure the concentration and purity of DNA.

PCR Amplification: The sequence of the particular pair of primers was used in Table (1). The PCR reaction was used to detect bacteria that possess GAPHD and swarming genes. A 25 µl volume was used for the PCR reactions, which included 12.5 µl of green master mix (Promega, USA), 1 µl of each primer (10 Pmol), and 2 µl of DNA template. The reaction volume was adjusted to 25 µl by using deionized distilled water. For the housekeeping gene (GAPDH), the annealing temperature was 53°C, while for the other gene, it was 56°C. Usually the annealing process takes between 30 and 60 seconds.

Table (1): Primers used in this study.

Id	Primer Name	Sequence	Product size (bp)	Reference
1	*GAPDH*-F	5'ACTTACGAGCAGATCAAAGC3'	190	(17)
2	*GAPDH*-R	5'AGTTTCACGAAGTTGTCGTT3		
3	*FlhC*-F	5'CCGGTTTGTGTAATGGCGTC3'	122	Designed in this study
4	*FlhC*-R	5'CAAACCGCACCAATGTCCAG3'		
5	*FlhD*-F	5'TTAGCGGCACTGACTCTTCC3'	87	
6	*FlhD*-R	5'TCGTCTGGTGGCTGTCAAAA3'		
7	*RecA*-F	5'GGGCCGTATCGTCGAAATCT3'		
8	*RecA*-R	5'GCGTCACAGATTTCCAGTGC3'	218	

Extension: The extension reaction is usually carried out between 72°C and 74°C, which is the ideal temperature for Taq DNA polymerase. The amplified DNA is given 1 minute per kilobyte. It is advised to extend for a final 5 minutes at 72°C–74°C. The amplified DNA is given 1 minute per kilobyte. It is recommended to continue at 72°C–74°C for the last five minutes. The PCR reaction products were stored at -20°C or immediately separated on 2% agarose gels.

Agarose gel electrophoresis of the PCR product: Agarose gel was prepared in 1% concentration as described by Lee *et al.*, (19). through dissolving 0.75 g of agarose powder in 75 ml 1X TBE buffer. Five microliters of a 100 base pair DNA ladder were aliquoted and put in the first well, on the left side of the agarose gel. After that, the DNA amplicons were cautiously put into the appropriate wells. The electrophoresis tank was then sealed with its unique lid. After that, the electrodes were positioned as directed, and 150 volts of electric current (corresponding to 5 V/cm) was applied for 40 minutes.

The loading buffer's bromophenol blue movement served as a monitor for the migration. Lastly, the gel documentation system was used to quickly take photos of the anticipated DNA bands once they had been inspected using an ultraviolet transilluminator.

Statistical analysis:

To determine the impact of different elements on research parameters, the IBM SPSS statistics program (29.0.2.0) was utilized. The chi-square test was used to determine the association between the studied variables.

Results:

Isolation and identification of bacteria: Out of the 143 samples (91 from urinary tract infections, 52 from catheters), 44 (30.8%) isolates were identified as *E.coli*, 35 (24.5%) in UTI, and 9 (6.3%) in catheters, by using MacConkey agar, EMB agar, and blood agar for characterization of colonies. The identification was conferred by biochemical test, Table (2).

Table (2): Biochemical Identification Results of *E.coli*

No.	Biochemicals test	Results
1	Catalase production	+ve
2	Oxidase test	-ve
3	Indole production	+ve
4	Urease production	-ve
5	Citrate	-ve
6	MR (Methyl red)	+ve
7	TSIA (Triple Sugar Iron Agar)	Acid/Acid, Gas +ve

The highest percentage of *E.coli* isolates was in urine samples 35/91 (38.5%) compared to catheters 9/52 (17.3%). In urine sample the predominant species was *E.coli* isolates 35 (38.5%), followed by 24 (26.4%) *Klebsiella* spp, 5 (5.5%) *Pseudomonas* spp, 2 (2.2%) *Proteus* spp. Out of the 52 samples of catheter, 22 (42.3%) isolates showed growth with 9 *E.coli* (17.3%) of catheter isolates, followed by *Klebsiella* spp 6 (11.5%), *Serratia* spp 4 (7.6%) and *Staphylococcus* spp 3 (5.8%), table (3). There was no significant association between the source of the sample and the number of *E,coli* isolates (P> 0.05). This was true for the other types of bacteria isolated (P> 0.05).

Table (3): The percentage of *E.coli* isolates and other bacteria from different sources

Sample source	No. of samples	No. and (%) of E.coli isolates	Other bacteria		
Urine	91	35 (38.5)	*Klebsiella* spp	*Pseudomonas* spp	*Proteus* spp
			24 (26.4%)	5 (5.5%)	2 (2.2%)
Catheter	52	9 (17.3)	*Serratia* spp	*Klebsiella* spp	*Staphylococcus* spp
			4 (7.6%)	6 (11.5%)	3 (5.8%)
P value	P<0.05		P<0.05		

Antibiotic susceptibility assays: The antibiotic susceptibility test results for *E. coli* are presented in Table (4), showing variations in susceptibility to the antibiotics. This test involved 44 isolates tested against eight antibiotics. Out of these, 41 isolates (93.2%) were sensitive to imipenem, 35 isolates (79.5%) to amikacin followed by 34 isolates (77.3%), which were sensitive to Azithromycin and 29 isolates (65.9%) to gentamicin, whereas, 32 isolates (72.7%) were resistant to Tetracycline and 30 isolates (68.2%) to cefotaxime and ceftriaxone. There is a statistically significant association between the type of antibiotic used and the bacterial sensitivity / resistance detected, ($P < 0.05$).

Table (4): Antibiotic susceptibility result of *E.coli*

Antibiotic	Number of isolates		
	Sensitive	Intermediate	Resistant
imipenem	41 (93.2%)	1 (2.3%)	2 (4.5%)
amikacin	35 (79.5%)	4 (9.1%)	5 (11.4%)
gentamicin	29 (65.9%)	5 (11.4%)	10 (22.7%)
ciprofloxacin	14 (31.8%)	2 (4.5%)	28 (63.63%)
Azithromycin	34 (77.3%)	0	10 (22.7%)
Tetracycline	11 (25.0%)	1 (2.3%)	32 (72.7%)
cefotaxime	14 (31.8%)	0	30 (68.2%)
ceftriaxone	14 (31.8%)	0	30 (68.2%)
P value	P > 0.05		

Swarming and swimming assay

Out of 44 isolates, 16 (36.4%) showed the ability to swarm on the two types of swarming media, as shown in Figure (1). Among these, 12 (27.3%) were UTI isolates and 4 (9.1%) were catheter isolates. Two isolates demonstrating swarming ability were sensitive to all antibiotics, while 14 were Multi-Drug Resistant (MDR). Additionally, 8 out of the 16 isolates that exhibited swarming ability also showed swimming ability on the modified medium. Not all isolates exhibited the same swarming strength. Each isolate swarming differed from the others in both dispersal strength and shape.

Figure (1): swarming of *E.coli*. A) Swarming on modified medium (0.4g% agar). B) Swarming on original medium (0.5g% agar). C) Swimming on a modified medium.

Genotypic identification and detection of swarming genes of *E. coli* isolates:Nine isolates of *E. coli* were selected because of the strong swarming and multi-resistance. The identification of these isolates was confirmed by using the housekeeping gene *GAPDH*, by conventional PCR. The result showed that all isolates were positive for the *GAPDH* gene, with an amplified size of 190 bp, using agarose gel electrophoresis, Figure 2. Swarming genes (flhC, flhD, and recA) were detected in 9 *E.coli* isolates by using conventional PCR. The results indicated that all isolates possessed the three tested swarming genes, with band sizes of 122 bp for flhC, 87 bp for flhD, and 218 bp for recA, Figure 2.

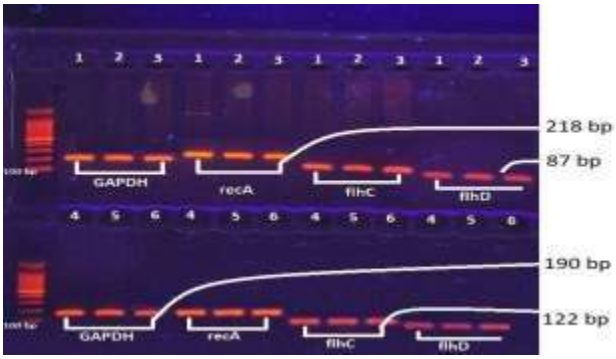

Figure (2): PCR detection of swarming genes of *E.coli* isolates were fractionated on 1.5% agarose gel electrophoresis stained with ethidium bromide, 40 minutes. Lane M: 100bp DNA ladder marker

Discussions:

Escherichia coli was the most prevalent in urine samples, followed by *Klebsiella* spp, *Pseudomonas* spp, and *Proteus* spp. The distribution of the types of bacterial isolates in the present study was consistent with those reported by other studies on UTIs. Khalaf and Flayyih (20) found that the urine samples revealed a majority of *E. coli* (70%) and that 75% of uncomplicated UTI cases were caused by UPEC. However, opportunistic UTIs are caused by other less prevalent pathogens such as group *B. streptococcus*, *K. pneumoniae*, *S. saprophyticus*, *E. faecalis*, *P. mirabilis*, *P. aeruginosa*, *S. aureus*, and other pathogenic bacteria. Over half of the cases of complex UTIs are caused by UPECs (10)**.** A study on 85 UTI cases reported a female preponderance (60%), and that 56.7% of the cases were under 40 years of age. All of the bacterial isolates demonstrated complete sensitivity to meropenem (13). In another study, *E. coli* was identified as the most common bacteria causing lower UTIs (46%), followed by *K. pneumoniae* (23%), *P. aeruginosa* (13%), *P. mirabilis* (10%), and *S. epidermidis* (8%). Female preponderance was also reported (62%), with males experiencing infections most frequently between the ages of 35 and 60 (21).

In a study conducted by Hameed *et al.* (22), it was found that out of 50 newborns, 6 (12%) had UTIs confirmed through urine culture and *E. coli* was the most commonly isolated microorganism. Notably, UTIs were more prevalent in female patients, with 66% of cases occurring in the first two months of life. Among the symptoms associated with UTIs, irritability was the most prevalent, observed in 83% of affected newborns. Interestingly, among jaundiced newborns with UTIs, a higher elevation in conjugated bilirubin was observed. However, it was found that jaundice resolved after appropriate antibiotics were administered.

The diagnosis of CAUTI requires more than just bacteriuria, and additional signs and symptoms like fever, flank discomfort, or suprapubic tenderness are necessary. Antibiotics are not recommended for patients with catheter-associated asymptomatic bacteriuria (CAASB) who are not at high risk of serious illness (4). The current study found that catheter isolates exhibited high resistance to cefotaxime, ceftriaxone, and ciprofloxacin (88.8%) followed by Tetracycline (66.6%), and showed high sensitivity to amikacin (88.8%) and imipenem (77.7%) El-Mahdy *et al*. reported that UPEC strains isolated from CAUTIs were found to be highly resistant to ampicillin (100%), amoxicillin-clavulanate and cefuroxime (86.7%), tetracycline (75.6%), ciprofloxacin, norfloxacin (71.1%), trimethoprim-sulfamethoxazole (66.7%), ceftazidime (55.6%), and aztreonam (53.3%). Likewise, the strains isolated from community UTIs showed an increased resistance to ampicillin (100%), cefuroxime (84.4%), tetracycline (75.6%), amoxicillin-clavulanate (73.3%), ciprofloxacin (66.7%), trimethoprim-sulfamethoxazole (62.2%), norfloxacin (60%), aztreonam, and ceftazidime (46.7%). Low resistance to amikacin, meropenem, and gentamicin was demonstrated by the isolates recovered from CAUTIs and community UTIs, which were 4.4%, 6.7%, and 26.7% in CAUTI and 2.2%, 4.4%, and 20% in community UTIs respectively. The resistance pattern of UPEC isolated from CAUTI and community UTIs did not significantly differ from one another (23).

Escherichia coli isolated from UTIs is becoming increasingly resistant to antibiotics and posing a serious public health concern. It is crucial to identify antibiotic resistance patterns in *E. coli* isolates for accurate prescriptions (24). The prevalence of bacterial pathogens producing extended-spectrum beta-lactamases (ESBLs) has led to a rise in UTI complications, presenting numerous management and epidemiological challenges, accounting for the majority of ESBL-producing organisms, most of which are nosocomial. However, the problem has recently become more severe due to the increased prevalence of MDR, *E. coli*, and ESBL. The majority of ESBL and *E. coli* are resistant to fluoroquinolones, trimethoprim, and gentamycin, as well as a variety of non-beta lactams, such as cephalosporins, penicillins, and piperacillin/tazobactam (25). According to Wang *et al*., sputum isolates exhibited higher resistance to 12 antibiotics compared to blood or urine isolates. Levofloxacin resistance was found to be higher in urine isolates. Additionally, urine isolates from young people displayed more resistance to certain antibiotics than those from older people. Furthermore, isolates from the elderly demonstrated greater resistance to most of the antibiotics tested compared to sputum strains isolated from children. (26).

The bacterial swarming on the modified media was generally stronger and better than on other media. The results indicated a wider spread of swarming on the modified medium, with a higher number of isolates demonstrating the ability to swarm. This suggests that the quantity of agar has a significant impact on the movement and spread of bacteria. This was further confirmed in swimming assays, where a lower agar concentration (0.3%) resulted in a wider spread for most isolates, considering an incubation temperature of 37°C for 24 hours. In a nutrient-rich plate with less than 0.3% agar (swimming assay), the bacteria exhibited chemotaxis, moving toward the nutrients through the agar pores and utilizing the nutrients. For the swarming assay, it is crucial to use an agar concentration higher than 0.3% to avoid swimming and accurately identify swarming motility (27)**.**

The ability of *E. coli* to move in urine isolates was found to be greater than in catheters, indicating that the environment plays a significant role in facilitating motility. Motility is a crucial factor for UPEC to travel

to the upper urinary tract, and it is one of UPEC's pathogenic traits. This ability allows the bacteria to ascend the ureters to the kidneys more quickly and efficiently (28). Many processes that occur on surfaces, such as adhesion, and interactions between bacteria and hosts, are influenced by the movement of bacteria's flagella. Thus, surface contact can control the expression of genes related to flagellar function and pathogenicity. Certain bacterial species have been shown to use their flagella as mechano-sensors (29).

In the current study, the identification of the *E. coli* isolates was confirmed using the housekeeping gene *GAPDH* with conventional PCR. Al-Imam and Flayyih used the *16SrRNA* gene to confirm the identification of *E. coli* O157:H7 isolates, which were positive for it, with 213bp (30).

In the current study, the presence of swarming genes (*flhC, flhD,* and *recA*) was confirmed in all tested *E. coli* isolates using conventional PCR. All isolates were found to have these three swarming genes, indicating that they are essential for swarming. The flagellar regulon is controlled by two master regulators, *flhD* and *flhC*. Mutant cells lacking these regulators are unable to move and do not have flagella. FlhDC activates specific promoters in response to environmental and metabolic signals (8). The flagellum is produced as a response to environmental stress, and the expression of *flhDC* is essential for its development. Catabolite suppression through cAMP affects the expression of *flhDC*. Acetyl phosphate inhibits flagellum development at high temperatures, likely due to OmpR phosphorylation, which suppresses *flhDC* production (31).

Escherichia coli recA has been found to play a new physiological role in promoting the bacterium's movement during swarming. In *E. coli* cells lacking the *recA* gene, swarming over a semi-solid surface is affected, while their swimming ability remains unchanged. These cells show reduced motility at the individual cell level when grown on a semi-solid surface and completely lose their collective swarming motion (9). Lane *et al*.'s research showed that a gene called *fliC* is important for this movement. Bacteria without this gene had reduced presence in the kidneys and disappeared from the spleen, indicating that wild-type UPEC use flagella to move and spread during UTI (32).

Conclusion:

Escherichia coli is more prevalent in UTIs than in catheters. The number of isolates demonstrating the ability to swarm was found to be higher in UTIs, and these isolates also exhibited the capability to swim. Most *E. coli* isolates are multidrug-resistant and can swarm.

Authors' declaration

We confirm that all the Figures and Tables in the manuscript are ours. Besides, the figures and images, which are not ours, have been given permission for re-publication attached to the manuscript.

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Authors' Contributions:

Study conception & design: (May Talib Flayyih. Literature search: (Hamza Ibrahim Kaittan). Data acquisition: (Hamza Ibrahim Kaittan. Data analysis & interpretation: (May Talib Flayyih). Manuscript preparation: (Hamza Ibrahim Kaittan). Manuscript editing & review: (May Talib Flayyih & Hamza Ibrahim Kaittan).

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انتشار جينات العج في الإشريكية القولونية المعزولة من التهاب المسالك البولية والتهاب المسالك البولية المرتبط بالقسطرة

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الخلاصة:
خلفية البحث: تعد إلتهابات المسالك البولية من بين الأمراض البكتيرية الأكثر شيوعا في كل من الأماكن العامة والمستشفيات. أحد أكثر أمراض المستشفيات إنتشارا هو عدوى المسالك البولية المرتبطة بالقسطرة. تزيد القسطرة البولية من خطر العدوى وتسهل على الإشريكية القولونية إستعمار الجهاز البولي. تم تكيف الإشريكية القولونية المسببة للأمراض البولية خصيصا للبقاء على قيد الحياة في الظروف الصعبة للمسالك البولية، حيث يمكن لهذه البكتيريا أن تلتصق بالمسالك البولية وتستعمرها، وتشكل مجتمعات بكتيرية داخل الخلايا باستخدام الشعيرات اللاصقة وتنشر عوامل الفوعة لتسبب العدوى. قد يكون علاج عدوى المسالك البولية المرتبطة بالقسطرة بفعالية ودقة أمرا صعبا. البكتيريا المقاومة للأدوية هي واحدة من أهم المخاوف الصحية في الوقت الحاضر.
الأهداف: تقييم مدى انتشار جينات العج (swarming genes) في الإشريكية القولونية التي تسبب التهاب المسالك البولية والتهاب المسالك البولية المرتبط بالقسطرة، وكذلك تحديد مدى مقاومتها للمضادات الحيوية.
المرضى والمنهجية: تم جمع 143 عينة سريرية من عينات الإدرار والقسطرة من مستشفيين تعليميين في مدينة بغداد خلال الفترة الممتدة من تشرين الأول - كانون الأول 2023. تم تشخيص البكتيريا بعد سلسلة من خطوات الزراعة والتنقية. تم إستخدام طرق تفاعل البوليميريز المتسلسل التقليدية للتعرف على البكتيريا والتحري عن وجود جينات العج (*recA*، *flhD*، و*flhC*). تم إتباع بروتوكول كيربي باورفي إعداد اختبار الحساسية المضادة للميكروبات.
النتائج: من 143 عينة، تم التعرف على 44 عزلة على أنها بكتيريا الإشريكية القولونية (35 من التهابات المسالك البولية، 9 من القسطرة). أظهرت هذه العزلات حساسية مختلفة للمضادات الحيوية، معظمها كانت متعددة المقاومة للأدوية، وأظهرت العزلات مقاومة عالية للتتراسيكلين (72.7%) وحساسية عالية للإيميبينيم (93.2%). كانت هناك 16 عزلة، 12 منها من التهابات المسالك البولية وأربع من القسطرة. تمتلك جميع عزلات الإشريكية القولونية القوية ومتعددة المقاومة للأدوية جينات العج الثلاثة التي تم اختبارها (*recA*، *flhD*، و *flhC*) بواسطة تفاعل البوليميريز المتسلسل التقليدي .
الإستنتاجات: تنتشر البكتيريا الإشريكية القولونية بشكل أكبر في حالات التهاب المسالك البولية مقارنة بالقسطرة. وقد وجد أن عدد العزلات التي أظهرت القدرة على التحشد أعلى في حالات التهاب المسالك البولية، كما أظهرت هذه العزلات أيضا القدرة على السباحة. معظم عزلات البكتيريا الإشريكية القولونية مقاومة للأدوية المتعددة ويمكنها التحشد.
مفتاح الكلمات: الاشريكية القولونية، حركة العج، حركة السباحة، recA، flhC، flhD

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Research Article

Correlation between Follicular Fluid Fatty Acids and Cell-Free Mitochondrial DNA in Women Undergoing Intra-Cytoplasmic Sperm Injections

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Abstract:

Background: Beta-oxidation of fatty acids takes place in the mitochondria to produce energy. This process is linked to the formation of free radicals. Previous researches propose that some fatty acids may be related to mitochondrial dysfunction, as they induce oxidative stress.

Objectives: To examine the correlation between follicular fluid fatty acids and relative cell-free mitochondrial DNA in the follicular fluid in women experiencing intra-cytoplasmic sperm injection (ICSI).

Methods: Fifty women subjected to ICSI participated in this cross-sectional research. Follicular fluid samples were obtained during oocyte pick-up. The samples were assessed for fatty acids, utilizing gas chromatography, and for relative cell-free mitochondrial DNA, real time polymerase chain reaction (PCR) was used.

Results: There was a strong significant positive correlation between follicular fluid margaric acid and follicular fluid relative cell-free mitochondrial DNA, as the correlation coefficient was 0.869, and the *P* value was 0.025. In addition, a strong significant inverse correlation was noticed, in women with diminished ovarian reserve, between follicular fluid oleic acid and relative cell-free mitochondrial DNA in the follicular fluid, as indicated by a correlation coefficient = - 0.9 and a *P* value = 0.037.

Conclusion: Margaric acid correlated positively with the relative cell-free mitochondrial DNA, which might reflect mitochondrial dysfunction, due to aggravation of oxidative stress. Whereas, oleic acid in women with diminished ovarian reserve, correlated negatively with relative cell-free mitochondrial DNA. However, more studies are required in this area of research.

Keywords: Follicular Fluid; Fatty acids; Intracytoplasmic Sperm injection; Mitochondrial DNA; Oxidative Stress.

Introduction:

Infertility is the inability to reproduce after twelve months of frequent and unprotected sexual activity (1). Male infertility can be due to decreased sperm count, motility, or normal morphology (2). Nevertheless, the actual reason can be unknown (3). In females, polycystic ovary syndrome (PCOS), a frequent endocrine disorder, may affect them during reproductive years (4). It may occur due to numerous factors (5). It can cause hyperandrogenism, irregular menses, infertility, obesity, and metabolic abnormalities (6-10). Diminished ovarian reserve is the reduction in the oocyte count and quality, which affects the reproductive potential negatively (11). Unexplained infertility is considered the reason for subfertility when all tests performed are normal (1). Fatty acids, comprising the carboxylic acid group and the hydrocarbon chain, are regarded as the primary building units of lipids (12). There are saturated, monounsaturated, and polyunsaturated fatty acids (13). Evidence states that fatty acids are a source of energy for sperm and oocytes (14,15). Oocytes need a high amount of energy to resume meiosis. In addition, fatty acids influence ovarian follicle growth by affecting prostaglandin and steroid synthesis in the granulosa cells (15). However, studies have shown discrepancies concerning the effects of fatty acids on oocytes and embryos (16, 17). Mitochondrial DNA (mtDNA) is a double-stranded DNA, circular in shape, located in the mitochondrial matrix, near the respiratory chain, which makes it liable for oxidation and mutations (18). Research has documented that the mtDNA copy numbers show a marked increment during oocyte maturation, due to the need for a considerable amount of energy (19). This energy, derived from the mitochondria, is crucial not only for growth of the oocytes but also for proper early embryonic development as glycolysis is blocked until embryos reaching morula- blastocyst stage. Mitochondrial dysfunction of the oocytes has been linked to energy deficiency and redox imbalance. Evidence showed that mitochondrial supplementation might improve oocyte quality as proposed (19) which possibly enhances fertility outcomes in assisted reproductive techniques (ART). Nonetheless, studies have cited contradictory findings about the

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relationship between the mtDNA and intra-cytoplasmic sperm injection (ICSI) outcomes (20,21). Fatty acid beta-oxidation occurs in the mitochondria and it produces reactive oxygen species (ROS) (22,23). It has been proposed that some fatty acids may aggravate the redox imbalance, a disturbed balance between free radicals and their scavengers (24), in the mitochondria (25). A link between fatty acids and mitochondrial dysfunction has been suggested as free fatty acids might potentiate ROS formation thus aggravating the oxidative stress damage. Furthermore, they can decrease mitochondrial membrane potential, they can also increase mitochondrial permeability and mtDNA expression causing mitochondrial dysfunction (25,26). Additionally, some fatty acids, namely, saturated fatty acids might activate apoptosis process (16) as a result of excessive ROS production. The need for ART has been increased (27,28). Assessing the follicular fluid (FF), which contains various substances, may be useful for finding markers that predict ICSI outcomes (29,30), as well as for a better understanding of the environment in which oocytes develop. This study was performed, due to the possible association between fatty acids and cf-mtDNA and the importance of understanding the microenvironment of the oocytes. This research aims to explore the correlation between fatty acids and relative cf-mtDNA in the FF of women, who experienced ICSI.

Patients and Methods:

This cross-sectional research involved fifty women who underwent ICSI at the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University, Baghdad in the period between December 2022 and May 2023. The study subjected included 11 cases with male factor infertility, 11 cases with PCOS, 10 cases with diminished ovarian reserve, 10 cases with unexplained infertility and 8 cases with tubal factor infertility. The inclusion criteria were women subjected to the ICSI program for various reasons of infertility. However, women having genital malformations, systemic diseases such as diabetes mellitus, thyroid gland disorders, renal or liver diseases, and females taking fibrates and statins (due to the potential impact of these medicines on fatty acid levels) were excluded. Male subfertility was diagnosed based on seminal fluid analysis (3). Tubal factor infertility was confirmed by hysterosalpingography (1). Cases of PCOS were diagnosed depending on the Rotterdam criteria (31-33), and cases of diminished ovarian reserve were diagnosed based on the Bologna criteria (34). Unexplained infertility was determined when all infertility investigations were normal (1). Body mass index (BMI) was calculated as weight in kilograms per height in square meters (35-37). Detailed history was obtained, full examination was carried out and hormone values were acquired from the women. On the 2nd day of the menstrual period, recombinant follicle stimulating hormone (r-FSH) (Gonal F, Merk Serono, Germany) injections were commenced with a dose of 150 to 300 IU per day based on the patient's clinical status. A flexible start protocol was used for the administration of gonadotropin releasing hormone (GnRH) antagonist, namely, Cetrorelix acetate (Cetrotide®, Merk, Switzerland), 0.25 mg per day. It was started when the leading follicles' size reached 13 to 14 mm. Serial ultrasound evaluations and frequent serum estradiol (E2) quantifications were performed to monitor ovarian follicles' size and number. Once the size of 3 follicles reached 17 mm, subcutaneous human chorionic gonadotropin (hCG) injection (Ovitrelle®; Merck international, Italy) was given to provoke the release of the oocytes. 35 to 36 hours after giving hCG, oocyte picking up was done via a single lumen ovum aspiration needle by Wallace (CooperSurgical, California, USA) under transvaginal ultrasound monitoring.

Assessment of oocytes and embryos:

Removal of cumulus cells was done before ICSI to assess oocytes' maturity. Oocytes were categorized into germinal vesicle (GV), metaphase I (MI) oocytes, and metaphase II (MII) oocytes depending on their maturity status. 18 to 20 hours after ICSI, evaluation of fertilization was done and fertilization rate was computed as the ratio of oocytes with 2 pronuclei to oocytes that were injected (38). Cleavage-stage embryos were categorized into grade 1, grade 2, and grade 3 based on the Istanbul Consensus Workshop (39).

Collection of the follicular fluid samples:

During oocyte retrieval, FF (containing no flushing media) was collected (pooled from multiple follicles) and divided into 2 parts. One part was centrifuged at 1500xg for 10 minutes and the supernatant was then used for assessing fatty acids. The other part was centrifuged at 3000xg for 15 minutes and the supernatant was then used for DNA extraction and relative cf-mtDNA evaluation. The supernatant samples were transferred into sterile tubes and kept at – 20 C till assessment time. FF samples that were cloudy or stained with blood were not included.

Evaluation of the follicular fluid samples using gas chromatography:

FF samples (1.5 ml for each) were vortexed for three minutes. Then, 3 ml of cold acetone was added to separate the proteins from the solution. The samples were shaken for seconds and were put at – 20˚C for fifteen minutes. The samples were centrifuged, and then the supernatant of each sample was mixed with three ml aliquots of hexane (Thomas Baker, India) and water. Following that, horizontal shaking of samples was done for 5 minutes. To isolate the solvent phase from the aqueous phase, centrifugation was carried out another time. The top layers (hexane) were taken into sterile tubes. A 0.25 ml aliquot of buffer (pH= 9), made by mixing 0.1 M Na3PO4 with 0.1 M Na2HPO4 in water, and 0.25 ml iodomethane (Fluka, Switzerland) in dichloromethane (Central drug house, India) (1:10 vol: vol) were added as well. Finally, shaking of samples was done for 5 minutes via the vortex to produce the fatty acid methyl esters (FAME) (16,40).

Identification of FAME was done by gas chromatography (7820A, Agilent Technologies, USA) equipped with the analytical column (Agilent HP-5ms ultra inert, USA) having dimensions of 30 m length, 0.250 mm inner diameter, and 0.25 µm film thickness. Helium (99.99 %) was the carrier gas. The beginning temperature was 60˚C (for 3 minutes), elevated to 180˚C (7˚C/ minute), then was raised to 280˚C (8˚C/ minute), which was kept for 3 minutes. Recognition of FAME was done depending on their retention times, and levels of fatty acids were calculated as the weight percentage of the whole fatty acids found (41).

Relative cell-free mitochondrial DNA evaluation:

FF samples were centrifuged again at 16000xg for 10 minutes before cell-free DNA extraction. After that, the supernatants of FF samples were transferred into new Eppendorf tubes. Each FF sample (200 µL) was processed for extracting cell-free DNA by the usage of the AddPrep Genomic DNA Extraction Kit (ADD BIO INC, Daejeon, Republic of Korea), corresponding to the guidelines of the producer.

Specific primers for the β-globin gene (represents the nuclear DNA) and ND1 gene (represents mitochondrial DNA) were used for the amplification; (Macrogen Co., Ltd., Republic of Korea) designed the primers. The following primers: 5′-CCCTAAAACCCGCCACATCT-3′ (forward) and 5′-GAGCGATGGTGAGAGCTAAGGT-3′ (reverse), which amplify a 69 base pair DNA piece, identified ND1. The primers: 5′-AAAGGTGCCCTTGAGGTTGTC-3′ (forward) and 5′-TGAAGGCTCATGGCAAGAAA-3′ (reverse), that amplify a 77 base pair DNA segment, were used for the detection of β-globin (20).

Relative quantification of cf-mtDNA was achieved by real time quantitative polymerase chain reaction (PCR) (Rotor-Gene Q, QIAGEN, Germany). 20 µL was used as a total volume to perform the reaction, which consists of the extracted DNA (2 µL), sense, and anti-sense primers (10 µM). SYBR Green master mix (10 µL) (PowerUp SYBR Green Master Mix, Applied Biosystems, Thermo Fisher Scientific Baltics UAB). PCR circumstances were 94 C (2 minutes), 40 cycles of 95 C (10 seconds), then 60 C (30 seconds).

Relative cf-mtDNA copy numbers were estimated via the Delta Delta CT method (Livak method), and fold changes were calculated using the equation: $2^{-\Delta\Delta Ct}$ (42).

Statistical Analysis:

Data analysis was accomplished utilizing Statistical Package for the Social Sciences (SPSS) version 29 (Chicago, IL, USA). Normally distributed data were expressed as mean ± standard deviation and non-normally distributed values were presented as median (interquartile range). Pearson correlation test was applied to test for correlations in normally distributed data. Spearman correlation test was utilized for data that are not normally distributed. The finding was assumed statistically significant when the *P* value is less than 0.05.

Results:

Fifty females were involved in the present research, 33 women complained of primary infertility, and 17 women experienced secondary infertility. Out of those 50 women, thirty-eight women performed no previous trials of ICSI, 10 women had failed one ICSI trial previously, 1 female had failed two previous trials and 1 participant underwent a successful previous trial. Characteristics of all patients concerning demographic data and ICSI parameters are illustrated in Table 1.

Table 1: Demographic data and ICSI parameters of the participants

Patients' characteristics (N= 50)	The value
Age (years)	32 ± 5.4
Body mass index (kg/m^2)*	28.8 (4.1)
Follicle stimulating hormone (mIU/ml)	6.4 ± 1.7
Luteinizing hormone (mIU/ml)	5.8 ± 2.9
Estradiol (pg/ml)	38.6 ± 14.7
Total count of collected oocytes*	11 (11)
Oocyte maturity rate (%)	65.1 ± 21.2
Fertilization rate (%)	67.1 ± 22.9
Percentage of high quality embryos (%)*	50 (48.7)

Data are reported as mean ± standard deviation, in normal distribution, or median (interquartile range), in non-normal distribution. ***** refers to variables in which the median was used. ICSI: Intra-cytoplasmic sperm injection; N: Number of patients.

In terms of correlations between fatty acids and relative cf-mtDNA in the FF, Table 2 shows the correlations between saturated fatty acids and relative cf-mtDNA. The correlation between margaric acid and cf-mtDNA was significant as illustrated by a *P* value less than 0.05.

Table 2: Correlations between saturated fatty acids and relative cf-mtDNA in the FF

Saturated fatty acids in the FF %	Relative cf-mtDNA in the FF	
Palmitic acid % (N= 50)	rho = 0.096	*P* = 0.509
Stearic acid %(N= 41)	rho = 0.212	*P* = 0.184
Margaric acid % (N= 6)	r = 0.869	*P* = 0.025
Acetic acid % (N= 5)	r = 0.426	*P* = 0.474
Propionic acid % (N= 19)	rho = 0.049	*P* = 0.842

Pearson correlation and Spearman correlation tests are used according to data distribution. cf-mtDNA: Cell-free mitochondrial DNA; FF: Follicular fluid; N: Number of patients.

Concerning the correlations between FF unsaturated fatty acids and relative cf-mtDNA in the FF, all correlations were non-significant as observed in Table 3.

Table 3: Correlations between unsaturated fatty acids and relative cf-mtDNA in the FF

Unsaturated fatty acids in the FF %	Relative cf-mtDNA in the FF
Oleic acid % (N= 21)	rho = - 0.313 *P* = 0.167
Linoleic acid % (N= 8)	rho = 0.548 *P* = 0.160
Palmitoleic acid % (N= 6)	rho = 0.086 *P* = 0.872

Spearman correlation test is applied. cf-mtDNA: Cell-free mitochondrial DNA; FF: Follicular fluid; N: Number of patients. When it comes to the correlations between FF fatty acids and FF relative cf-mtDNA in different causes of subfertility, it has been shown that palmitic acid correlated positively with relative cf-mtDNA in women having reduced ovarian reserve and in cases of male infertility as rho = 0.564, *P* = 0.090 and rho = 0.545, *P* = 0.083, respectively. A positive, non-significant correlation was also identified between stearic acid and relative cf-mtDNA, in male factor infertility, since rho was 0.612 and *P* value was 0.060. It was found that oleic acid in the diminished ovarian reserve patients correlated significantly and negatively with cf-mtDNA in the same group as the correlation coefficient (rho) was -0.9 and the *P* value was 0.037.

Discussion:

This study aims to correlate FF fatty acids and relative cf-mtDNA in the FF of women undergoing ICSI. Owing to the relationship between the level of cf-mtDNA in body fluids and oxidative stress (43), and the correlation between some fatty acids and oxidative stress (44), we proposed that there might be a correlation between fatty acids and cf-mtDNA in the FF of females subjected to ICSI. In our study, a significant positive correlation has been detected between FF margaric acid and FF relative cf-mtDNA. This may be attributed to the relationship between margaric acid, being a saturated fatty acid, and cf-mtDNA with oxidative stress, which indirectly causes this positive correlation between margaric acid and cf-mtDNA. In a previous study, a positive correlation was shown between plasma mtDNA and the level of H2O2 (which reflects the level of ROS) and illustrated a possible link between elevated mtDNA and oxidative stress (43). It has been documented that ingestion of saturated fat, in women with PCOS, can result in the induction of oxidative stress; the researchers have noticed that ROS production, $p47^{phox}$ gene expression, and circulating thiobarbituric acid-reactive substances increase after consumption of a diet rich in saturated fat (45). Likewise, saturated fatty acids may lead to severe apoptosis, which probably modifies the constitution of the mitochondrial membranes, altering the mitochondrial function (46). Endoplasmic reticulum stress may be induced by lipotoxicity, as well (47). Besides that, free fatty acids have a role in lowering mitochondrial membrane potential, elevating mitochondrial permeability, and mtDNA expression (25). Furthermore, accumulation and increment of excess fatty acids might result in impairment of mitochondrial function by increasing the production of toxic metabolites and elevating oxidative stress, thus affecting mitochondrial performance (48). This might be another explanation of the positive correlation between margaric acid and cf-mtDNA. In addition, impaired mitochondrial function can occur secondary to insulin resistance that might happen due to ingestion of high amount of saturated fat (for example, margaric acid), therefore, raised saturated fatty acids might be associated with increased cf-mtDNA due to mitochondrial dysfunction (48).

FF palmitic acid in diminished ovarian reserve and male factor subfertility, and FF stearic acid, in male factor infertility show a positive correlation with FF relative cf-mtDNA in the current research. This can reflect mitochondrial dysfunction due to exacerbating oxidative stress, as agreed to in numerous studies (46-48). Although these correlations have not reached statistical significance, they are approaching that stage. This study detected a significant inverse correlation between oleic acid and relative cf-mtDNA in the FF of women with low ovarian reserve. This finding was in accordance with that mentioned in a recent study about the effects of monounsaturated fatty acids as they promoted mitochondrial oxidation, elevated antioxidant ability, and reduced inflammatory and peroxidation markers (49). This probably explains their favorable effects on mitochondrial function and illustrates that oleic acid, being a monounsaturated fatty acid, might have a protective role by improving the metabolic profile (49). Meanwhile, the excess level of FF cf-mtDNA possibly reflected the mitochondrial functional impairment of ovarian granulosa cells that led to the elevation of FF cf-mtDNA, secreted from these cells (50). Therefore, these opposite effects of oleic acid, suggested to have a protective role, and cf-mtDNA (linked to oxidative stress and mitochondrial dysfunction) might explain the significant negative correlation that was found between them. In the current research, no correlation was identified between linoleic acid and cf-mtDNA. This might be due to the small number of participants. This finding disagreed with another study done by Xu et al. (2019) which revealed that omega-6 polyunsaturated fatty acids, owing to their pro-oxidative effects, might cause mitochondrial dysfunction (26).

Limitations:

Some limitations that should be addressed are the small number of participants, the sample size especially for certain fatty acids with very small numbers, the cross-sectional design of the study, and the lack of involvement in the exact nutritional status, and the eating habits of the women included in the study. It is recommended to include more participants and to take into consideration the nutritional condition in future studies. Moreover, our suggestion for future research is exploring the exact role of oxidative stress biomarkers in conjunction with fatty acid profiles or cf-mtDNA levels.

Conclusion:

This research demonstrated a possible link between fatty acids and relative cf-mtDNA in the FF. Margaric acid showed a significant direct correlation with relative cf-mtDNA reflecting mitochondrial dysfunction due to oxidative stress. Oleic acid correlated significantly and inversely with relative cf-mtDNA in patients with diminished ovarian reserve. Therefore, different fatty acids exert various effects, which might be related to their degree of saturation.

Nonetheless, further research is justified to unveil other unknown mechanisms and factors that explain the associations between fatty acids and cf-mtDNA in the FF.

Authors' declaration:
We confirm that all the Tables in the manuscript belong to the current study. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in (the University of Baghdad/ College of Medicine) according to the code number (197) on (09/ October /2023).
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Authors' contributions:
Study conception & design: (Zainab M. Alawad and Hanan L. Al-Omary). Literature search: (Zainab M. Alawad). Data acquisition: (Zainab M. Alawad). Data analysis & interpretation: (Zainab M. Alawad and Hanan L. Al-Omary). Manuscript preparation: (Zainab M. Alawad). Manuscript editing & review: (Hanan L. Al-Omary).

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العلاقة بين الأحماض الدهنية والحمض النووي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي لدى النساء اللاتي يخضعن للحقن المجهري

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الخلاصة:
الخلفية: أكسدة بيتا للأحماض الدهنية يحدث في المايتوكوندريا لإنتاج الطاقة. ترتبط هذه العملية بتكوين الجذور الحرة. تقترح الأبحاث السابقة أن بعض الأحماض الدهنية قد تكون ذات صلة بالاختلال الوظيفي للمايتوكوندريا لأنها تحفز الاجهاد التأكسدي.
الأهداف: لدراسة الارتباط بين الأحماض الدهنية في السائل الجريبي والحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي عند النساء اللاتي يخضعن للحقن المجهري.
المرضى والطرق: تمت مشاركة خمسين امرأة خضعت للحقن المجهري في هذا البحث المقطعي. تم الحصول على عينات السائل الجريبي أثناء التقاط البويضات. تم تقييم العينات للأحماض الدهنية باستخدام كروماتوغرافي الغاز وللحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا تم استخدام تفاعل البلمرة المتسلسل في الوقت الحقيقي.
النتائج: كانت هناك علاقة ارتباط قوية معنوية موجبة بين حمض المارجريك في السائل الجريبي والحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي لأن معامل الارتباط كان 0.869 وكانت القيمة الاحتمالية 0.025. إضافة الى ذلك لوحظت علاقة قوية معنوية عكسية، في النساء اللاتي لديهن انخفاض في مخزون المبيض، بين حمض الأوليك في السائل الجريبي والحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي كما موضح بمعامل الارتباط = - 0.9 والقيمة الاحتمالية = 0.037.
الاستنتاج: من المحتمل ان تظهر الأحماض الدهنية المختلفة في السائل الجريبي ارتباطات مختلفة مع الحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا في السائل الجريبي. هذا ممكن أن يحدد بمستوى التشبع لان حمض المارجريك ارتبط بشكل إيجابي مع الحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا، الذي قد يعكس خلل المايتوكوندريا بسبب تفاقم الاجهاد التأكسدي. بينما، حمض الأوليك، في النساء اللاتي لديهن انخفاض في مخزون المبيض، ارتبط سلبا مع الحمض النووي النسبي للمايتوكوندريا الخالي من الخلايا. ومع ذلك هناك حاجة الى مزيد من الدراسات في هذا المجال البحثي.
الكلمات المفتاحية: السائل الجريبي، الأحماض الدهنية، الحمض النووي للمايتوكوندريا، الحقن المجهري، الاجهاد التأكسدي.

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Case Series

What Governs Immediate or Delayed Cardioversion of Atrial Fibrillation by Direct Current Shock?

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Abstract:

Background: Aftershock delivery and dierct current DC-cardioversion of atrial fibrillation may be immediate or delayed.

Objective: To characterize the immediate or delayed reversion of atrial fibrillation.

Methods: The study was conducted at Alhassani Heart Centre from October 2018 to February 2022**.** Patients diagnosed with persistent atrial fibrillation and who reverted to sinus rhythm after DC-Cardioversion were included in this case series study. Some patients showed immediate conversion to sinus rhythm while others showed delayed conversion after shock delivery. The duration of the atrial fibrillation, the ventricular rate range before the intervention, the preceding drug therapy, patient weight, and left atrial size were measured to illuminate the factors that affect the reversion format.

Results: From a total of 86 patients with persistent atrial fibrillation treated with DC-cardioversion, 77 (89%) patients reverted into sinus rhythm and were included in the study. Fifty patients reverted immediately and 27 patients reverted late. The mean ventricular rate was higher in the immediate group 138 ± 22 compared to 75 ± 18 in the delayed group. The post-conversion appearance of atrial premature beats was more in the delayed group. The left atrial size was slightly larger in the delayed group. The role of taking a preceding drug was not significant in both groups.

Conclusion: The pattern of reversion in atrial fibrillation patients undergoing DC shock is governed by the ventricular rate before the reversion and the appearance of atrial premature complexes after DC shock.

Keywords: Atrial fibrillation; Cardioversion; DC shock; Delayed; Immediate.

Introduction:

Electrical cardioversion for atrial fibrillation (AF) by synchronized direct current (DC) shock is a common and efficient procedure to convert atrial fibrillation to normal sinus rhythm (SR)1-12. Achieving SR improves hemodynamics, especially in patients with heart failure (HF)13,14.

DC shock usually converts AF into SR immediately after the application of the shock5,11,15,16. It is not uncommon that the reconversion is delayed for a few beats before SR is achieved16.

Occasionally this delay may be long enough to make the physician decide to deliver another unnecessary DC shock.

The etiologies behind this delayed cardioversion phenomenon are not clear. Residual wavelets in the atrium may still be present after the DC shock to shortly maintain AF, but not enough to sustain it16-20.

This case series study aims to clarify what factors govern the immediate or delayed cardioversion of AF.

Patients and Methods:

Patients who received synchronized DC Shock and reverted into SR at Alhassani Cardiology February 2022, were included in the study. AF was classified as early persistent when it was sustained for > 7 days and < 3 months; late persistent if it was sustained for > 3 months and < 1 year, and chronic if the symptoms persisted for more than a year. The decision of rhythm control strategy was made for all patients with persistent AF. In the chronic AF, the strategy was chosen after discussing the pros and cons with the patient. Direct oral anticoagulant (DOAC) was started 48-72 hours before DC shock. Transthoracic echocardiography was done before DC shock to exclude LA thrombi.

The patient arrived at the hospital in a fasting state. Pre-procedural assessment of the physical status, risk evaluation, and discussion with the patient and family was done. An intravenous line is set and the patient is put in a supine position with shoulder support. Oxygen is administered via nasal cannula. The patient is connected to cardiac rhythm monitoring facilities. Ketamine 25 mg plus fentanyl 25-37 ugm are administered intravenously and a calculated dose of propofol 80-140 mg according to the weight and built of the patient aiming for heavy sedation. Synchronized DC Shock is delivered once the patient loses consciousness. This method is sufficient even if another shock is needed. The heart rhythm is recorded by the ECG monitor of the defibrillator (Cardioserv

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from GE). One ECG lead is chosen with the highest voltage R wave and clearly shows synchronization sign. The positive electrode of the defibrillator's paddle (or patch) is put at the left mid-axillary line and the negative one at the right mid infra-clavicular line. Under the satisfactory sedation state, a first DC Shock of 200 J is delivered and the rhythm is instantly observed. If the rhythm is still in AF, a second shock of 300 J is delivered. If needed, a third last dose of 300 J is given. The interval between the shocks is 2-3 minutes. The procedure was labelled as successful if SR was achieved, and a failure if the rhythm was still in AF after the third shock. The patient is labeled as immediate cardioversion if SR is achieved immediately after the shock, and as delayed cardioversion if the cardioversion is delayed after delivering the DC shock. Accordingly, the cardioverted patients are classified into one of two groups, the immediate group (IG) and the delayed group (DG). In the DG the number of beats in AF and time in seconds before restoring SR was calculated. The ventricular rate before and after the cardioversion was measured in both groups. The appearance of atrial premature complexes after cardioversion was noticed in both groups. The energy needed for the cardioversion was compared between the two groups. The time in minutes for the first response to command after reconversion was assessed as also the time until full consciousness recovery. A full neurological assessment was performed immediately after recovery of consciousness. The patient was observed at the recovery unit for 2-3 hours and discharged home 2 hours after regaining full consciousness. DOAC and oral antiarrhythmic drugs with either amiodarone or flecainide were started at a minimal dose for six months or according to the clinical status of the patient. Follow-up was done after 4 weeks and then every 3 months or with recurrence of symptoms. At the outpatient (OP) clinic, patients were assessed by clinical symptoms, ECG, and 48 hours of Holter monitoring. Echocardiography was repeated to assess LVEF and LA size.

Results:

From a total of 86 patients with persistent AF, 77 patients (89%) were converted into SR with synchronized DC shock. These 77 patients were included in this case series study. The ages of the participants ranged from 35 to 84 years. There were 36 males and 41 females. The weight of the patients ranged from 75-98 kg. The underlying structural heart diseases (SHDs) in the group were ischemic cardiomyopathy (iCMP) in 16 patients (21%), Tachycardia induced cardiomyopathy (TIC) in 11 patients (14%), Thyrotoxicosis in two patients and repaired atrial septal defect (ASD) in one patient. No structural heart disease was found in 47 patients (61%). The duration of AF based on symptoms and available previous ECGs was as follows: 1-2 months in 11 patients (14.3%), 3 months – one year in 41 patients (53.2%), and more than one year in 25 patients (32.5%). The presenting symptoms were palpitation in 62 patients (80.5%), dyspnea in 38 patients (49.4%), dizzy spells in 18 patients (23.4%), syncope in 10 patients (13%), and chest pain in 8 patients (10.4%). Many patients had more than one symptom. The anticoagulant drugs given were Rivaroxaban in 65 patients (84.4%), dabigatran in seven patients (9%), and five patients (6.5%) patients received warfarin, Table 1.

Table 1: Demographic and clinical features of the study group

Variable	Category	Number	%
Gender	Male	36	46.8
	Female	41	53.2
SHD	Yes	30	39.0
	No	47	61.0
Assessed duration of AF	1-2 months	11	14.3
	3 months – 1year	41	53.2
	> 1 year	25	32.5
Presenting symptoms	Palpatation	62	80.5
	Shortness of breath	38	49.4
	Dizziness	18	23.4
	Syncope	10	13.0
	Chest pain	8	10.4
Anticoagulant drugs	Rivaroxaban	65	84.4
	Dabigatran	7	9.0
	Warfarin	5	6.5
Total		77	100,0

Fifty patients (64.9%) were converted immediately after the DC shock (the immediate group – IG) and 27 patients (35.1%) had a delayed conversion (the delayed group – DG).

Where the comparison of the two groups in terms of a number of parameters. The mean age was higher for the Immediately converted than the delayed converted group, but statistically not significant ($p>0.05$). There were more males in the DG and more females in the IG, but they were not significantly associated. The mean weight of the cases in the two groups does not seem to be statistically significant also there are no significant differences in weight among immediate and delayed groups ($p>0.05$), on the other hand, the Pre-conversion ventricular mean, Pre-conversion AF beats and Number of beats to achieve SR

were significantly differing between immediate and delayed groups ($p<0.05$), while statistically the significant differences ($p>0.05$) were not observed

for DC shock dose needed, LA size, Time for the response, Time for full recovery, SR during follow-up and Recurrence of AF. Table 2

Table 2: Distribution of the immediate and delayed conversions groups

Parameter	Category	Immediate group	Delayed group	Statistical test	*P*-value
Age (years)	Mean±SD	62±9	60±10	T-test	0.37
Gender	Male	22 (44%)	16 (59.3%)	Chi-square	0.20
	Female	28 (56%)	11 (40.7%)		
Weight (Kg)	Mean±SD	89±7.7	90±10.2	T-test	0.63
Pre-conversion ventricular mean (rate/minute)	Mean±SD	138±22	75±18	T-test	0.0001
Pre-conversion AF beats	Range	0	4 – 22		0.0001
	Mean±SD	0	13±6	T-test	
	Time (seconds)	0	3.4 - 11	T-test	0.0001
	Mean±SD	0	7.2±4		
DC shock dose needed	200J – 1st dose	38 (76%)	18 (66.7%)	Chi-square	0.44
	300J - 1st dose	11 (22%)	7 (25.9%)		
	300J – 2nd dose	1 (2%)	2 (7.4%)		
The post-conversion appearance of APCs	Yes	12 (24%)	24 (88.9%)	Chi-square	0.00001
	No	38 (76%)	3 (11.1%)		
LA size (cm)	Mean±SD	4.3±2	4.5±3	T-test	0.7
Time for response (minutes)	Range	10 - 16	7.5 - 16	T-test	0.5
	Mean±SD	13±6	12±8		
Time for full recovery (minutes)	Range	15 - 26	12 – 26	T-test	0.6
	Mean±SD	22±9	23±10		
SR during follow-up	No.(%)	32 (64%)	19 (70.4%)	Chi-square	0.57
Recurrence of AF	No. (%)	18 (36%)	8 (29.6%)	Chi-square	0.57
Number of beats to achieve SR	<5	0 (0%)	3 (11.1%)	One way ANOVA	0.00001
	5-10	0 (0%)	8 (29.6%)		
	11-15	0 (0%)	3 (11.1%)		
	16+	0 (0%)	13 (48.1%)		

The synchronized DC shock reverts the AF into sinus rhythm immediately with the appearance of few atrial premature beats after the reversion which usually disappears within a few seconds (Figuer: 1),

Figure 1: SDCS reverting the AF immediately into SR with frequent APC appearance

another immediate reversion of AF into sinus rhythm with no atrial premature beats appearance in figure (2).

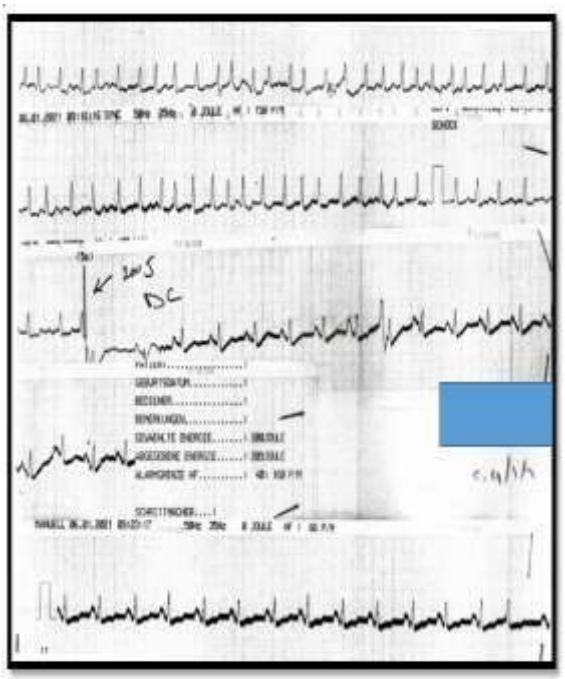

Figure 2: AF reverted immediately into SR after 200J SDCS

The delayed pattern of reversion of AF into sinus rhythm where about for 10 beats AF continued before stabilizing into sinus rhythm (figure 3),

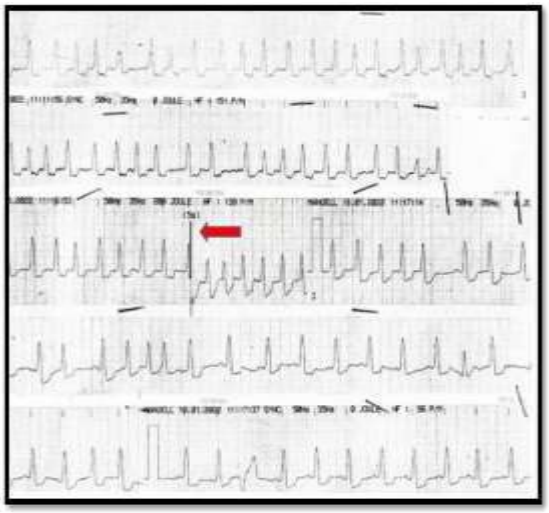

Figure 3: Delayed reversion of AF into SR by SDCS (red arrow) after 11 AF (3.6 seconds) betas with frequent APCs

while the delayed reversion pattern of AF into sinus rhythm with limited duration of AF after the DC shock before stable sinus rhythm achievement were found in some patients (fig.4),

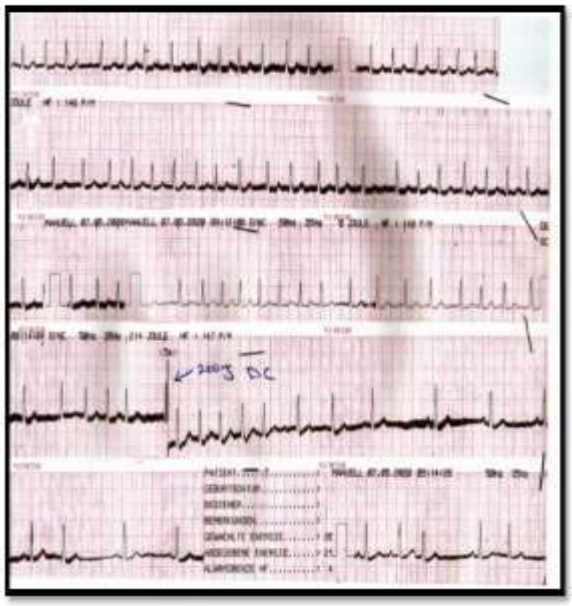

Figure 4: Delayed reversion of AF into SR by SDDCS after 7 beats (3.4 seconds) in AF

and The remarkable delayed reversion of AF into sinus rhythm after DC shock delivery where we can see AF continued for about 36 beats (11seconds) before stable sinus rhythm appearance (fig.5). In this situation in specific the physician may consider unnecessarily deliver another DC shock.

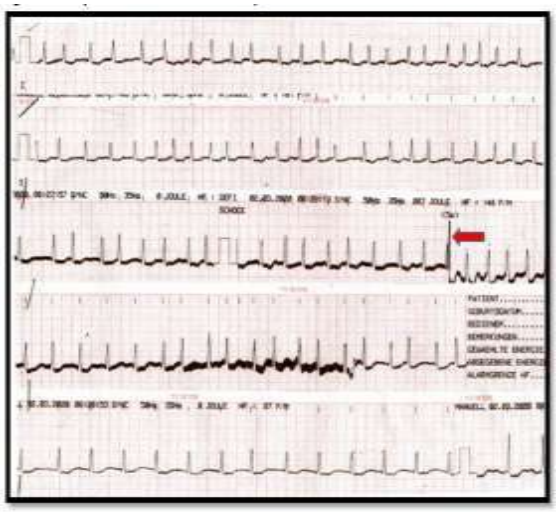

Figure 5: Delayed reversion of AF by SDCS (red arrow) after 24 AF beats (11 seconds)

Discussion:

The potential mechanism of AF is multiple micro-reentry circuits firing together in a variable time and cycle length. Cardioversion depolarizes cardiac tissues and makes them refractory. Depolarisation of all micro-reentry circuits involved in AF will lead to the termination of AF.

The termination of AF by DC shock can be immediate after shock delivery or delayed for a few beats16. Waiting for a short period before delivering a second shock is practically and clinically significant. The factors which govern these conversion patterns were looked for in this study. Wong et al16, (the only study we found in the literature discussing the delayed and the immediate reversion of AF into SR with DC shock) used coronary sinus catheters to record atrial potentials in 171 patients. They found that 54% of their AF patients converted immediately after cardioversion and 45% had a delayed pattern, compared to the current study with 65% with an immediate conversion and 35% with a late pattern and the ventricular rate was faster in the IG than the DG group. These findings are in contradiction with those of Wong et al. In our study, a slower ventricular rate just before the cardioversion predicts a delayed pattern of reversion. The number of APCs within SR after reversion was significantly more frequent in the DG than the IG. This may suggest that there is still an active atrial discharge after reversion but not sufficient to continue the AF which is consistent with Wong's explanation. The duration of AF after delivering the DC shock in the delayed group can be long enough to make the treating physician intend another DC shock but according to our findings it is advisable to wait even for 1-2 minutes for the AF to revert into SR before considering delivering another DC shock.The successful dose of DC shock to revert AF into SR in the two groups was not significantly different. The recurrence rate of atrial fibrillation in the IG was almost similar to that in the DG, a finding that does not help to predict recurrence.

Limitations: The number of patients is relatively small.

Conclusion:
The slow VR before DC shock is the main predictor for delayed conversion of atrial fibrillation by DC shock. Another predictor of delayed conversion is the appearance of APCs after DC shock delivery. In clinical practice delayed reversion should be carefully observed before delivering another DC shock.

Authors' declaration:
We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in (Place where the research was conducted or samples collected and treated) according to the code number **(123 on (15.08.2018)**
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The manuscript should mention the contribution of each author to the research done:
Study conception & design: (Amar T. Al-Hamdi). Literature search: (All authors). Data acquisition: (All authors). Data analysis & interpretation: (All authors). Manuscript preparation: (Azad J. Ali & Becker S. Alzand). Manuscript editing & review: (All authors).

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ماذا يحكم إرجاع فرفرة أذين القلب إلى النبض الطبيعي المباشر أو المتأخر؟

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الخلاصة

الخلفية: إن إرجاع النبض الطبيعي بواسطة الرجة الكهربية في فرفرة أذين القلب قد يكون مباشرا أو متأخرا.

الهدف: بحث خصائص كل من الإرجاع المباشر أو المتأخر لفرفرة أذين القلب.

المرضى والمنهجية: أجريت الدراسة في مركز الحسني لأمراض القلب في الفترة من تشرين الأول 2018 الى شباط 2022. أدرج في هذه الدراسة مرضى فرفرة أذين القلب المستمر الذين أرجعوا الى النبض الطبيعي بواسطة الرجة الكهربية. أظهرت مجموعة منهم إرجاعا مباشرا وأظهرت الأخرى إرجاعا متأخرا بعد عمل الرجة. درست الظواهر التالية: فترة فرفرة أذين القلب، مدى سرعة البطين قبل الإرجاع، العلاج السابق للرجة، وزن المرضى، وحجم الأذين الأيسر في كلا المجموعتين اعلاه.

النتائج: من المجموع الكلي ل 86 مريضا لفرفرة أذين القلب المستمرة عرضوا للرجة الكهربية، أرجع 77 مريضا (89%) إلى النبض الطبيعي وأدرجوا في هذه الدراسة. أرجع خمسون مريضا مباشرة و 27 أرجعوا متاخرا. كان معدل سرعة البطين أسرع في المجموعة المباشرة 138± 22 مقارنة ب 75±18 في المجموعة المتأخرة. ان ظهور ضربات أذينية هاجرة بعد الرجة كان أكثر في المجموعة المتأخرة. لم يكن تأثير ألعلاج الدوائي المسبق ذو أهمية في كلا المجموعتين. كانت فترة اللانضمية أقصر في المجموعة المباشرة.

الإستنتاج: إن الإرجاع المتأخر أو المباشر لفرفرة أذين القلب إلى النسق الطبيعي بواسطة الرجة الكهربية يحكم بسرعة البطين السابقة للإرجاع وظهور ضربات أذينية هاجرة بعد الرجة الكهربية .

الكلمات المفتاحية: إرجاع فرفرة؛ أذين القلب المباشر؛ المتأخر.

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Review Article

From Global Insights to National Impact: Advancing Cardio-Oncology in Iraq

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Introduction

Cancer remains a leading cause of mortality and morbidity worldwide. Advances in cancer therapies—including immunotherapies (e.g., checkpoint inhibitors, gene-targeted therapies), antibody-based cancer toxins, chemotherapy, radiotherapy, and surgery—have significantly improved survival rates (1, 2). However, this progress has led to a surge in the prevalence of cardiovascular disease (CVD) among cancer survivors, now recognized as a leading cause of mortality in this population (3, 4). These intersecting burdens highlight the growing need to prevent, detect, and manage cardiovascular complications in cancer care pathways and call for important initiatives in establishing Cardio-oncology services globally (5, 6).

Cardio-oncology (CO), a multidisciplinary specialty, focuses on optimising cardiovascular risk stratification, prevention, and treatment among cancer survivors (7). The field has addressed the cardiotoxicity associated with cancer treatments (see Table 1), such as heart failure, arrhythmias, and coronary artery disease. Baseline risk assessment and early identification of high-risk patients are central to mitigating treatment-related cardiac complications and ensuring uninterrupted cancer care in this population.

Table 1: Summary of cardiovascular effects of commonly used cancer therapies (8,9)

Drug	Cardiovascular effect
Anthracyclines (e.g., Doxorubicin)	Induces DNA damage in cardiomyocytes and vascular endothelium leading to cardiotoxicity, cardiomyopathy and heart failure.
HER2 Inhibitors (e.g., Trastuzumab)	Interferes with myocardial survival pathways, with enhanced cardiotoxicity when combined with anthracyclines.
VEGF Inhibitors (e.g., Bevacizumab)	Impairs vascular remodeling and increases vascular resistance.
Immune Checkpoint Inhibitors (e.g., Nivolumab)	Causes myocarditis, arrhythmias, vasculitis, and pericardial diseases.
Tyrosine Kinase Inhibitors (e.g., Imatinib)	Causes QT prolongation, ischemic heart disease, and pulmonary hypertension.
Anti-Microtubule Agents (e.g., Paclitaxel)	Enhances cardiotoxicity when combined with trastuzumab, but if used alone then it has low cardiac risk.
Platinum-based therapy (e.g., Cisplatin)	Increases risk of ischemic heart disease, myocarditis and arterial thrombosis.
Antimetabolites (e.g., 5-FU)	Causes coronary artery spasm, leading to angina and myocardial infarction.
Radiotherapy	Causes vascular damage and fibrosis resulting in long-term complications include coronary artery disease, valvular disease, and pericardial disease.

Recent advancements in the field, coupled with the development of specialist CO guidelines, have provided structured protocols to manage therapy-related cardiotoxicities and improve long-term outcomes for cancer survivors. The relevance of this subspeciality extends globally and locally, where the dual burden of cancer and CVD continues to pose significant challenges for healthcare systems.

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Global trends in cardio-oncology:

The Cardio-Oncology (CO) field emerged in the 1960s when anthracyclines were first used to treat cancers and saw its first dedicated service at MD Anderson, USA, in 2005. Over the last few years, numerous dedicated centers providing CO services were established in the USA, Europe, some Asian countries while low- and middle-income countries (LMICs) still lack such centers and services due to financial constraints and infrastructure constraints (5,7, 10).

In an important step to translate the accumulating science from the CO experiences into clinical application, the 2022 ESC clinical practice guidelines on CO emerged as an important tool to define the cardiotoxicities of anticancer therapies, and to minimize the potential cardiovascular complications of different cancer therapies in a huge effort to improve survival and quality of life of cancer survivors (10, 11). However, these guidelines highlighted the main knowledge gaps in the field of CO including but not limited to; the role of CO services, dedicated clinics and networks on the prognosis of patients with cancers and survivors, it also raised the importance of exploring the role of different healthcare providers and heart teams including clinical pharmacists in CO services and their impacts on prognosis (10). These knowledge gaps represent future opportunities for scientists and clinicians interested in the field of CO.

Now, CO represents more than only the field of preventing and managing cardiovascular effects of cancer therapies, but there is increasing evidences that both cancer and CVD have the same stems like smoking, dyslipidemia, obesity, diabetes and genetic backgrounds (12), thus, CO is now considered a novel platform for translational scientists, clinicians and researchers to answer many research questions and build new hypotheses in this innovative field.

The Role of Big Data in Cardio-Oncology Research

A significant challenge in global CO (CO) guidelines is the reliance on Level C evidence, which primarily based on expert opinion. This limitation is further compounded by the exclusion of cancer patients from cardiology trials and cardiovascular patients from oncology trials (13, 14), resulting in a significant lack of real-world evidence to inform clinical management. Big data from national administrative databases and registries can address these gaps, elevate evidence in CO to Level B, and ultimately improve clinical decision-making in this growing field.

In the United Kingdom (UK), the Keele Cardiovascular Research Group has led CO research using big data. By employing datasets obtained from the National Institute for Cardiovascular Outcomes Research (NICOR) in the UK, the group developed earlier this year the updated PRECISE-DAPT cancer score to enhance bleeding risk stratification for cancer survivors undergoing percutaneous coronary intervention (PCI), offering a tailored approach to managing the delicate balance between thrombotic and bleeding risks in this vulnerable population (15). Beyond this, the group has contributed to advancing the level of evidence in CO literature through a range of other impactful studies over the last several years (16–22).

National status of Cardio-Oncology field in Iraq:

In 2019, the Iraqi Cardio-Oncology Programme (ICOP) was founded by a senior consultant cardiologist and his mentee; the cardiology clinical pharmacist, they initiated the first CO services that were very crucial to provide during the very critical time of COVID-19 era (23). This service was founded first at Medical City hospitals (Al-Amal Oncology Centre and Baghdad Oncology Hospital), in Baghdad. Since then, data from this Iraqi CO Clinic started to be published (23–26), such model provides an example to facilitate establishing CO clinics in other LMICs saving many trials and errors. In Iraq, other CO clinics were established in Babylon and Basrah, while two other CO clinics are under planning in Al-Anbar and Salahuddin. To date, the primary challenges for CO service to be integrated into routine care of cancer patients in Iraq are the limited awareness of healthcare providers and decision-makers of this important initiative, and shortage of trained specialists in this field. Lack of proper training can deprive cancer survivors from the simple and vital initial cardiovascular risk stratification before proceeding with their chemotherapy protocol. Lack of multidisciplinary team approach and limited access for regular follow up are other barriers in the field of CO in Iraq, not to mention the lack of infrastructure including high-cost diagnostic tools like cardiac imaging and biomarkers. Finally, as in other subspecialities of cardiology, there is lack of uniform global guidelines tailored to low-resource settings which further exacerbates the issue.

Recommendations and Future Directions:

-Cardio-Oncology Clinics: Proper infrastructure is needed to establish CO clinics. Streamlining these services within already established oncology centers will be a pragmatic and feasible approach. Multidisciplinary team is the cornerstone for CO clinics, so, cardiologists, oncologists, hematologists, clinical pharmacists, nurses, dieticians and psychosocial workers can collaborate to make decisions regarding risk stratification and management of CO patients. When referring cancer patients for CO services, a structured and comprehensive approach is required to assess both their cardiovascular risk and the potential impact of cancer treatments on cardiovascular system. The standard of care should include baseline assessments before starting potentially cardiotoxic therapies, regular monitoring during therapy, and long-term follow-up care after completing the cancer therapies.

-Training in Cardio-Oncology: Training in CO needs to combine the expertise of both cardiology and oncology to address the cardiovascular needs of cancer survivors. CO training requires in-depth understanding of the impacts of cancer therapies on cardiovascular system as well as the ability to provide both preventive measures and therapeutic interventions. A structured postgraduate training program in CO should provide a comprehensive foundational education, hands-on

clinical experience, and advanced training and interdisciplinary collaboration (27). This ensures that the CO-trained providers are fully equipped to manage the complex cardiovascular needs of cancer survivors, both during treatment and in long-term survivorship. Collaboration with international societies like International Cardio-Oncology Society (ICOS) can also help in building a global network of specialists in this field.

Research infrastructure: Establishing proper research infrastructure is vital in CO to identify potential ways to prevent, diagnose, and treat cardiovascular complications of different cancer therapies. CO is still an emerging field in Iraq with many gaps in knowledge and evidence in the time that the cancer survivors keep increasing. Establishing electronic health records and data registries is essential to improve observational research output in this field to explore the long-term effects of cancer therapies, predictors of these effects and to investigate the potential preventive and therapeutic interventions.

Equally important is working on funding and establishing dedicated research groups within universities. These initiatives would expand knowledge, inform national policy decisions, and guide clinical practices in CO with evidence-based approaches, ultimately enhancing the quality of care for cancer survivors across the country, see Figure 1.

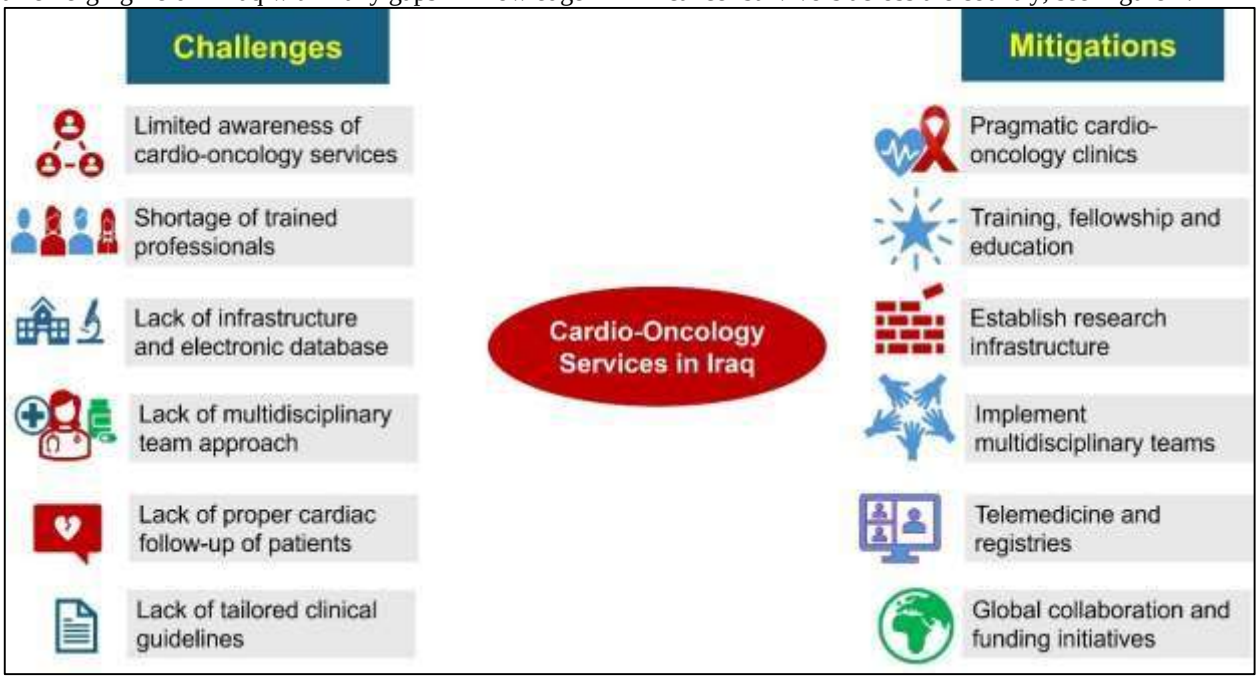

Figure 1: Main challenges for establishing cardio-oncology services in Iraq and suggested mitigations

Conclusion:

Developing cardio-oncology services in Iraq presents a unique opportunity to improve outcomes for cancer survivors with cardiovascular risks. While challenges such as limited awareness, inadequate infrastructure, and training gaps persist, prioritising the establishment of multidisciplinary clinics, bespoke training programmes, and research infrastructure can help overcome these barriers. This effort could also position Iraq as a regional leader in advancing cardio-oncology, ultimately improving survivorship and the quality of care for cancer population.

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Letter to the Editor

Prostate cancer screening; is it recommended in 2024?

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As we are living the month of Men's Health awareness, November, we thought of touching base regarding screening for prostate cancer; an intriguing topic indeed, and the jury is out as of yet regarding whether to formalize a screening platform for prostate cancer. The fact remains that prostate cancer is one of the most common cancers in men and the fifth leading cancer-related death globally (1).

However, it remains to be determined whether mass screening is recommended for such an important health issue.

Over the past few decades, the pendulum has swung with many trials attempting to come up with the sensible conclusion as to whether screening is recommended for prostate cancer.

For screening for any medical condition to be effective according to Wilson and Jungner criteria, the condition has to constitute an important health problem with no natural history. Also, it has to have a recognizable latent or early symptomatic stage (2).

More importantly, there has to be an easy, reliable, and acceptable test for the screening along with an acceptable treatment. Speaking of which, the treatment has to be effective, especially if commenced early.

When it comes to prostate cancer, it ticks most of the boxes regarding this criteria, especially when it comes to knowing the natural history of the disease.

We know for a fact that prostate cancer has a wide spectrum of pathology ranging from the low risk that in many cases does not require any intervention, and active surveillance or watchful waiting would be the way to go. On the other end of the spectrum, there are the aggressive cancers of the prostate that require immediate attention.

If we look into the incidence of prostate cancer per hundred thousand males all around the world, the United States of America will be at the top of the list with the highest incidence and very low mortality. If we compare that with another country like Zimbabwe, the observer would see that the incidence is a quarter of that in the United States; however, the mortality is slightly higher. In other words, almost every single man diagnosed in Zimbabwe with prostate cancer will eventually succumb to the disease (3).

There are two possible conclusions to draw from this observation: either the healthcare system in the United States is so brilliant that prostate cancer cases are diagnosed very early and mortality is low! The other explanation, which is probably more plausible, is the over diagnosis of prostate cancer.

So what if there was an over diagnosis of cancer cases of the prostate? An audit carried out in Belfast City Hospital a few years back showed that of 470 low-risk prostate cancer patients on active surveillance, 17% decided to go for intervention simply because of anxiety (unpublished series).

Therefore, in order to draw a conclusion about whether or not the screen for prostate cancer, it would be worthwhile looking into two famous prospective randomized controlled trials.

Let's take the American one first: Prostate, Lung, Colorectal, Ovarian Trials (PLCO): This trial recruited 77,000 men aged between 55 and 74 years. Those were equally divided into two groups of 38,500 men in each. Men in the control arm were to be tested at the start and end of the trial. The screening arm men would be involved in an annual PSA and digital rectal examination (4).

At the end of 10 years, followed by secondary analysis at 13 years, the prostate cancer incidence was 4250 men in the screening arm as opposed to 3815 men in the control arm, with a relative risk of increase of detection of only 12%. Therefore, the trial concluded that screening is not important for prostate cancer.

However, a major drawback of this trial was the significant contamination of around 50% of men in the control group jumping across and into the screaming arm to get their PSA checked! This has contributed to the under powering of the trial.

At about the same time, the European Randomized Study of Screening for Prostate Cancer (ERSPC) recruited 182,000 men aged between 50 and 74 years, equally divided into two groups containing 91,000 men each.

The screening arm subjects were offered digital rectal examination and PSA every four years. At the end of

the first analysis at nine years, the cancer incidence in the screening arm was 8.2% as opposed to 4.8% in the control arm.

This has shown 20% less mortality with screening. However, it also concluded that in order to save one life, around 1500 men would have to be screened, and 48 would have to be treated. These results did not support screening as a justifiable tool for prostate cancer prevention (5).

However, the Europeans persevered with collecting data from the ERSPC recruits, and with time, the numbers needed to screen dropped to 979 men at 11 years, then 781 men at 13 years, eventually dropping to 570 men at 16 years after the initial trial. Similarly, the numbers needed to treat dropped from 48 men at 9 years to 35 men at 11 years, then 27 men at 13 years, and eventually 18 men at 16 years.

Despite the fact of comparing different pathologies, if we compare that to other cancers, we will see that the numbers needed to screen are much higher in cervical cancer (n = 2250 women), colorectal cancer (n = 1250 people), and breast cancer (n = 465 women).

With these updated numbers, prostate cancer screening might be justified in certain circumstances. Therefore, I would conclude this editorial by saying that for mass prostate cancer screening, over-detection still weighs marginally against the benefit. However, targeting high-risk populations would undoubtedly increase the benefits of screening.

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