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Address: Maslak Mah. AOS 55. Sok. No: 2, 42 Maslak A Blok Daire: 231 Sarıyer, İstanbul-Türkiye
Phone: +90 212 241 26 53
Fax: +90 212 241 26 54
e-mail: www.kbud.org.tr
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- Technical Reports Tschantz BA, Moran B. Modeling of the hydrologic transport of mercury in the Upper East Fork Poplar Creek (UEFPC) watershed. Technical Report for Lockheed Martin Energy Systems: Bethesda, MD, September 2004.
- Supplement Castelli WP. Lipids, risk factors and ischaemic heart disease. Atherosclerosis 1996;124 Suppl:S1-9.
- Epub ahead of print Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. [Epub ahead of print] Clin Chem February 6, 2009 as doi:10.1373/clinchem.2008.113035.
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Research Article

Serum ceramide and meteorin-like protein as potential biomarkers of type 2 diabetes mellitus

Hussein Saeed Sfayyih[1], Abdulkareem Mohammed Jewad[1], Hasan Abd Ali Khudhair[2]

[1]College of Health and Medical Techniques/Al-Basrah, Southern Technical University, Ministry of Higher Education and Scientific Research, Basra, Iraq

[2]Al-Nasiriyah Technical Institute, Southern Technical University, Ministry of Higher Education and Scientific Research, Basra, Iraq

Abstract

Objectives: The recent research aims to detect ceramide and meteorin-like proteins as potential markers for identifying type 2 diabetes and monitoring its progression.

Methods: A cross-sectional study included three groups: type 2 diabetes without hypertension, type 2 diabetes with hypertension, and healthy control groups. Serum ceramide, meteorin-like protein, insulin, fasting blood glucose, lipid profile, and hemoglobin A1c levels were measured.

Results: Higher concentrations of ceramide, fasting blood glucose, hemoglobin A1c, and the homeostatic model assessment of insulin resistance were observed in both type 2 diabetes groups compared to the healthy control group. In the type 2 diabetes group with hypertension, total cholesterol was elevated compared to the other study groups; however, the concentration of low/very-low-density lipoprotein was statistically higher than in the healthy control group. Serum meteorin-like protein was statistically lower in the type 2 diabetes group with hypertension than in the other study groups and positively correlated with fasting blood glucose in type 2 diabetes with hypertension. The ceramide level showed a significant positive correlation with meteorin-like protein across all study groups and with systolic blood pressure in the type 2 diabetes group with hypertension. In type 2 diabetes without hypertension, ceramide negatively correlated with the homeostatic model assessment of insulin resistance and fasting blood glucose.

Conclusion: Elevated ceramide levels could accelerate type 2 diabetes progression. Meteorin-like protein levels were lower in type 2 diabetes with hypertension and higher in type 2 diabetes without hypertension. It positively correlated with fasting blood glucose in type 2 diabetes with hypertension, suggesting that meteorin-like protein may play a potential role in glycemic and blood pressure control.

Keywords: Ceramide, hypertension, IR, meteorin-like protein, T2D

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Type 2 diabetes (T2D) is a chronic, multisystem disorder and a major global public health issue that steadily declines the quality of life. In the twenty-first century, T2D has become one of the most significant epidemics due to its continually rising incidence. T2D patients often remain untreated for years because of the lack of early severe symptoms and the gradual progression of the disease, which lacks typical hyperglycemia manifestations, such as weight loss and dehydration [1]. Hypertension and dyslipidemia are some of the most common cardiovascular disease (CVD) risk factors [2]. An important area in clinical and public health research is understanding the correlation between various phases of glucose intolerance and serum lipid patterns, which has the potential to inform future prevention strategies for diabetes mellitus (DM) and related outcomes.

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Address for correspondence: Hasan Abd Ali Khudhair, MD. Al-Nasiriyah Technical Institute, Southern Technical University, Ministry of Higher Education and Scientific Research, Basra, Iraq
Phone: 9647821711810 **E-mail:** hasanabdali89@stu.edu.iq **ORCID:** 0000-0002-2612-615X
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Fasting blood glucose (FBG) and hemoglobin A1c (HbA1c) tests are unable to accurately guide the best possible treatment for T2D, as they fail to account sufficiently for variations in diabetes causes, insulin resistance, and insulin production. These tests are only useful post-disease manifestations [3]. Therefore, it is crucial to find biomarkers for rapid and precise DM outcome detection and to develop more accurate non-invasive markers that may be used for T2D diagnosis across various disease stages.

Ceramides (Cer) are involved in numerous pathophysiological processes, including cancer, inflammation, neurodegenerative diseases, obesity, diabetes, and CVD. Ceramides exhibit pro-inflammatory and pro-apoptotic properties, and their levels in the bloodstream are positively associated with age. However, the impact of ceramide alterations on T2D remains uncertain. Previous research suggests that ceramides play a role in T2D development by decreasing insulin production, disrupting insulin signaling, and impairing glucose transporter activity [4]. A better understanding of ceramide levels and T2D risk could direct the development of new pharmacologic treatments for both primary and secondary prevention of T2D.

Meteorin-like protein (Metrnl) has a diverse impact on metabolism, immunology, and inflammation. Various physiological activities, such as exercise, temperature changes, bariatric surgery, and high-fat diets, may also affect Metrnl levels. Studies report differing findings about Metrnl levels in patients; one study observed reduced Metrnl in diabetics [5], while another found elevated levels in diabetic patients [6]. Meteorin-like protein attracts eosinophils into adipose tissue, which is a primary producer of interleukin (IL)-13 and IL-4, which activate thermogenic genes. This suggests that Metrnl may regulate temperature and energy consumption [7]. Thus, Metrnl is a compelling subject to examine in the context of metabolic conditions. The current research aims to detect lipid abnormalities in T2D subjects and to examine the relationship between various serum lipid patterns and T2D, as well as address knowledge gaps regarding the profiles of ceramide and Metrnl in T2D patients. The hope is that these observations will translate into new screening assays and therapies to alleviate and potentially prevent or cure T2D.

Materials and Methods

1. **Subjects/study design:** Between August 2023 and December 2023, cross-sectional research was conducted at the Al-Nasiriyah Teaching Hospital in Thi-Qar governorate, Iraq. Three study groups were enrolled in this research (15 males and 15 females for each group): the T2D without hypertension group (first group), which consisted of 30 T2D patients without hypertension aged 34 to 56 years; the T2D with hypertension group (second group), which included 30 T2D patients with hypertension aged 34–56 years; and the healthy control (HC) group (third group), comprising 30 subjects aged 35 to 55 years.

   Patients in the first and second groups had a confirmed T2D diagnosis from the Al-Nasiriyah Teaching Hospital. Exclusion criteria for this study included patients with recent blood transfusion, cancer, chronic or autoimmune diseases, pregnant or lactating mothers, patients under corticosteroid or biological therapy, recent surgery, or any diabetes complications. Eligible patients had to fast for at least 12 hours and meet none of the above criteria. Additionally, all T2D patients were diagnosed within the past six months, ensuring that case group participants were relatively newly diagnosed to allow for a more consistent comparison of early disease biomarkers and progression.

   Eligibility criteria for the HC group included no family history of DM, non-smoking status, non-pregnancy or lactation (for females), absence of inflammation or infection, no chronic or autoimmune diseases, no recent blood transfusions or surgeries, and no use of biological agents.

   The present study received full approval from the Ethics Committee of the Training and Human Development Unit, Thi-Qar Health Department, Ministry of Health, Iraq. This approval was granted under committee number 153/2023 on July 27, 2023, in line with the Helsinki Declaration, and informed consent was obtained from each participant to meet international research ethical standards.

2. **Blood pressure determination:** Blood pressure (BP) was measured using a digital patient monitor (UTAS Technologies, Slovakia). Normal systolic blood pressure (SBP) was defined as <140 mmHg, and normal diastolic blood pressure (DBP) was <90 mmHg [8].

3. **Sample collection:** Three to five milliliters (mL) of peripheral blood (PB) were collected from each subject after 12 hours of fasting. Two mL of collected PB was used for the HbA1c test, while the remaining PB was used for serum separation. Collected sera were stored at -80 degrees Celsius if not used immediately for subsequent analysis.

4. **Biochemical assays:** Quantitative determination of HbA1c in PB and FBG in serum was performed *in vitro* using the Roche/Cobas c111 system (Roche, Germany) with HbA1c and glucose kits (Roche, Germany). Findings were expressed as percentages (%) for HbA1c and in milligrams (mg) per deciliter (dL) for FBG.

   Human insulin was detected and quantified in micro-international units (µIU)/mL using an insulin enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, USA) based on sandwich-ELISA technology. The homeostatic model assessment (HOMA)-IR was determined using the following formula:

HOMA-IR=Fasting insulin (µIU)×FBG (mg/dl)/405 [9]

Serum TG, total cholesterol (TC), and high-density lipoprotein (HDL) were detected and titrated in mg/dl by Roche/Cobas c111 devices (Roche, Germany) using TG, cholesterol, and HDL kits, respectively (Roche, Germany). Serum very low-density lipoprotein (vLDL) and low-density lipoprotein (LDL) were determined by the formulas number (1) and number (2), respectively.

VLDL (mg/dl)=TG/5 (1) [10]

LDL (mg/dl)=TG–(HDL + VLDL (2) [11]

Serum cer and metrnl detection

Serum ceramide (Cer) and meteorin-like protein (Metrnl) levels were detected in nanograms (ng)/mL using Cer and Metrnl ELISA kits (Bioassay Technology Laboratory, China), based on sandwich-ELISA technology. The kits have sensitivities of 0.023 ng/mL for Metrnl and 0.62 ng/mL for Cer, allowing detection at very low levels. The detection range for Metrnl is 0.05–15 ng/mL and for Cer is 1–400 ng/mL, which defines the range within which the assay can accurately measure these biomolecules. Cer and Metrnl levels were categorized as follows: below-normal (<40 ng/mL and <1 ng/mL), normal (40–70 ng/mL and 1–3 ng/mL), and above-normal (>70 ng/mL and >3 ng/mL), respectively.

For all the above-mentioned assays, the test protocol was followed according to the manufacturer's instructions, and all scoring values were based on levels observed in the HC group.

Statistical analysis

For data presentation and analysis, the Statistical Package for Social Sciences version 22 was used. Frequencies, relative frequencies, means, and standard deviation (SD) were calculated as descriptive statistics. The Chi-Square test was employed to compare categorical parameters, while the significance of differences in continuous variables with a non-normal distribution was evaluated using the Kruskal-Wallis test. Multiple comparisons within groups were tested using the Dunn test as a post hoc test. Spearman's correlation (r) was utilized to assess correlations between variables. Statistical significance was determined when the p-value was below 0.05.

Results

The findings revealed a significant elevation in age, LDL, and vLDL mean values in T2D patients with hypertension (48.2±5.13 years, 168.6±67.17 mg/dL, and 50.8±32.65 mg/dL, respectively) compared to the HC group (42.7±6.13 years, 103.5±43.45 mg/dL, and 26.7±12.27 mg/dL, respectively). The levels of FBG, HbA1c, HOMA-IR, and TG were significantly higher in both T2D without hypertension (223.9±107.67 mg/dL, 8.5%±1.87, 8.17±6.23, and 189.2±97.32 mg/dL, respectively) and T2D with hypertension (236.4±129.82 mg/dL, 8.4%±1.62, 12.50±9.75, and 254.3±163.3 mg/dL, respectively) compared to the HC group (98.47±13.24 mg/dL, 5.4%±0.57, 2.85±2.63, and 133.9±61.36 mg/dL, respectively). For TC, there was a significantly higher level in T2D with hypertension (208.8±42.81 mg/dL) compared to T2D without hypertension (184.9±34.09 mg/dL) and the HC group (162.1±34.77 mg/dL). Mean SBP in T2D patients with hypertension was significantly higher (140.9±15.34 mmHg) compared to T2D patients without hypertension (123.06±7.13 mmHg) and controls (118.4±5.26 mmHg), with P-values of <0.001 for both. Similarly, mean DBP was elevated in T2D patients with hypertension (89.6±8.55 mmHg) compared to T2D patients without hypertension (83.1±6.30 mmHg) and the HC group (81.3±5.19 mmHg), with significant P-values of 0.001 for both. All other comparisons revealed non-significant differences (Table 1).

Table 2 demonstrated that T2D without hypertension and T2D with hypertension groups had significantly higher percentages of above-normal Cer levels (53.3% and 40%, respectively) compared to the HC group (20%). However, there was no statistical difference between T2D without hypertension and T2D with hypertension. The mean Cer level was highest in T2D without hypertension (66.99±14.4 ng/mL) and in T2D with hypertension (61.66±20.69 ng/mL) compared to the control group (45.41±13.9 ng/mL), with a significant difference observed. No significant difference was found in mean Cer levels between T2D without hypertension and T2D with hypertension.

The percentage of Metrnl below-normal levels was significantly lower in T2D without hypertension (20%) compared to T2D with hypertension (60%) and the HC group (56.7%). No significant difference was observed in Metrnl levels between T2D with hypertension and the HC group. The mean Metrnl titer was significantly higher in T2D without hypertension (1.71±0.86 ng/mL) than in T2D with hypertension (1.12±0.96 ng/mL) and the HC group (1.55±1.80 ng/mL), and it was statistically elevated in the HC group compared to T2D with hypertension (Table 3).

The percentage of Metrnl below-normal levels was significantly higher in T2D without hypertension, T2D with hypertension, and HC subjects with below-normal Cer levels (50%, 100%, and 77.8%, respectively) compared to those with above-normal Cer levels (18.75%, 50%, and 0%, respectively). Metrnl concentration was statistically significant in T2D with hypertension and HC subjects with above-normal Cer levels (1.27 ng/mL and 3.70 ng/mL, respectively) compared to those with below-normal Cer levels (0.62 ng/mL and 0.76 ng/mL, respectively). However, no significant differences were observed in mean Metrnl levels for T2D without hypertension individuals with below-normal, normal, or above-normal Cer levels (Table 4).

A significant positive relationship was observed between Cer and SBP among T2D patients with hypertension. Additionally, a significant negative correlation was found between Cer, HOMA-IR, and FBG in T2D patients without hypertension. Among T2D patients with hypertension, Metrnl positively correlated with FBG, while in the HC group, it inversely correlated with FBG (Table 5). All other correlations were not significant, as shown in the Table.

Discussion

Individuals with T2D often exhibit insulin resistance (IR), impaired glucose tolerance, dyslipidemia, and hypertension [12]. These findings align with the current study, which showed elevated levels of TG, TC, HOMA-IR, LDL, and vLDL in both T2D groups compared to the HC group (Table 1). Hyperglycemia and elevated HbA1c levels negatively impact lipid profiles and increase the risk of CVD and dyslipidemia. Increased free fatty acids (FFA) stimulate triglyceride (TG) formation, leading to higher secretion of apolipoprotein B (ApoB) and LDL. Insulin typically promotes ApoB breakdown by activating phosphatidylinositol-3 kinase, but this

Table 1. Comparison of demographic and biochemical parameter between study groups

Variables	G1 (n=30) Mean±SD	G2 (n=30) Mean±SD	G3 (n=30) Mean±SD	p
Age (years)	45.7±6.88	48.2±5.13	42.7±6.13	G1/G2: 0.256 G2/G3: 0.002* G1/G3: 0.155
SBP (mmHg)	123.06±7.13	140.9±15.34	118.4±5.26	G1/G2: <0.001* G2/G3: 0.001* G1/G3: 0.06
DBP (mmHg)	83.1±6.30	89.6±8.55	81.3±5.19	G1/G2: 0.001* G2/G3: 0.001* G1/G3: 0.566
FBG (mg/dl)	223.9±107.67	236.4±129.82	98.47±13.24	G1/G2: 0.991 G2/G3: <0.001* G1/G3: <0.001*
HbA1c (%)	8.5±1.87	8.4±1.62	5.4±0.57	G1/G2: 0.969 G2/G3: <0.001* G1/G3: <0.001*
HOMA-IR	8.17±6.23	12.50±9.75	2.85±2.63	G1/G2: 0.292 G2/G3: <0.001* G1/G3: 0.001
TG (mg/dl)	189.2±97.32	254.3±163.3	133.9±61.36	G1/G2: 0.168 G2/G3: <0.001* G1/G3: 0.035*
TC (mg/dl)	184.9±34.09	208.8±42.81	162.1±34.77	G1×G2: 0.04* G2/G3: <0.001* G1/G3: 0.053
HDL (mg/dl)	86.5±21.91	91.04±16.28	85.3±19.03	G1/G2: 0.641 G2/G3: 0.486 G1/G3: 0.966
LDL (mg/dl)	136.1±48.04	168.6±67.17	103.5±43.45	G1/G2: 0.057 G2/G3: <0.001* G1/G3: 0.055
vLDL (mg/dl)	37.8±19.46	50.8±32.65	26.7±12.27	G1/G2: 0.132 G2/G3: <0.001* G1/G3: 0.073

The Dunn test is a post hoc test for multiple comparisons within groups, and the Kruskal-Wallis test is used to compare non-parametric continuous variables. *: Significant differences. G1: T2D without hypertension; G2: T2D with hypertension; G3: Control group; n: Number; SD: Standard deviation; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBG: Fasting blood glucose; HbA1c: Hemoglobin A1c; HOMA-IR: Homeostatic model assessment-insulin resistance; TG: Triglyceride; TC: Total cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; vLDL: Very low density lipoprotein; mmHg: Millimeters of mercury; mg: Milligram; dl: Deciliter.

process is impaired in IR conditions [13], possibly explaining the elevated TG levels under IR conditions.

The association between high blood pressure and dyslipidemia in T2D enhances cardiovascular and metabolic disease risk factors [12]. Consistent with these observations, the present research found a significantly higher level of TC in T2D with hypertension compared to T2D without hypertension (Table 1). A previous cross-sectional study [14] also reported significant differences in LDL levels among individuals diagnosed with T2D and hypertension. This investigation supports findings of increased TC levels in T2D patients with hypertension, indicating the presence of dyslipidemia.

Research has shown that as individuals with both hypertension and diabetes age, they face a higher risk of experiencing macrovascular and microvascular events [15]. The findings of this previous research support our current investigation, which shows an increasing trend in age among patients with both high BP and DM. The current study revealed significantly higher FBG levels and HbA1c percentages in both T2D groups compared to the HC group (Table 1). Consistent with recent findings, Khan et al. [16] observed a notable elevation in FBG and HbA1c levels in diabetic individuals. Previous studies have shown that individuals with T2D exhibit elevated levels of FBG, TC, LDL, and TG compared to control groups [17]. This study

Table 2. The result of ceramide in all study group

Biomarker/ groups	G1 (n=30)		G2 (n=30)		G3 (n=30)		Total (n=90)	
	n	%	n	%	n	%	n	%
Cer (ng/ml) FR (%)								
Below N (<40)	2	6.7	3	10	9	30	14	15.6
Normal (40–70)	12	40	15	50	15	50	42	46.7
Above N (>70)	16	53.3	12	40	6	20	34	37.8
Total	30	100	30	100	30	100	90	100
Mean±SD	66.99±14.40		61.66±20.69		45.41±13.90		58.02±16.33	
p-value	G1/G2: >0.05, G1/G3: <0.05*, G2/G3: <0.05*							

The Chi-square test is used to compare categorical variables, and the Kruskal-Wallis test is used to compare the mean of non-parametric continuous variables. *: Significant differences. G1: T2D without hypertension; G2: T2D with hypertension; G3: Control group; n: Number; Cer: Ceramide; ng: Nanogram; ml: Milliliter; FR: Frequency; N: Normal; SD: Standard deviation.

Table 3. The result of meteorin-like protein in all study group

Biomarker/ groups	G1 (n=30)		G2 (n=30)		G3 (n=30)		Total (n=90)	
	n	%	n	%	n	%	n	%
Metrnl (ng/ml) FR (%)								
Below N (<1)	6	20	18	60	17	56.7	41	45.6
Normal (1–3)	19	63.3	10	33.3	8	26.7	37	41.1
Above N (>3)	5	16.7	2	6.7	5	16.7	12	13.3
Total	30	100	30	100	30	100	90	100
Mean±SD	1.71±0.86		1.12±0.96		1.55±1.80		1.38±1.14	
p-value	G1/G2: <0.05*, G1/G3: <0.05*, G2/G3: <0.05*							

The Chi-square test is used to compare categorical variables, and the Kruskal-Wallis test is used to compare the mean of non-parametric continuous variables. *: Significant differences. G1: T2D without hypertension; G2: T2D with hypertension; G3: Control group; n: Number; Metrnl: Meteorin-like protein; ng: Nanogram; ml: Milliliter; FR: frequency; N: normal; SD: standard deviation.

confirms that diabetic patients, with or without hypertension, experience elevated FBG and HbA1c levels, highlighting the importance of these variables in assessing glycemic control.

The findings of this study showed no significant differences in HDL levels between the study groups (Table 1), consistent with other research [18] that reported no link between HDL concentrations and T2D occurrence.

The current study found that T2D patients with hypertension had significantly higher SBP and DBP compared to those without hypertension and the HC group (Table 1). This finding is consistent with recent research, such as a study by Rapsomaniki et al. [19], which demonstrated that hypertension is more severe and prevalent in T2D patients, contributing to a higher cardiovascular risk. Even normotensive T2D patients tend to have higher BP than healthy individuals, suggesting possible subclinical hypertension, as noted by Zaccardi et al. [20]. These findings highlight the importance of strict BP management in T2D patients to reduce the risk of complications, a point underscored in recent guidelines and reviews on the management of hypertension in diabetes by De Boer et al. [21].

Elevated levels of Cer in the bloodstream can result in IR and metabolic syndrome; however, disruption of Cer biosynthesis through drugs or genetics can promote glucose metabolism and improve insulin sensitivity [22]. Consistent with these findings, the current study showed a significantly higher Cer level among both T2D groups compared to the HC group (Table 2). Evidence suggests that several metabolic conditions, such as DM and CVD, can be ameliorated by inhibiting Cer formation and promoting Cer breakdown in humans [23]. These findings confirm that Cer could potentially serve as a specific T2D biomarker.

Meteorin-like protein (Metrnl) is a newly identified adipomyokine that may assist in managing metabolic and inflammatory diseases, such as T2D. Since its discovery, many researchers have investigated the link between blood concentrations of Metrnl and T2D, although findings have yet to reach a consensus [24]. Multiple studies on the role of serum Metrnl in T2D have report-

Table 4. The comparison between ceramide and meteorin-like protein levels

Biomarkers	Metrnl (ng/ml)								p
	Below N (<1)		Normal (1–3)		Above N (>3)		Total		
	FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	FR (%)	Mean	
Ceramide (ng/dml)									
Group 1									
Below N (n=2)	1 (50)	0.68	0 (0)	0	1 (50)	3.09	2 (100)	1.88	<0.05*
Normal (n=12)	2 (16.7)	0.71	9 (75)	1.62	1 (8.3)	3.19	12 (100)	1.60	
Above N (n=16)	3 (18.75)	0.85	10 (62.5)	1.55	3 (18.75)	3.45	16 (100)	1.77	
Total (n=30)	6 (20)	0.78	19 (63.3)	1.58	4 (16.7)	3.33	30 (100)	1.71	
Group 2									
Below N (n=3)	3 (100)	0.62	0 (0)	0	0 (0)	0(0)	3 (100)	0.62	<0.05*
Normal (n=15)	9 (60)	0.71	6 (40)	1.17	0 (0)	4.38	15 (100)	1.11	
Above N (n=12)	6 (50)	0.76	4 (33.3)	1.20	2 (16.7)	4.65	12 (100)	1.27	
Total (n=30)	18 (60)	0.71	10 (33.3)	1.2	2 (6.7)	4.51	30 (100)	1.12	
Group 3									
Below N (n=9)	7 (77.8)	0.48	2 (22.2)	1.73	0 (0)	0	9 (100)	0.76	<0.05*
Normal (n=15)	10 (66.7)	0.54	5 (33.3)	1.29	0 (0)	0	15 (100)	0.90	
Above N (n=6)	0 (0)	0	1 (16.7)	1.46	5 (83.3)	4.09	6 (100)	3.70	
Total (n=30)	17 (56.7)	0.51	8 (26.6)	1.42	5 (16.6)	4.09	30 (100)	1.55	

The Chi-square test is used to compare categorical variables, and the Kruskal-Wallis test is used to compare the mean of non-parametric continuous variables. *: Significant differences. Metrnl: Meteorin-like protein; N: Normal; FR: Frequency; ng: nanogram, ml: Milliliter; n: Number.

Table 5. Illustrates the Pearson correlation between ceramide, meteorin-like protein, and anthropometric parameters

Biomarkers/ groups	Ceramide (ng/ml)						Metrnl (ng/ml)					
	Group 1		Group 2		Group 3		Group 1		Group 2		Group 3	
	r	p	r	p	r	p	r	p	r	p	r	p
Age (year)	0.09	0.62	-0.02	0.90	0.15	0.42	-0.09	0.67	-0.14	0.46	0.08	0.69
SBP (mmHg)	0.21	0.26	0.49	0.01	-0.10	0.60	-0.14	0.50	0.14	0.45	0.09	0.64
DBP (mmHg)	0.22	0.24	0.34	0.07	-0.22	0.25	0.14	0.50	-0.07	0.70	-0.33	0.07
HbA1c (%)	-0.30	0.10	0.20	0.28	-0.02	0.91	0.35	0.08	-0.33	0.07	0.00	0.98
HOMA-IR	-0.35	0.04	-0.07	0.68	0.201	0.28	-0.004	0.98	0.21	0.25	-0.28	0.13
FBG (mg/dl)	-0.35	0.04	-0.08	0.66	-0.17	0.36	-0.26	0.15	0.36	0.04	-0.52	0.00
TG (mg/dl)	-0.09	0.63	-0.11	0.58	-0.14	0.48	0.08	0.71	0.03	0.87	-0.10	0.61
TC (mg/dl)	0.26	0.16	0.06	0.76	0.04	0.84	-0.08	0.69	-0.14	0.45	-0.26	0.17
HDL-c (mg/dl)	0.29	0.12	0.09	0.65	0.09	0.64	0.09	0.66	-0.08	0.69	0.01	0.97
LDL (mg/dl)	0.03	0.88	0.01	0.96	-0.06	0.77	-4.10	0.98	-0.09	0.63	-0.12	0.52
vLDL (mg/dl)	-0.09	0.63	-0.11	0.58	-0.14	0.48	0.08	0.71	0.03	0.87	-0.10	0.61

Spearman correlation is used for correlation between non-parametric continuous variables. Metrnl: Meteorin-like protein; ng: Nanogram; ml: Milliliter; r: Correlation coefficient; mg: Milligram; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HbA1c: Hemoglobin A1c; HOMA-IR: Homeostatic model assessment-insulin resistance; FBG: Fasting blood glucose; TG: Triglyceride; TC: Total cholesterol; HDL-c: High density lipoprotein cholesterol; LDL: Low density lipoprotein; vLDL: Very low density lipoprotein; dl: Deciliter; mmHg: Millimeter of mercury.

ed controversial results. In the current research, Metrnl levels were markedly increased in T2D subjects without hypertension compared to T2D patients with hypertension and the HC group. Additionally, Metrnl levels were statistically lower in T2D subjects with hypertension than in the HC group (Table 3). Similar to these findings, another study found that Metrnl levels in the bloodstream were inversely correlated with BP [6]. According to our data, we speculate that Metrnl is elevated in T2D without hypertension and decreased in T2D with hypertension; however, further clinical studies are required to verify this speculation.

Pancreatic beta (β)-cells produce Cer in response to lipotoxicity and hyperglycemia, and β-cell death can be prevented by inhibiting Cer synthesis. Individuals with high lipid accumulation in the liver have higher Cer levels than those with healthy livers [25]. This literature indicates that Cer has a crucial role in lipid-associated IR. Consistent with these observations, the current research found a significant positive correlation between Cer and Metrnl across all study groups (Table 4), indicating that a higher Cer level is associated with elevated lipid levels and higher Metrnl levels.

Metabolomics studies have shown varying relationships between Cer levels and HOMA-IR, ranging from positive to negative or no associations [26]. The current research reported a significant inverse relationship between Cer and HOMA-IR in T2D patients without hypertension (Table 5). Plasma Cer species exhibit a correlation with insulin sensitivity in small cross-sectional investigations involving fewer than 50 individuals [27]. Together, these data suggest that certain Cer species may have either deleterious or protective roles in DM and could offer future treatment targets. Several studies have evaluated the role of sphingolipids on phosphatidylinositol-3 kinase, phosphoinositide-dependent protein kinase-1, phosphoinositide, and glucose transporter-4 (GLUT4) [28, 29]. However, most researchers have not identified a direct role. The current research reported a significant inverse relationship between Cer and FBG in T2D patients without hypertension (Table 5). While cell culture experiments have shown that Cer limits GLUT4 translocation and suppresses insulin-induced glucose uptake [30], human research is limited, and the precise impact of sphingolipids on glucose homeostasis remains unclear. Population-based research has found correlations between various Cer species and insulin levels, but it is still uncertain if sphingolipids are related to subsequent biomarkers of metabolic disorders, such as glucose intolerance or the occurrence of diabetes itself [31].

Moreover, a statistically significant positive correlation was found between Cer concentrations and SBP in T2D subjects with hypertension (Table 5). By increasing oxidative stress in blood vessels, Cer may exacerbate hypertension, leading to impaired vasodilation and endothelial dysfunction [32]. Ceramides, suggested as potential biomarkers for hypertension, have been linked to inflammation and vascular dysfunction. An investigation by Vozella et al. [33] revealed a positive correlation between age and plasma Cer in 164 individuals (84 women). However, the current investigation did not find a clear correlation between age and serum Cer levels across study groups (Table 5). This discrepancy may stem from the previous study's focus on women only, whereas our study included both sexes. Additional factors, such as study quality, sample collection conditions, and measurement materials, may have contributed to this variation.

Al Khairi et al. [7] reported no significant link between Metrnl, FBG, HbA1c, and HOMA-IR in T2D subjects. Similarly, the current research found that Metrnl was positively correlated with FBG in T2D with hypertension and negatively correlated with FBG in the HC group (Table 5). An observational study also reported a significant elevation in serum Metrnl in newly diagnosed T2D, with a notable correlation with glucose profile and IR [34]. The elevated Metrnl concentrations in T2D patients may indicate a protective compensatory response to metabolic stress, including IR. Thus, a strong correlation may exist between circulating Metrnl and glucose regulation in the body. To our knowledge, this research is the first to establish the valuable roles of Cer and Metrnl as possible biomarkers for Iraqi patients with T2D.

Additionally, no significant relationship was reported between Cer and DBP, HbA1c, or lipid profiles in any study group (Table 5). This aligns with an observational cross-sectional study of 84 T2D participants and 75 controls, which reported no significant relationship between Cer and DBP, lipid profiles, or HbA1c in T2D patients [35]. Furthermore, there was no statistical relationship between Metrnl and other variables listed in Table 5 across all study groups. This is consistent with a cross-sectional study of 150 subjects, which found no significant correlation between Metrnl and biochemical parameters such as lipid profile, SBP, and DBP [36]. Another study on 80 T2D patients revealed no significant correlation between Metrnl and biochemical tests involving HbA1c, TG, TC, HDL, LDL, SBP, and DBP in T2D groups [37].

Table 5 also presents correlations between Metrnl concentrations and age, HbA1c, and HOMA-IR across three groups, with no significant correlations found in any group. The negative correlation between Metrnl and age in T2D groups aligns with findings by Wu et al. [38], while the lack of a significant correlation with HbA1c is in line with mixed results reported by Phuong et al. [39]. The negligible correlation between Metrnl and HOMA-IR in T2D without hypertension echoes findings from Paczkowska et al. [40]. These results suggest complex interactions between Metrnl, metabolic factors, and hypertension.

In the control group, the table indicates a positive correlation between Metrnl levels and age, though this correlation is not statistically significant. These findings align with previous research, such as a study by Raschke et al. [41], which reported no significant correlation between Metrnl and age in a healthy population. The control subjects also show no significant relationship between Metrnl and HbA1c. The correlation between Metrnl and HOMA-IR is a non-significant negative. These results are consistent with studies such as Ding et al. [42], which found no significant correlation between Metrnl and HOMA-IR in obese individuals.

This study has several limitations. First, the sample size was relatively small, and only Iraqi individuals were included; thus, results may not be generalizable to other ethnic groups. Additional confounding factors, particularly exercise, cannot be ruled out, as Cer and Metrnl levels may fluctuate with activity. Future studies with larger, more diverse populations should be conducted to address these aspects.

Conclusion

The current study found a significant increase in Cer levels, which could potentially accelerate T2D progression. Cer levels exhibited an inverse correlation with both FBG and HOMA-IR, while SBP and Metrnl showed a positive relationship with Cer. Relationships were observed between Cer, BP, and IR. Metrnl levels were lower in T2D patients with hypertension and higher in T2D without hypertension. The positive correlation between Metrnl and FBG in T2D patients with hypertension suggests that Metrnl may play a potential role in glycemic and BP control. Enhancing knowledge of Metrnl's role in DM development may aid in identifying potential therapeutic targets for managing DM and its complications.

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Ethics Committee Approval: The study was approved by The Ethics Committee of the Training and Human Development Unit, Thi-Qar Health Department, Ministry of Health, Iraq (No: 153/2023, Date: 27/07/2023).

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Research Article

Rheumatoid Factor and ASO assessment by immunoturbidimetry and immunonephelometry

Berrak Guven[1], Havva Buyukyavuz[1], Ishak Ozel Tekin[2]

[1]Department of Biochemistry, Zonguldak Bulent Ecevit University Faculty of Medicine, Zonguldak, Türkiye
[2]Department of Immunology, Zonguldak Bulent Ecevit University Faculty of Medicine, Zonguldak, Türkiye

Abstract

Objectives: In this study, ASO and RF immunoturbidimetric assays determined on the Roche Cobas analyzer were evaluated against an immunonephelometric method.

Methods: ASO and RF assays were performed with the immunonephelometric method using the Beckman Coulter Immage 800 analyzer and the immunoturbidimetric method using the Cobas c501 analyzer. Precision values of both assays were calculated using internal quality control (IQC) samples provided by the test manufacturers. In addition, to assess bias, IQC and external quality control (EQA) data were used. Method comparison studies were performed using serum specimens randomly selected from routine hospital orders.

Results: Both assays demonstrated good precision for ASO, with precision values of 3.2% CV in the immunoturbidimetric assay and 5.0% CV in the immunonephelometric assay. Although the immunoturbidimetric assay for RF showed good precision, the precision of RF exceeded the desired limits in the immunonephelometric assay. Bias obtained from EQA data was excellent in both ASO and RF for the immunoturbidimetric assay. The Passing–Bablok regression equation was obtained as y=1.65x - 20, r=0.98 for ASO, and as y=1.02x - 10.9, r=0.85 for RF.

Conclusion: In conclusion, ASO and RF tests on the Cobas analyzer are suitable for routine use because they meet the requirements for accuracy and precision. The imprecision of the RF assay should be improved, especially for the immunonephelometric assay.

Keywords: Antistreptolysin-O, nephelometry, rheumatoid factor, turbidimetry

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The utilization of specific protein tests to predict risks associated with infection and autoantibody presence has increased over the last few years. Antistreptolysin O (ASO) and rheumatoid factor (RF) tests are among the most requested tests. ASO antibodies are produced by the host after an infection with group A beta-hemolytic streptococcus. ASO is used as a serological marker to indicate a past infection, even though evidence of its usefulness is limited. ASO titers are frequently requested, especially in cases of acute tonsillitis in the pediatric population [1]. RF is an autoantibody directed against gamma globulins. Rheumatoid arthritis (RA) patients have high RF levels in serum [2]; its titer mostly correlates with disease severity and predicts a poor prognosis [3, 4].

Specific proteins are analyzed using specialized methods such as radial immunodiffusion, immunoelectrophoresis, ELISA, dedicated immunonephelometers, or immunoturbidimeters [5]. Nephelometry has traditionally been considered a reference method. The most frequently used methods for the routine measurement of serum ASO and RF are based on immunonephelometry or immunoturbidimetry. Turbidimetry and nephelometry are photometric assays commonly used to quantify immune-complex precipitates by their ability to interact with incident light [6]. A special analyzer is required for nephelometric measurements, whereas turbidimetric measurements can be easily performed on a clinical chemistry analyzer. Thus, clinical laboratories may opt to shift some analyses

Address for correspondence: Berrak Guven, MD. Department of Biochemistry, Zonguldak Bulent Ecevit University Faculty of Medicine, Zonguldak, Türkiye
Phone: +90 372 261 28 39 **E-mail:** berrak_guven@hotmail.com **ORCID:** 0000-0003-4073-3164
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to the clinical chemistry analyzer for cost savings. Some studies in the literature compare specific protein levels between turbidimetry and nephelometric methods [7, 8]. These studies compared the turbidimetric system of the Abbott Architect ci8200 with the nephelometric systems of Beckman Immage or Dade Behring. To the best of our knowledge, there is no comparative study in the literature with the turbidimetric assay of the Roche Cobas analyzer, so it is necessary to evaluate the performance of specific protein assays on the Cobas system.

In this study, we aim to evaluate the analytical performance of ASO and RF assays conducted on the Cobas analyzer and to compare them with nephelometric assays.

Materials and Methods

Analyzers and assays

Assays for ASO and RF were performed with the immunonephelometric method using the Beckman Coulter Immage 800 (Beckman Coulter Inc., USA) analyzer and the immunoturbidimetric method using the Cobas c501 (Roche Diagnostics GmbH, Mannheim, Germany) analyzer. The Cobas c501 module performs photometric assays, with a throughput of up to 1000 tests per hour for a combination of photometric and ion-selective electrode (ISE) tests. The Immage 800 is a nephelometer that performs approximately 180 tests per hour. Reagents, calibrators, and quality control (QC) materials were of the same origin as the instruments. The sample volume is 2 µL for ASO and 3 µL for RF in the Cobas system, while it is 3.5 µL for ASO and 5 µL for RF in the Immage system. The analytical ranges were 20–600 IU/mL (Cobas) and 25–800 IU/mL (Immage) for ASO, and 10–130 IU/mL (Cobas) and 20–800 IU/mL (Immage) for RF. The declared within-run imprecision for Cobas was below 2% for the ASO assay and below 1% for the RF assay, whereas for Immage, it was above 2% for both the ASO and RF assays.

Precision and bias

Precision study was performed according to the CLSI EP05-A3 protocol using the manufacturers internal quality control (IQC) (low and high levels) materials [6]. The cumulative coefficient of variation (CV%) measured on different days (20 days/month, twice a day), was calculated for both IQC levels, designated as CV1% (low-level) and CV2% (high-level). Total CV% was calculated as the following formula:

$$CV\% = \sqrt{(CV1^2 + CV2^2)}$$

Biases were calculated from the difference between the laboratory results and the target values of the IQC samples in the control inserts. To obtain bias of the turbidimetric method, the external quality assurance (EQA) program data were collected from BIORAD EQAS schemes (Monthly specific proteins) in 2023 year. Bias% values were calculated as following formula:

Bias% = Lab EQAS result- Peer group mean / Peer group mean

The desirable specifications for imprecision and bias were presented as 4.3% and 6.5% for RF test [10]. Hoewever, there were no desirable specifications in the literature for ASO test.

Method comparison

For comparison, fresh serum samples from patients whose ASO and RF levels were ordered in the routine laboratory were used. No additional samples were collected, no medical records were reviewed, and no contact with patients was made. For method comparison experiments, samples were analyzed on the two analyzers on the same day. Abnormal samples, such as those indicating hemolysis, icterus, or lipemia, were excluded.

Statistical analysis

The MedCalc for Windows statistical package (MedCalc Software, Ostend, Belgium) was used to perform method comparison. Method comparison results were analyzed using Passing-Bablok regression analysis and presented as y=bx+a. Intercept (a) and slope (b) values were considered significantly different from 0 and 1, respectively.

Results

The results of the precision and bias studies are summarized in Table 1.

We compared random patient samples, which included 99 for ASO and 61 for RF results. The patient sample-based method comparison data is presented in Figures 1 and 2. Strong correlations were determined between the turbidimetric and nephelometric methods for ASO and RF (r=0.977 and r=0.854, respectively). Passing-Bablok regression analysis gave a slope of 1.64 and an intercept of -20.0 for ASO, and a slope of 1.02 and an intercept of -10.9 for RF (Figs. 1, 2).

Discussion

Recently, growing test volumes for infection and autoantibody detection, along with the need for rapid turnaround times, have led to the increased use of turbidimetric analyzers rather than nephelometric analyzers. Turbidimetric measurements are easily performed on photometers or spectrophotometers and require minimal optimization [11]. In this study, we found that the imprecision of the ASO and RF assays on the Cobas analyzer was acceptable. Tur-

Table 1. Performances obtained IQC and EQA results of two methods

Test	CV1%	CV2%	Total CV%	Bias% (IQC)	Bias% (EQA)
ASO					
Turbidimetry	2.5	2.0	3.2	-3.6	-1.6
Nephelometry	3.8	3.3	5.0	-1.1	NA
RF					
Turbidimetry	2.0	1.5	2.5	1.4	-3.6
Nephelometry	5.6	3.9	7.3	1.6	NA

IQC: Internal quality control; EQA: External quality assurance; CV: Coefficient of variation; ASO: Antistreptolysin O; RF: Rheumatoid factor; NA: Not available

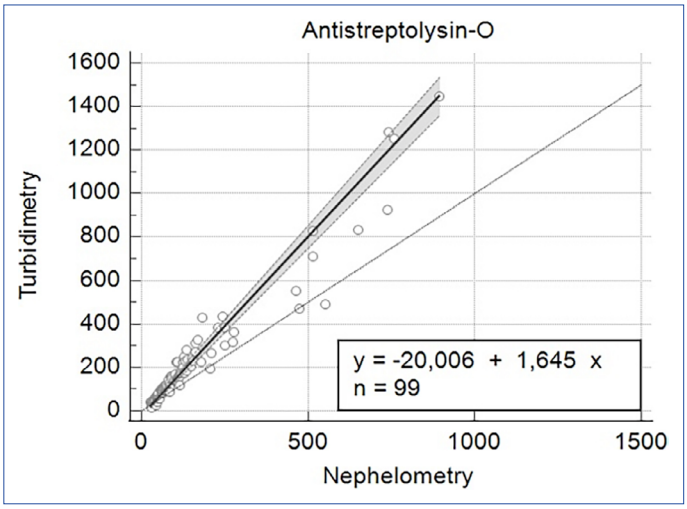

Figure 1. Comparison of ASO results using regression analysis.
ASO: Antistreptolysin O.

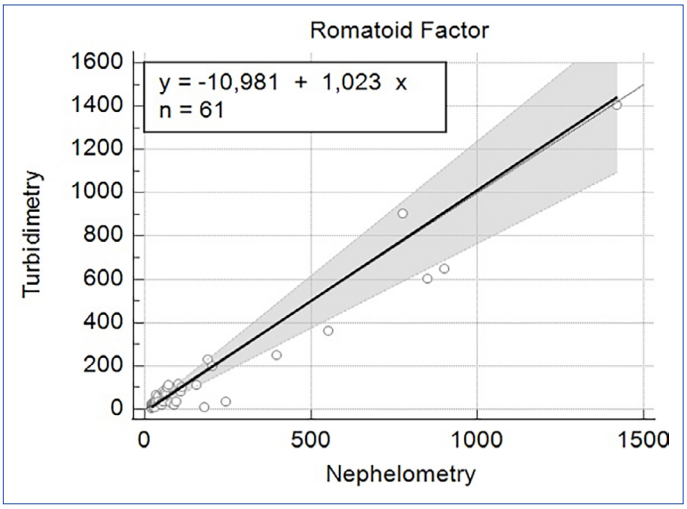

Figure 2. Comparison of RF results using regression analysis.
RF: Rheumatoid factor.

bidimetry appeared to perform well in ASO and RF assays when also evaluated for accuracy. When comparing the two systems, imprecision was lower with the turbidimetry-based instrument than with nephelometry. Our results align with studies reporting that turbidimetric assays are rapid, automated, applicable, and more reproducible than nephelometric assays [12, 13]. However, considering the acceptability of analytical imprecision, RF nephelometric analysis fell outside the desirable range.

The ASO and RF turbidimetric tests compared well with the corresponding nephelometric assays, based on the observed correlation coefficients. Interestingly, although the method bias for ASO and RF was within acceptable ranges, the slope performance indicated a proportional bias for ASO and a constant bias for RF. The regression line slopes from method comparison studies were 1.65 for ASO and 1.02 for RF. Specifically, the slope for ASO was outside the acceptable range; however, this was not evaluated as unexpected, due to antibody specificity in immune measurements based on antigen-antibody complexes. For this reason, the reference ranges declared by the two systems for these tests may differ. The reference interval of the Immage analyzer for ASO is 25–116 IU/mL, while the reference interval of the Cobas analyzer is higher (20–150 IU/mL).

In this comparison, we initially accepted nephelometry as the reference method for serum ASO and RF determination. However, by the end of our study, we believe that this traditional acceptance in the measurement of specific proteins should be reconsidered. Some studies in the literature comparing these two methods already express doubt about which one is more accurate [14–16]. Therefore, we suggest that further improvement in the standardization of nephelometric methods is beneficial, specifically for the RF assay.

There are limitations to our study, as we did not investigate the effects of interference and different clinical conditions, including normal and pathological levels.

Conclusion

In conclusion, ASO and RF tests on the Cobas analyzer are suitable for routine use, as they meet the requirements for accuracy and precision. The imprecision of the RF assay should be improved, especially for the immunonephelometric assay.

Ethics Committee Approval: The study was approved by The Zonguldak Bulent Ecevit University Non-interventional Clinical Research Ethics Committee (No: 2023/09, Date: 03/05/2023).

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Research Article

Correlation between insulin resistance and serum irisin levels in polycystic ovary syndrome

Gulsan Karabay[1*], Umut Karabay[2], Durmus Ayan[3], Sebnem Cigerli[4], Ayse Ender Yumru[1]**

[1]Department of Gynecology and Obstetrics, University of Health Sciences, Sisli Hamidiye Etfal Training and Research Hospital, Istanbul, Türkiye
[2]Department of Internal Medicine, Gulhane Training and Research Hospital, Ankara, Türkiye
[3]Department of Medical Biochemistry, Nigde Omer Halisdemir University Faculty of Medicine, Nigde, Türkiye
[4]Department of Medical Biochemistry, University of Health Sciences, Sisli Hamidiye Etfal Training and Research Hospital, Istanbul, Türkiye

Abstract

Objectives: Polycystic Ovary Syndrome (PCOS) is a common endocrine disorder affecting women of reproductive age, often characterized by insulin resistance, hyperandrogenism, and metabolic disturbances. This study aimed to investigate the relationship between serum irisin levels, a myokine involved in energy regulation, and insulin resistance in women with PCOS.

Methods: A prospective study was conducted with 90 women diagnosed with PCOS, divided into two groups: 45 with insulin resistance and 45 without. Insulin resistance was evaluated using the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR). Serum irisin levels were measured using an Enzyme-Linked Immunosorbent Assay (ELISA). Statistical analyses, including correlation and regression tests, were used to assess the relationships between serum irisin levels and various metabolic and hormonal parameters.

Results: No significant difference in serum irisin levels was found between PCOS patients with insulin resistance (3.66±2.69 ng/mL) and those without insulin resistance (2.77±1.72 ng/mL) (p=0.065). Weak correlations were identified between serum irisin levels and insulin, HOMA-IR, free testosterone, and total testosterone levels. Significant positive correlations were observed with insulin (p<0.001) and HOMA-IR (p=0.008), while negative correlations were found with free testosterone (p=0.029) and total testosterone (p=0.013). Additionally, no significant differences in serum irisin levels were detected between patients with and without metabolic syndrome.

Conclusion: Although weak correlations between serum irisin levels and insulin resistance markers were observed, no significant difference was found between PCOS patients with and without insulin resistance. These findings suggest that serum irisin may not be a key factor in the pathophysiology of PCOS related to insulin resistance. Larger studies are needed to further explore the role of irisin in PCOS and its potential as a therapeutic target.

Keywords: Hyperandrogenism, insulin resistance, irisin, metabolic syndrome, Polycystic Ovary Syndrome (PCOS)

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Polycystic Ovary Syndrome (PCOS) is a complex endocrine illness affecting 4% to 12% of women of reproductive age, making it one of the most prevalent hormonal disorders in this demographic [1]. It is characterized by hyperandrogenism, chronic anovulation, and polycystic ovaries, and may be associated with irregular periods, hirsutism, and infertility [2]. Though PCOS is widespread, the underlying mechanisms of its development are not fully understood. Genetic, environmental, and lifestyle factors are thought to contribute to its development, with insulin resistance playing a central role in its metabolic manifestations [3].

An estimated 50% to 70% of women with PCOS exhibit insulin resistance, irrespective of their body mass index (BMI) [4, 5]. This insulin resistance exacerbates hyperinsulinemia, which in

*The current affiliation of the author: Division of Perinatology, Department of Gynecology and Obstetrics, Ankara Etlik City Hospital, Ankara, Türkiye
**The current affiliation of the author: Department of Gynecology and Obstetrics, University of Health Sciences, Gaziosmanpasa Training and Research, Istanbul, Türkiye

Address for correspondence: Gulsan Karabay, MD. Department of Gynecology and Obstetrics, University of Health Sciences, Sisli Hamidiye Etfal Training and Research Hospital, Istanbul, Türkiye
Phone: +90 553 533 79 31 **E-mail:** drgulsankarabay@gmail.com **ORCID:** 0000-0003-2567-2850
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turn stimulates ovarian androgen production, further complicating the hormonal imbalance characteristic of PCOS [4]. Furthermore, the condition of insulin resistance makes women with PCOS more susceptible to the development of long-term problems, including type 2 diabetes, metabolic syndrome, and cardiovascular disease [4, 6].

Recent attention has turned to irisin, a myokine first identified in 2012, which is released during physical activity and plays a key role in energy homeostasis [7–9]. Irisin is synthesized by the breakdown of the membrane protein FNDC5 and stimulates the process of browning in white adipose tissue, increasing heat production and total energy expenditure [8, 10]. Additionally, irisin has been shown to improve glucose metabolism, reduce insulin resistance, and potentially mitigate obesity-related complications [10–12]. These effects suggest that irisin could be a crucial link between exercise and metabolic improvements in conditions such as obesity, diabetes, and PCOS [13].

Given the central role of insulin resistance in the pathophysiology of PCOS and the effects of irisin on metabolic regulation, we aimed to investigate the relationship between serum irisin levels and insulin resistance in women with PCOS. Gaining a comprehensive understanding of this correlation could offer fresh perspectives on the mechanisms that regulate PCOS and emphasize potential treatment targets for controlling its metabolic effects.

Materials and Methods

This study was performed prospectively from August 2017 to February 2018 at the Gynecology and Obstetrics Clinic of the Health Sciences University Şişli Hamidiye Etfal Training and Research Hospital Perinatology Department. The first group consisted of 45 individuals diagnosed with both PCOS and insulin resistance, while the second group consisted of 45 patients diagnosed with PCOS but without insulin resistance.

Patients meeting two or more criteria, such as oligomenorrhea-amenorrhea, polycystic ovaries on ultrasound, and hyperandrogenism, were diagnosed with PCOS [14]. The exclusion criteria encompassed chronic illnesses (e.g., diabetes mellitus, thyroid dysfunction, kidney disease, hypertension), chronic drug usage, smoking, alcohol intake, and ovarian proliferation. Study participants were selected from women aged 18 to 45 who were still fertile. Demographic characteristics of the patients were recorded in the study. Insulin, glucose, free testosterone (F-TES), high-density lipoprotein (HDL), dehydroepiandrosterone sulfate (DHEA-S), low-density lipoprotein (LDL), white blood cells (WBC), hemoglobin, platelets, total testosterone (T-TES), sex hormone-binding globulin (SHBG), and prolactin levels were analyzed in venous blood samples from the patients.

The HOMA-IR approach, an acronym for Homeostatic Model Assessment of Insulin Resistance, was used to evaluate the insulin resistance of the patients. Blood glucose and insulin levels were measured after a fasting period of 8–10 hours. The study entailed the computation of HOMA-IR and Free Androgen Index (FAI) values for the patients, along with the implementation of Ferriman-Gallwey grading. HOMA-IR was calculated as follows: Fasting glucose (mg/dL) × Fasting insulin (uIU/mL) / 405. Patients with a HOMA-IR score ≥2.5 were classified as insulin resistant [15]. The Free Androgen Index (FAI) was calculated using the formula: total testosterone × 100 / SHBG [16]. The degree of hirsutism was assessed using the Ferriman-Gallwey scale, which is based on nine androgen-sensitive areas, with scores from 0 (no terminal hair growth) to 4 (severe hair growth). A score of >8 indicated a diagnosis of hirsutism [17].

Metabolic syndrome was diagnosed according to the American Heart Association (AHA) criteria, determined by the presence of three or more of the following: fasting serum glucose (FSG) ≥ 100 mg/dL, serum HDL<50 mg/dL, serum triglycerides ≥150 mg/dL, waist circumference ≥88 cm, and blood pressure ≥130/85 mm/Hg [18].

The study was conducted in compliance with the principles stated in the Declaration of Helsinki. Each patient was informed about the trial and gave informed consent. The local ethics commission granted ethical permission for this project, with approval number 849-22.08.2017.

Sample collection

The patients' venous blood samples were transferred into biochemical tubes fitted with separating gel. The biochemical tubes were spun in a centrifuge at +4°C, separating the blood cells and serum (4000 rpm, 10 minutes). The serum from the centrifuged blood samples was added to Eppendorf tubes, which were stored at -80°C until the irisin kits were obtained. The serum samples were slowly thawed the day before the test and homogenized by vortexing. The serums were prepared by keeping them at room temperature the day before the study.

Laboratory measurement and assay performance parameters

The spectrophotometric assay was used to analyze serum glucose, HDL, and triglyceride levels on a Roche Cobas c701 autoanalyzer (Mannheim, Germany). The LDL-C level was calculated using the Friedewald formula: [Total cholesterol - (HDL-C + (triglycerides / 5))]. An electrochemiluminescence assay (ECLIA) was used to analyze serum levels of F-TES, T-TES, prolactin, DHEA, SHBG, and insulin on a Roche Cobas e601 autoanalyzer (Mannheim, Germany). WBC and hemoglobin values were measured using a SYSMEX XT2000 Hematology Analyzer (Sysmex, Germany) via the fluorescence flow cytometry method.

The Enzyme-Linked Immunosorbent Assay (ELISA) was used to assess serum irisin levels. A Biotek ELX800 microplate reader was used to detect the absorbance of irisin (Human Irisin ELISA kit, catalog number: E-EL-H6120, www.elabsicience.com). Calculations were performed using Gen 5 software. Blood samples were analyzed in duplicate, with a dilution rerun procedure for values higher than the measuring range. The measurement range was 0.015–1 ng/mL. Three different mean values were used for intra-assay precision (0.04±0.002 ng/mL, CV%=5.34; 0.1±0.005 ng/mL, CV%=4.96; 0.47±0.23 ng/mL, CV%=5.02) and for inter-assay precision (0.04±0.002 ng/mL, CV%=5.1; 0.1±0.004, CV%=4.55; 0.4±0.02, CV%=4.56). All performance values were provided by the manufacturer's insert.

Table 1. Demographic characteristics of all patients included in the study

	Group with insulin resistance n=45 Mean±SD	Group without insulin resistance n=45 Mean±SD	p
Age (year)	24.4±5.4	24±4.5	0.974
Weight (kg)	72.2±17.2	62.3±11.6	0.004*
Height (cm)	162.7±7.1	165.7±6	0.032*
BMI (kg/m^2)	27.2±5.8	22.7±4.1	<0.001*
Waist circumference (cm)	87.9±12.6	79.1±12	0.001*
Hip circumference (cm)	92.9±17.6	94±12.8	0.875

*: The group without insulin resistance. SD: Standard deviation; BMI: Body mass index.

Table 2. Laboratory values of all patients included in the study

	Group with insulin resistance n=45 Mean±SD/SEM	Group without insulin resistance n=45 Mean±SD/SEM	p
Hemoglobin (g/dL)	12.7±1.4[a]	12.6±1.1[a]	0.781
WBC (10^3/mm^3)	9.7±0.12[b]	8.3±0.05[b]	0.184
Platelet (10^3/mm^3)	305±85.6[a]	264.3±64.1[a]	0.012*
Glucose (mg/dL)	101.3±0.87[b]	85.7±1.90[b]	<0.001*
Insulin (uIU/mL)	21.5±0.26[b]	7±0.05[b]	<0.001*
HOMA-IR	5.8±0.14[b]	1.5±0.01[b]	<0.001*
HDL (mg/dL)	48.4±8.3[a]	54.4±11.9[a]	0.019*
LDL (mg/dL)	103.2±27.4[a]	102.8±28.4[a]	0.940
Triglyceride (mg/dL)	107.4±1.62[b]	97.3±1.86[b]	0.150
F-TES (pg/mL)	2.00±0.03[b]	2.44±0.02[b]	0.012*
T-TES (ng/dL)	2.54±0.03[b]	2.50±0.02[b]	0.460
Prolactin (µg/L)	22.5±0.22[b]	18.5±0.20[b]	0.045*
DHEA (µg/dL)	295.1±2.14[b]	311±2.65[b]	0.490
SHBG (nmol/L)	64.2±0.96[b]	53.9±0.81[b]	0.390
FAI	5.6±0.08[b]	7.4±0.14[b]	0.388
Ferriman gallwey score	4.3±0.15[b]	7.6±0.16[b]	0.008
Serum irisin (ng/mL)	3.66±0.06[b]	2.77±0.04[b]	0.065

*: No difference between the two groups. [a]: Standard deviation; [b]: Standard error of mean. SD: Standard deviation; SEM: Standard error of mean; WBC: White blood cell; HOMA-IR: Homeostatic model assessment of insulin resistance; HDL: High-density lipoprotein; LDL: Low-density lipoproteins; F- TES: Free testosterone; T-TES: Total testosterone; DHEA: Dehydroepiandrosterone; SHBG: Sex hormone binding globulin; FAI: Free androgen index.

Statistical analysis

The statistical analysis was conducted using SPSS 15.0 software for Windows. Descriptive statistics were presented as the mean, standard deviation, and standard error of the mean for numerical variables, and as numbers and percentages for categorical variables. Skewness and kurtosis values were initially used to evaluate normal distribution, followed by the Shapiro-Wilk test to confirm normality. The Student's t-test was used to compare two independent groups, as the numerical variables met the normal distribution requirement. Since the parametric test condition was met in all tests, Pearson correlation analysis was applied. Multivariate linear regression analysis was performed to determine if there were independent risk factors in the relationship between irisin and HOMA-IR. The statistical significance level was set at a p-value of less than 0.05.

Results

Table 1 presents the comprehensive characteristics of the individuals participating in the clinical investigation. The results showed no statistical difference in age and hip circumference between the two groups (p=0.974, p=0.875). The cohort with insulin resistance demonstrated significantly higher weight, BMI, and waist circumference compared to the cohort without insulin resistance. Conversely, the group without insulin resistance showed greater height (p=0.004, p<0.001, p=0.001, and p=0.032, respectively).

The laboratory values of the participants are presented in Table 2. Analysis of the laboratory data indicated no difference in hemoglobin, WBC, LDL-C, triglyceride, total testosterone, DHEA-S, SHBG, and FAI values between the two groups (p=0.781,

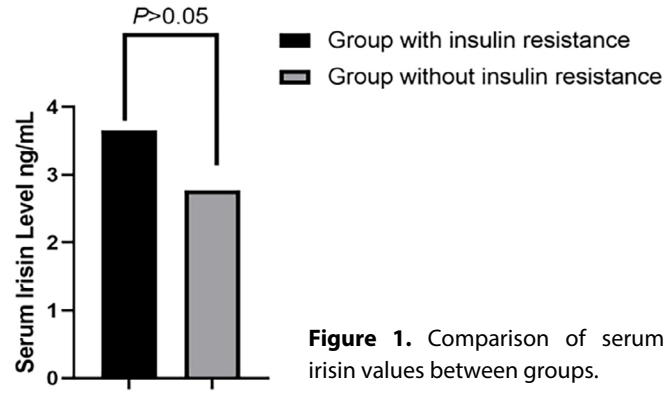

Figure 1. Comparison of serum irisin values between groups.

p=0.184, p=0.940, p=0.150, p=0.460, p=0.490, p=0.390, p=0.388, respectively). PCOS patients with insulin resistance had higher levels of platelets, insulin, glucose, HOMA-IR, and prolactin compared to PCOS patients without insulin resistance (p=0.012, p<0.001, p<0.001, p<0.001, p=0.045, respectively). The group with insulin resistance exhibited significantly lower levels of HDL-C, free testosterone, and Ferriman-Gallwey scores (p=0.019, p=0.012, p=0.008). Serum irisin levels were comparable between both groups. The concentration was 3.66±0.06 ng/mL in the group with insulin resistance and 2.77±0.04 ng/mL in the group without insulin resistance (p=0.065) (Fig. 1).

Table 3 displays the relationships between serum irisin concentration and the laboratory values of the study participants. Serum irisin levels were correlated with hemoglobin, insulin, HOMA-IR, F-TES, and T-TES levels (r=0.287, P=0.006; r=0.376, p<0.001; r=0.279, p=0.008; r=-0.230, p=0.029; r=-0.261, p=0.013, respectively). However, these relationships were weak and correlations were incomplete. Positive correlations were significant for hemoglobin, insulin, and HOMA-IR, while negative correlations were significant for F-TES and T-TES.

Table 4 shows serum irisin levels in the control and case groups with a Ferriman-Gallwey score >8. The average blood irisin level in PCOS patients with a high Ferriman-Gallwey score and insulin resistance was 3.70±0.06 ng/mL. In PCOS patients with a high Ferriman-Gallwey score but without insulin resistance, the average irisin concentration was 2.71±0.04 ng/mL. No substantial difference in irisin levels was found between the two groups (p=0.561).

The patients in the study had no prior chronic illness but were later diagnosed with metabolic syndrome. These patients were not excluded from the study. Among patients with insulin resistance, 12 (26.7%) were diagnosed with metabolic syndrome, compared to 3 (6.7%) in patients without insulin resistance. Serum irisin levels in patients with metabolic syndrome are shown in Table 5. The mean serum irisin concentration in PCOS patients with both metabolic syndrome and insulin resistance was 3.99±0.07 ng/mL, whereas in PCOS patients with metabolic syndrome but without insulin resistance, the mean was 2.37±0.02 ng/mL. There was no substantial distinction between the two groups (p=0.410).

According to multivariate linear regression analysis, age, BMI, and waist circumference were not identified as independent factors in the relationship between irisin and HOMA-IR (Beta=0.136, p=0.169; Beta=0.160, p=0.092; Beta=0.150, p=0.145, respectively).

Table 3. The relationship between laboratory findings and serum irisin in all patients included in the study

	Serum Irisin	
	r	p
Hemoglobin (g/dL)	0.287	**0.006**
WBC (10^3/mm^3)	0.104	0.328
Glucose (mg/dL)	0.141	0.183
Insulin (uIU/mL)	0.376	**<0.001**
HOMA-IR	0.279	**0.008**
HDL (mg/dL)	-0.161	0.130
LDL (mg/dL)	0.143	0.179
Triglyceride (mg/dL)	0.061	0.567
F-TES (pg/mL)	-0.230	**0.029**
T-TES (ng/dL)	-0.261	**0.013**
Prolactin (µg/L)	0.052	0.627
DHEA (µg/dL)	0.009	0.934
SHBG (nmol/L)	0.149	0.160

Student t-test was used to examine whether there was a significant difference between the means of two groups. WBC: White blood cell; HOMA-IR: Homeostatic model assessment of insulin resistance; HDL: High-density lipoprotein; LDL: Low-density lipoproteins; F- TES: Free testosterone; T-TES: Total testosterone; DHEA: Dehydroepiandrosterone; SHBG: Sex hormone binding globulin.

Table 4. Serum irisin levels of the control and Case groups with Ferriman-Gallwey score>8

	Ferriman gallwey score>8	
	Serum Irisin Mean±SEM	p
Insulin resistance		
Yes n=17	3.70±0.06	0.561
No n=29	2.71±0.04	

SEM: Standard error of mean.

Table 5. Serum irisin levels diagnosed in patients with metabolic syndrome

	Metabolic syndrome	
	Serum Irisin Mean±SEM	p
Insulin resistance		
Yes n=12	3.99±0.07	0.410
No n=3	2.37±0.02	

SEM: Standard error of mean.

Discussion

Our study investigated the relationship between the hormone irisin and insulin resistance in PCOS patients. While the serum irisin level in PCOS patients with insulin resistance was 3.66±0.06 ng/mL, it was 2.77±0.04 ng/mL in PCOS patients without insulin resistance, with no significant difference between them. Insulin, HOMA-IR, F-TES, and T-TES levels were weakly correlated with serum irisin levels. Hemoglobin, insulin, and HOMA-IR values showed a positive correlation with irisin levels, whereas F-TES and T-TES values were negatively correlated.

Insulin resistance affects 50–70% of women with PCOS and is strongly linked to metabolic syndrome, hypertension, dyslipidemia, diabetes mellitus, and long-term cardiovascular disease [5]. The complexity of these comorbidities highlights the importance of understanding and managing insulin resistance in PCOS patients. The hormone irisin has been shown to improve hepatic glucose metabolism by promoting glucose uptake in skeletal muscle [19]. Due to its association with improved glucose tolerance and reduced insulin resistance, irisin is an attractive molecule to study in PCOS patients.

Chang et al. [20] conducted a comparison of serum irisin levels between PCOS and control groups, finding abnormally elevated serum irisin levels in PCOS patients. Additionally, PCOS patients exhibited elevated BMI and insulin levels. Increased serum irisin levels were linked to the development of insulin resistance and hyperandrogenemia. Bayraktar et al. [21] observed elevated serum irisin levels in individuals with PCOS compared to control patients; however, insulin resistance and HOMA-IR levels were similar in both the PCOS and control groups. Conversely, Masaeli et al. [22] found that in patients with PCOS, blood irisin levels were significantly elevated compared to those without the condition. They also noted a reduction in insulin resistance and serum irisin levels following three months of metformin therapy in PCOS patients. Similarly, Farhan et al. [23] examined serum irisin levels in PCOS patients before and after therapy, observing a reduction in serum irisin levels and insulin resistance following four months of metformin treatment compared to pretreatment levels in the control group.

Since not all PCOS patients exhibit insulin resistance, we selected participants from both groups of PCOS patients to allow for a more controlled comparison of serum irisin levels within a similar patient population, minimizing potential confounding factors. Serum irisin levels were not significantly different between PCOS patients with and without insulin resistance. Given that insulin resistance is not the only determinant in PCOS development, further research with larger patient cohorts is necessary.

Hyperandrogenism, although not necessarily present in every patient, is one of the diagnostic criteria for PCOS and can influence the treatment modality to be planned. This consideration has prompted the examination of the correlation between irisin and hyperandrogenemia. Among PCOS patients, Li et al. [24] examined the correlation between hyperandrogenism, the FAI index, and serum irisin. PCOS patients with a high FAI exhibited elevated serum irisin levels compared to both PCOS and control patients with a low FAI index. Additionally, these patients had a higher incidence of insulin resistance when hyperandrogenemia was present. Zhang et al. [25] observed elevated serum irisin levels in individuals with PCOS compared to a control group. They further analyzed these levels based on PCOS characteristics. While serum irisin levels in patients with a normoandrogenic phenotype were nearly equivalent to those in control participants, patients with a hyperandrogenic phenotype had higher serum irisin levels. Based on these findings, it has been shown that therapy with insulin sensitizers is not effective for PCOS patients with a normoandrogenic phenotype.

The results of our investigation indicated that PCOS patients without insulin resistance had higher FAI rates and lower serum irisin levels, though this difference did not reach statistical significance. In the studies mentioned above, patients with hyperandrogenemia were not divided into groups with and without insulin resistance for comparison. In our study, we had the opportunity to compare patients with hyperandrogenemia in two groups: those with and those without insulin resistance. We also investigated the relationship between hirsutism, a clinical manifestation of hyperandrogenemia, and serum irisin. In our comparison of patients with hyperandrogenemia, the group with insulin resistance had elevated serum irisin levels; however, there was no statistically significant difference observed between the group with insulin resistance and the group without it.

While commonly referred to as a myokine, irisin is also secreted from adipose tissue [11, 12]. A study by Arhire et al. [26] revealed a significant correlation between blood irisin levels and both obesity and metabolic syndrome. Furthermore, Elizondo-Montemayor et al. [27] demonstrated that an elevated serum irisin concentration is associated with a twofold increase in the risk of metabolic syndrome. To investigate the correlation between serum irisin and metabolic syndrome, the study included patients who were not initially diagnosed with metabolic syndrome but were subsequently diagnosed with it. The serum irisin levels of these patients were then compared. No statistically significant difference in serum irisin levels was observed between patients with PCOS and insulin resistance diagnosed with metabolic syndrome and patients without insulin resistance diagnosed with metabolic syndrome.

A primary limitation of our study is the restricted sample size. Additionally, we were unable to evaluate changes in serum irisin levels post-treatment for PCOS. However, our study provided an opportunity to compare serum irisin levels in patients who were diagnosed with metabolic syndrome after their PCOS diagnosis and who had elevated Ferriman-Gallwey scores due to hyperandrogenemia.

Conclusion

The objective of this study was to investigate the correlation between serum irisin levels and insulin resistance in women diagnosed with PCOS. Our findings revealed no statistically significant difference in serum irisin levels between PCOS patients with and without insulin resistance. While modest correlations were found between serum irisin and insulin, HOMA-IR, free testosterone, and total testosterone levels, these relationships were not sufficiently robust to establish conclusive findings on the involvement of irisin in the pathogenesis of PCOS.

In the metabolic complications of PCOS, insulin resistance remains a crucial determinant. Although irisin may play an essential role in enhancing insulin sensitivity and glucose metabolism, our results suggest that insulin resistance alone may not have a direct impact on serum irisin levels in PCOS patients. This underscores the complexity of the condition, in which various factors, such as hyperandrogenism and metabolic syndrome, likely interact to influence clinical outcomes.

Further research is necessary to elucidate the role of irisin in PCOS and its potential as a therapeutic target. Future studies should include larger patient cohorts and carefully evaluate the impact of treatment on serum irisin levels to gain a deeper understanding of its therapeutic significance in the management of PCOS.

Ethics Committee Approval: The study was approved by The University of Health Sciences Şişli Hamidiye Etfal Training and Research Hospital Clinical Research Ethics Committee (No: 849, Date: 22/08/2017).

Authorship Contributions: Concept – G.K., U.K., D.A., S.C., A.E.Y.; Design – G.K., U.K., D.A., S.C., A.E.Y.; Supervision – G.K., U.K., D.A., S.C., A.E.Y.; Funding – G.K.; Materials – S.C., D.A., G.K.; Data collection &/or processing – G.K., D.A.; Analysis and/or interpretation – G.K., U.K.; Literature search – G.K., U.K.; Writing – G.K., S.C.; Critical review – A.E.Y., G.K.

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Research Article

Effect of temperature changes on the expression of cancer stem cell protein CD-44 and TAU protein in AMGM-5 cancer cell line: An immunocytochemistry study

Zaynab Saad Abdulghany[1], Noah A. Mahmood[1], Noora Mustafa Kareem[2], Firas Subhi Salah[1]

[1]Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, Baghdad, Iraq
[2]Department of Pathology and Forensic Medicine, Alnahrain College of Medicine, University of Alnahrain, Baghdad, Iraq

Abstract

Objectives: Glioblastoma multiforme (GBM) has long been one of the most common and particularly invasive malignant gliomas. High-grade gliomas are highly prone to relapse and associated with poor prognosis. This study tests the hypothesis that hyper-thermal conditions could influence TAU and CD-44 protein expression by increasing temperature in glioblastoma cancer cell line culture.

Methods: AMGM cancer cells were cultured and maintained under normal growth conditions, then separated into two groups: one group was cultured at 37°C, and the other at 40°C. After 24 hours of growth, cells underwent immunocytochemistry (ICC) to visualize the localization of TAU and CD-44 markers.

Results: The results show that fewer AMGM cells remained stable enough to grow at 40°C; these cells lost their fusiform shape and became spherical compared to cells grown under normal conditions. Additionally, an increase in microenvironmental temperature significantly affected TAU protein expression in the nucleus of AMGM cells, with a 71.4% increase at 40°C. In contrast, the expression of CD-44, typically expressed on the cell membrane of AMGM cells, decreased by 42.9% at 40°C.

Conclusion: Changes in the microenvironment may affect glioblastoma cell line development by influencing the cancer stem cell marker CD-44 and the microtubule-stabilizing protein TAU. These markers could serve as potential targets for the treatment and prevention of glioblastoma.

Keywords: AMGM cancer cell line, CD-44, hyper-thermal, TAU

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The tumor microenvironment (TME) refers to the conditions under which tumor cells grow, communicate, and die [1, 2]. In 2011, Ungefroren and his team defined TME as the context that controls tumorigenesis, including processes such as epithelial-mesenchymal transition (EMT), migration, invasion, metastasis, apoptosis, and chemotherapeutic drug resistance [3].

Various types of microenvironments are commonly used and identified in *in vitro* cell cultures, including thermal and serum conditions. Studies have shown that hyper-thermal treatment can enhance tumor shrinkage and decrease oxygen consumption [4]. Hyperthermia is one of the few promising strategies among alternative therapies for cancer treatment [5].

For years, gliomas have been recognized as highly heterogeneous tumors at the molecular level, with varying survival times, even among patients with the same grade [6]. According to the American Association of Neurological Sur-

Address for correspondence: Zaynab Saad Abdulghany, Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, Baghdad, Iraq
Phone: 9647812218857 **E-mail:** zaynab.saad@iccmgr.org **ORCID:** 0000-0002-1388-5245
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geons (2023), glioblastoma (grade IV glioma) is the highest-grade glioma, clinically representing the most common and aggressive primary brain tumor with poor patient survival rates. Experimental data suggest that the low survival rate may partially be due to the presence of glioma stem cells (GSCs) [7].

The quiescence of GSCs in their niches, effective DNA damage repair, drug transporter activity, and Notch signaling are factors contributing to therapy resistance [8]. To improve glioblastoma treatment outcomes, GSCs must be eradicated. Hyperthermia, particularly in combination with irradiation, is emerging as a promising therapeutic approach, as it appears that multiple DNA repair pathways in GSCs are sensitive to hyperthermia [9].

The cancer stem cell marker CD-44 is a transmembrane glycoprotein that functions as a hyaluronic acid receptor. CD-44 has been implicated in EMT and tumor invasion [10]. A recent review highlighted CD-44 as a predictor of chemotherapy resistance in mesenchymal-like glioma [11] and as a factor in GBM prognosis. CD-44 inhibition has been suggested as a therapeutic strategy for several malignant tumors [12].

Tau protein, also known as microtubule-associated protein (MAPT), was identified in 1986 as a protein that binds to and stabilizes microtubules [13]. Under normal physiological conditions, phosphorylation regulates Tau's binding to microtubules and other functions [14]. However, hyperphosphorylation of Tau protein leads to its aggregation and the formation of neurofibrillary tangles [15], which are key features in the development of Alzheimer's disease (AD). Tau pathology is correlated with neurodegeneration and AD progression [16, 17], making Tau phosphorylation a viable target for treating AD and other tauopathies, though no treatments currently exist [18].

Studies on the effect of hyper/hypo-thermal conditions on cancer stem cell markers *in vitro* have suggested that hyperthermia at 46°C for 10 minutes can induce high levels of cancer cell death in pancreatic ductal adenocarcinoma compared to non-malignant cells [19].

The combined effect of chemotherapy and hyper-thermal treatment on cancer cell proliferation was explored in a 2014 study by Lee. Metformin alone, or in combination with hyperthermia, showed cytotoxicity against cancer stem cells (CD-44/CD24) in MCF-7 human breast cancer cells and MIA PaCa-2 human pancreatic cancer cells. The authors applied heating at 42°C for 1 hour, finding it partially toxic to cancer cells and CSCs and that it enhanced metformin's efficacy in reducing both cancer cells and CSCs [20]. Severe hyperthermia (45°C for 1 hour) was also found to reduce viability and induce apoptosis in MG-63 osteosarcoma cells, with increased activities of caspases 3/7, 4, and 12 after 72 hours at 37°C [21].

This study aims to determine the presence of TAU (MAPT) and CD-44 markers in glioblastoma cancer cell lines before and after exposure to hyper-thermal conditions at 40 °C for 24 hours using the immunocytochemistry (ICC) technique.

Materials and Methods

Cell culture maintenance and heat treatment

The glioblastoma AMGM cancer cell line was obtained from the Cell Bank Unit at the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) and cultured in RPMI-1640 (Sigma-Aldrich, Germany) supplemented with 10% calf bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cancer cell line falcons were passaged using Trypsin-EDTA (US Biological, USA) and incubated at 37°C.

To determine the expression of TAU and CD-44 in AMGM cells, cells were seeded at 4×10^5 cells per 60-mm dish in 3 mL of medium 24 hours before experiments at 37°C and 40°C. Each experiment was performed in triplicate and repeated twice (Fig. 1).

Immunostaining of TAU and CD-44 proteins

The primary antibodies used were mouse monoclonal anti-TAU (A-10, Abcam, 1:20 from 1 mg/mL) and anti-CD-44 (sc-9960, 1:20 from 1 mg/mL; Santacruz). Immunostaining was performed overnight with primary antibodies. Cells were incubated for 1 hour at room temperature in the dark with secondary conjugated antibodies (dilution 1:200 from 1.5 mg/mL; Pathinsitu). Slides were then covered with DPX mounting medium containing DAPI to counterstain the nuclei for CD-44 and the cytosol for TAU. A coverslip was applied, and the slides were analyzed under an Olympus light microscope at 400x magnification. Microscopy images were captured using a MICROS CAM 500 "PREMIUM" camera with Microvisible software.

Scoring

The number of stained cells was examined under microscopy and scored as follows: 0=no expression or stained cells; 1=5% of cells; 2=≥5% of cells; 3=≥25% of cells; 4=≥50% of cells; and 5=≥75% of cells.

Statistical analysis

Statistical comparisons between groups were conducted using the one-tailed Mann-Whitney U-test. A probability value of $p<0.05$ was considered statistically significant. Analyses were performed with SPSS-24 statistical software and Microsoft Excel.

Results

Maintenance and culturing of AMGM glioblastoma cell line

The AMGM glioblastoma cell line was initially established at the Iraqi Center for Cancer and Medical Genetics, Mustansiriyah University [22]. The specific staining criteria for the definition of primary glioblastoma cell lines and to check whether the glioblastoma-derived primary cell lines have similarities to glioblastoma tissue, we performed immunocytochemistry staining for markers commonly used in glioma diagnosis. We first observed the morphology and colony formation of cells

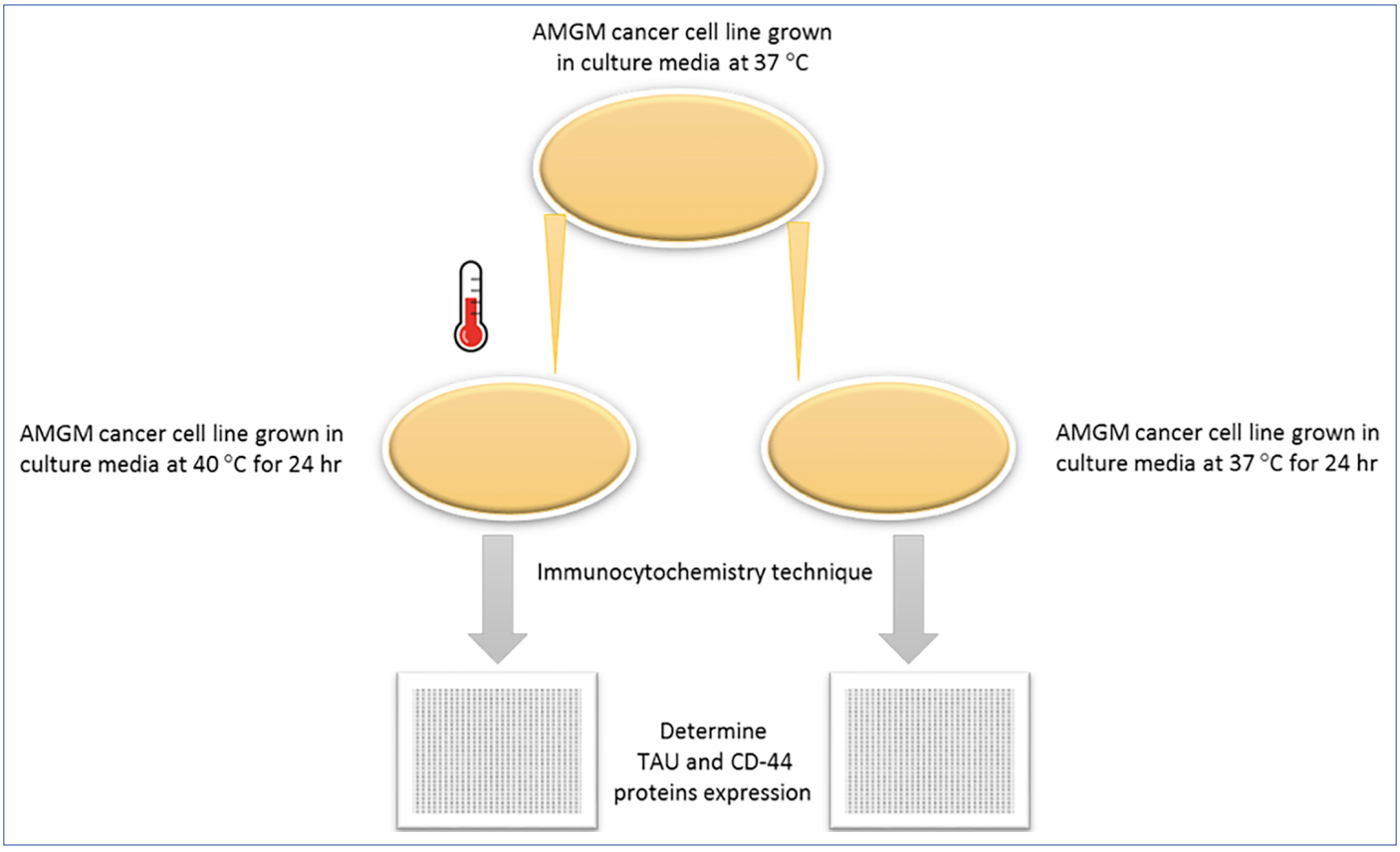

Figure 1. Summary determines the effect of incubation AMGM cancer cells under temperature (40°C) on TAU and CD-44 protein expression compared to normal incubation culture conditions (37°C).

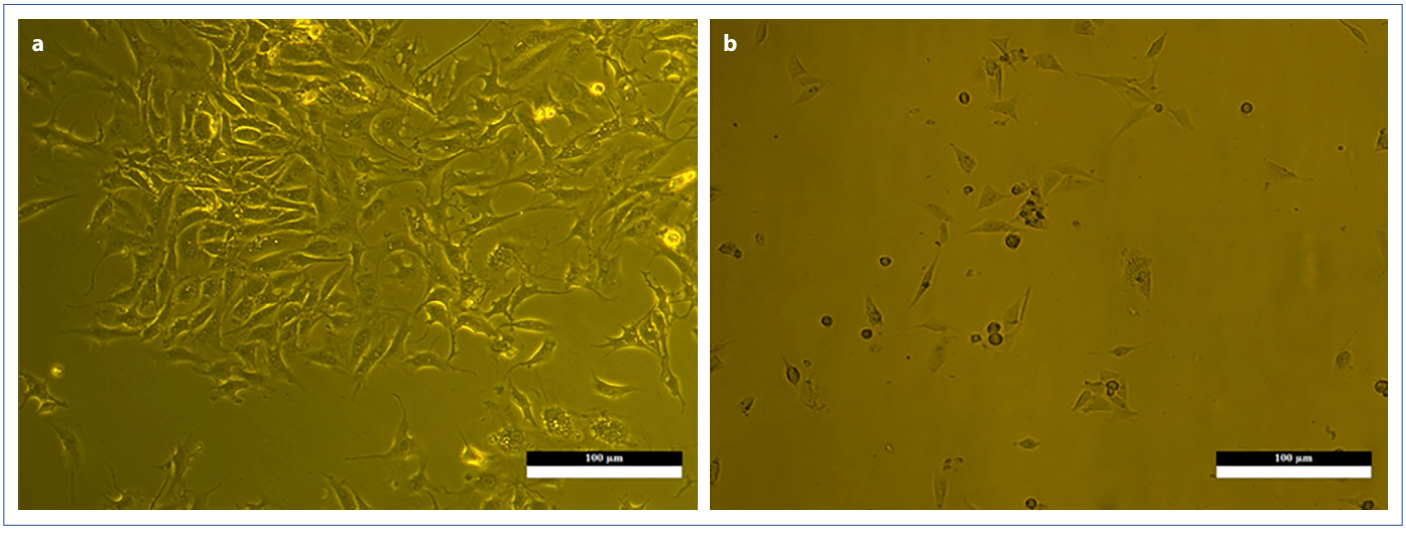

Figure 2. Growing AMGM cancer cell line culture at two different incubation temperatures: (a) 37°C and (b) 4°C under an inverted microscope. This shows how the glioblastoma cells were monolayer and had a fusiform at optimal incubation temperature, compared to cells cultured in high atmosphere temperature (40°C) the cells lose their unity and start to circle in shape an indication of apoptosis.

under both incubation conditions before staining (Fig. 2). Under standard incubation conditions, cells formed a single flattened layer, appeared elongated, and had a fusiform shape (Fig. 2a). In contrast, at 40°C, fewer cells retained the fusiform shape, and cell numbers were reduced (Fig. 2b).

CD-44 and MAPT staining: ICC

The results of Immunocytochemistry of both markers the CD-44 and MAPT in AMGM cancer cells cultured in two different incubation temperatures 37°C and 40°C were shown in Figures 3 and 4 respectively. In Figure 3a, TAU protein

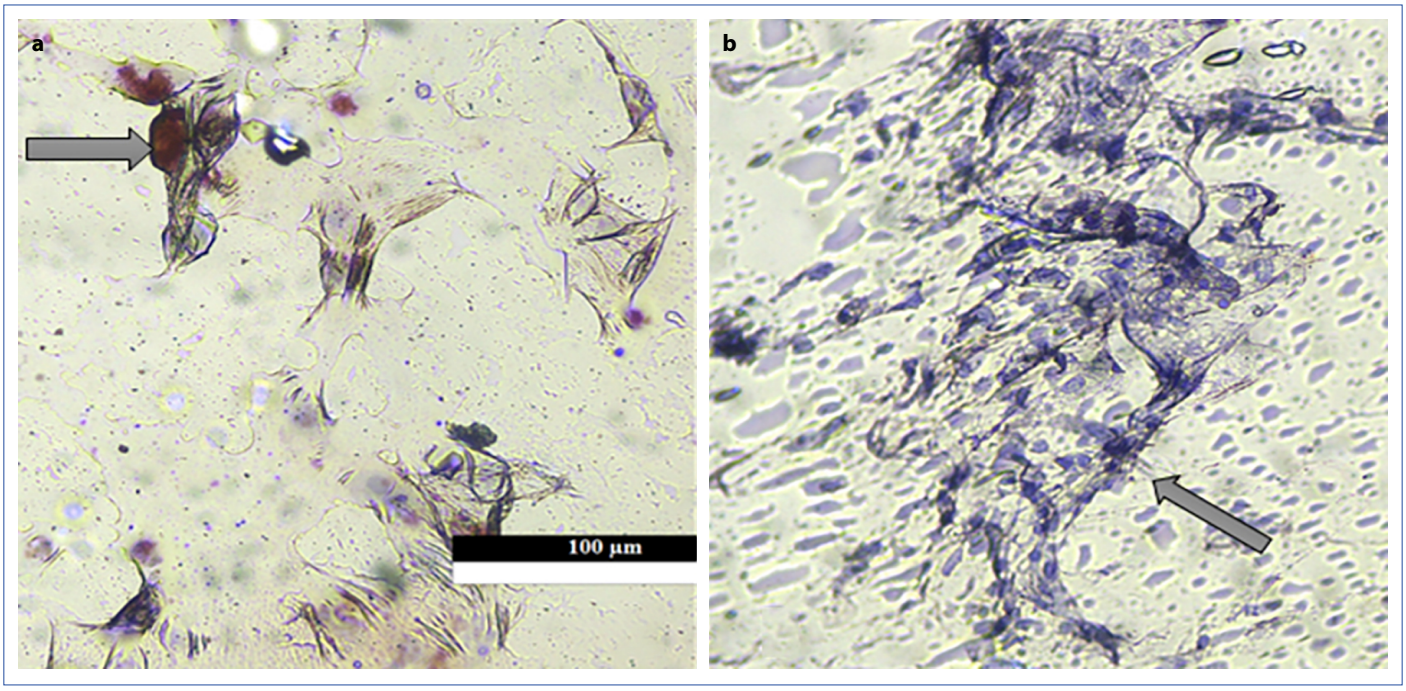

Figure 3. Immunocytochemistry staining of tau in glioblastoma cancer cell line. (a) Represents cells incubation culture at 37°C while (b) Represents culture cells at 40°C. the arrows show TAU protein expression in the nucleus of the glioblastoma cell line.

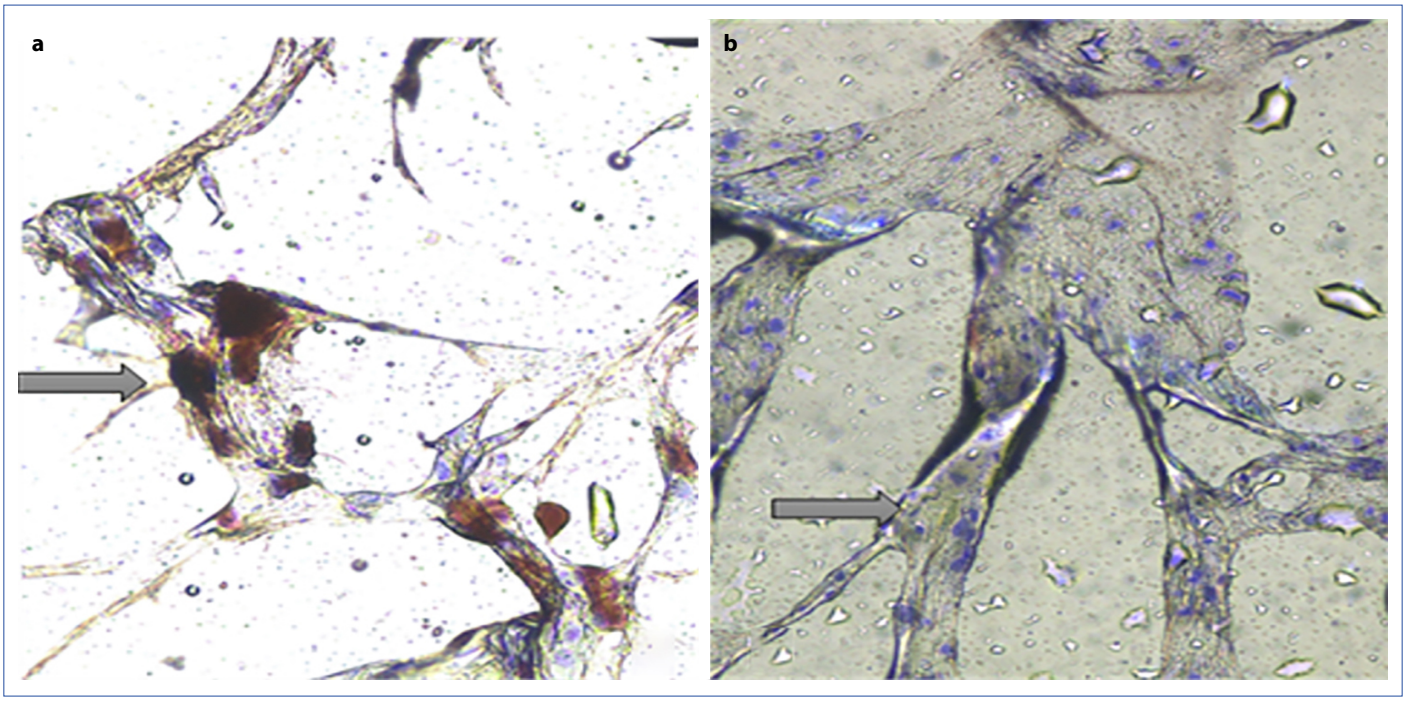

Figure 4. Immunocytochemistry staining of CD-44 in glioblastoma cancer cell line. (a) Represents cells incubation culture at 37°C while (b) Represents culture cells at 40°C. the arrows show CD-44 protein in the nucleus of the glioblastoma cell line.

was poorly expressed in the nucleus of glioblastoma cancer cells growing at 37°C compared to B- expressed highly when growing at 40°C.

Next, in Figure 4c, CD-44 protein was expressed in the cell membrane of glioblastoma cancer cells growing at 37°C compared to D- expressed highly when growing at 40°C.

Tables 1 and 2 show the scoring of both TAU and CD-44 proteins in the growing glioblastoma AMGM cancer cell line in two different culture conditions 37°C and 40°C respectively. In Table 1, TAU protein expression was affected by increasing the temperature where the scoring was highly +3 in 71.4 % at 40°C significantly compared to

Table 1. Tau marker expression in selected AMGM cell line while cultures in both 37°C and 40°C respectively

AMGM cells cultured conditions	Tau 1+ (%)	Tau 2+ (%)	Tau 3+ (%)	p
37°C	-	57	42.9	0.001
40°C	28.6	-	71.4	

Table 2. CD-44 marker expression in selected AMGM cell line while cultures in both 37°C and 40°C respectively

Cells cultured conditions	CD-44 1+ (%)	CD-44 2+ (%)	CD-44 3+ (%)	p
37°C	-	-	100	0.02
40°C	28.6	28.6	42.9	

growing the cells at 37°C the score +3 was determined in 42.9% of the cells with p<0.001.

In Table 2, CD-44 protein expression was affected by increasing the temperature where the scoring highly +3 in 100 % of the glioblastoma AMGM cell line at 37°C significantly compared to growing the cells at 40°C the score of +3 was determined in 42.9% of the cells with p<0.02.

Discussion

Glioma is known for its high heterogeneity, influenced by genetic, epigenetic, and tumor microenvironmental factors. This heterogeneity is linked to the adaptive response, treatment resistance, and overall behavior of brain tumors [23]. Understanding the mechanisms underlying this heterogeneity is critical for advancing glioma diagnosis, treatment, and potentially prevention.

This study aimed to explore whether changes in the microenvironment affect the development of glioblastoma in the AMGM cell line by examining the expression of TAU protein and the cancer stem cell marker CD-44. The microenvironmental change tested here was an increase in incubation temperature, with AMGM cells cultured at 37°C and 40°C. Immunohistochemistry staining revealed that elevated temperature increased TAU protein expression while decreasing CD-44 levels. A limitation of our study was the lack of a normal cell line for comparison, as initially planned.

According to a study by Lim et al. [24], glioblastoma cell aggressiveness increases with tumor growth and migration. These cells secrete various molecules, including soluble CD-44 and adhesion molecules, into the extracellular matrix, which can induce neuronal degeneration by activating TAU protein.

TAU protein is encoded by the MAPT gene on chromosome 17 and is primarily expressed in neuronal axons. It normally facilitates the polymerization and stabilization of microtubules [25]. TAU is extensively post-translationally modified, and in Alzheimer's disease (AD), it detaches from microtubules and aggregates to form plaques [26].

Recent research has shown that TAU depletion significantly inhibits *in vitro* spheroid growth of the glioblastoma cell line U87-MG and reduces 2D cell proliferation [27]. A correlation between TAU expression and heat shock proteins has also been documented, with HSP70, a chaperone protein, mediating the ubiquitinylation of aberrant TAU species for selective elimination [28].

Another recent study utilizing RNA sequencing data suggested that CD-44 could serve as a biomarker for M2 tumor-associated macrophages (TAMs), promoting immune suppression and glioma progression in the tumor microenvironment [29]. Johansson et al. [30] demonstrated that CD-44 is activated under hypoxic conditions by interacting with HIF-2α to enhance hypoxia in glioma stem cells. They proposed that blocking CD-44-ligand interactions through antibodies, inhibiting external cleavage of CD-44, or using gamma-secretase inhibitors to inhibit internal cleavage could mitigate these effects.

A separate transcriptomic study noted that a reduction in CD-44 levels in U251MG glioblastoma cells suppressed cell growth and induced cellular senescence. The authors proposed that CD-44 could serve as a marker for hypoxia, glycolysis, and anti-tumor immune responses [31].

In summary, our study demonstrates that an elevated microenvironmental temperature of 40°C affects the expression of the cancer stem cell marker CD-44 and increases TAU protein expression in glioblastoma AMGM cancer cell cultures.

Conclusion

In conclusion, this preliminary study found that hyperthermia at 40°C increased TAU protein and decreased CD-44 expression in the AMGM glioblastoma cancer cell line. These findings suggest that controlling the cellular environment's temperature could be a potential therapeutic approach for glioblastoma. Further studies examining chronic or repeated mild hyperthermia exposure in glioma mouse models are required to assess its effects on cancer progression and related diseases.

Ethics Committee Approval: The local Institutional Review Board deemed the study exempt from review.

Authorship Contributions: Concept – Z.S.A., N.A.M.; Design – Z.S.A., N.A.M.; Supervision – Z.S.A., N.A.M.; Data collection &/or processing – Z.S.A., N.A.M.; Analysis and/or interpretation – N.A.M., N.M.K.; Literature search – Z.S.A.; Writing – Z.S.A.; Critical review – Z.S.A., F.S.S.

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Research Article

Evaluation of measurement uncertainty of coagulation parameters according to two different current guidelines

Merve Zeytinli Aksit

Department of Medical Biochemistry, Izmir Bakircay University, Cigli Training and Research Hospital, Izmir, Türkiye

Abstract

Objectives: This study aims to calculate the measurement uncertainty values of prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer, and fibrinogen tests according to ISO/TS 20914 and Nordtest 2017 guidelines and to compare these values with the total allowable error (TEa%) and maximum expanded allowable measurement uncertainty (MAU) values established by international organizations.

Methods: Normal and pathological level internal quality control data for PT, APTT, D-dimer, and fibrinogen tests performed on the Sysmex CS2100 device between January and May 2024 were obtained from the Laboratory Information System. External quality control data for October 2023 and September 2024 were sourced from the external quality control system. Measurement uncertainty was calculated following ISO/TS 20914 and Nordtest 2017 guidelines.

Results: According to the ISO/TS 20914 guideline, the measurement uncertainty values for PT, APTT, D-dimer, and fibrinogen tests were 10.42%, 3.49%, 4.81%, and 19.10%, respectively. According to the Nordtest guideline, the measurement uncertainty values were 10.44%, 12.64%, 17.94%, and 21.69%, respectively.

Conclusion: Based on the ISO/TS 20914 guideline, it was observed that the measurement uncertainty values for all coagulation tests met the TEa% analytical targets. According to the Nordtest guideline, all tests except fibrinogen met these targets. When evaluated against the MAU criterion, it was determined that D-dimer met the targeted quality specification according to both guidelines; however, the measurement uncertainty values for PT, APTT, and fibrinogen exceeded the allowed targets. Standardization of the measurement uncertainty calculation model and the determination of analytical targets based on laboratory priorities can ensure reliable monitoring of analytical performance.

Keywords: Coagulation, Nordtest, measurement uncertainty, total allowable error

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Since laboratory results are crucial in the diagnosis, treatment, follow-up, and risk assessment of diseases, accurate and reliable measurements are essential. Measurement uncertainty (MU) is a concept that characterizes the distribution of values that can be attributed to a measurement to evaluate the reliability and accuracy of the analysis result [1]. MU is not a doubt about the validity of the measurement but rather a defined confidence limit. MU can also provide laboratory users with confirmation that patient results meet performance specifications [2]. MU components must be identified throughout the entire traceability chain, starting with reference material providers, through *in vitro* diagnostic manufacturers, processes for assigning calibrator values, and finally, the result. In addition to uncertainties in the steps of the metrological chain, test results are also affected by uncertainties arising from random effects in laboratory measurements [3].

Apart from uncertainties due to matrix effects, interferences, environmental factors, reference materials, and calibrators, differences in the methods and procedures used in calculating MU also contribute to MU. The ISO/TS 20914:2019 guideline recommends calculating MU after each of the three main MU sources has been estimated. According to this guideline, the MU value should primarily be calculated based on long-term uncertainty (uRW) and calibrator uncertainty (uCAL),

Address for correspondence: Merve Zeytinli Aksit, MD. Department of Medical Biochemistry, Izmir Bakircay University, Cigli Training and Research Hospital, Izmir, Türkiye
Phone: +90 544 514 25 40 **E-mail:** mervezeyt@gmail.com **ORCID:** 0000-0003-0212-1167
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and bias (uBias) should be included in the calculation only when a significant medical difference is observed [4].

There is limited literature investigating MU in coagulation parameters. Therefore, the aim of our study was to calculate the MU values of prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer, and fibrinogen tests according to ISO/TS 20914 [4] and Nordtest 2017 [5] guidelines and to compare them with the total allowable error (TEa%) and maximum expanded allowable measurement uncertainty (MAU) values determined by international organizations.

Materials and Methods

Approval for our retrospective study was received from the Ethics Committee of Bakircay University Cigli Training and Research Hospital, with the decision dated 03 July 2024 and numbered 1648. Normal and pathological level internal quality control (IQC) data for PT, APTT, D-dimer, and fibrinogen tests (Control Plasma N (Lot: 507924), Control Plasma P (Lot: 556741), Dade Ci-Trol 2 (Lot: 548527), INNOVANCE D-Dimer Controls (Lot: 575611-575506, Siemens Healthcare Diagnostics, Marburg, Germany)) run on the Sysmex CS2100 (Sysmex Corporation, Kobe, Japan) device between January and May 2024 were obtained from the Laboratory Information System. The reagents used for PT, APTT, D-dimer, and fibrinogen tests were Thromborel S, Dade Actin FS, INNOVANCE D-Dimer, and Multifibren U, respectively.

External quality control (EQC) data (External Quality Assurance Services (EQAS) Coagulation Program, Lot: 281000–281100, Bio-Rad Laboratories Inc., Irvine, CA, USA) for October 2023 to September 2024 were obtained from the EQC system.

MU calculation according to the ISO/TS 20914 guide [4]

The standard deviation (SD) of the IQC results was calculated. The SD was accepted as long-term precision (uRW). The uRW was calculated using equation:

$uRW = \sqrt{(SD\ (Level\ 1)^2 + SD\ (Level\ 2)^2)} / 2$

Calibrator uncertainty (uCAL) data was obtained from the manufacturer (Table 1). The mean bias (%) was calculated according to equation:

Mean bias (%): Σ bias(%)(EQC) / Number of EQCs

Since the mean bias (%) values calculated from the EQC data were lower than the desirable bias (%) values, ubias was not included in the uncertainty calculation. The combined uncertainty was calculated according to the following equation:

$Combined\ uncertainty = \sqrt{(uRW^2 + uCAL^2)}$

The expanded uncertainty was calculated using equation:

Expanded uncertainty = k x u (At 95% confidence interval, k=2 was taken).

The expanded uncertainty values were compared with TEa% values of international organizations [Clinical Laboratory Improvement Amendments (CLIA), Wisconsin State Laboratory of Hygiene (WLSH), Wadsworth Center of the New York State Department of Health (NYS), and American Association of Bioanalysts (AAB)] and MAU values in the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) Biological Variation database [6,7]. 50% of the total allowable error was taken as desirable bias.

MU calculation according to Nordtest guide [5]

The intermediate precision standard deviation ($S_{RW(Absolute)}$) was calculated from IQC data. The relative intermediate precision standard deviation ($S_{RW(Relative)}$) was calculated according to equation:

$S_{RW(Relative)} = S_{RW(Absolute)} \times 100) / mean$

The uRW is equal to the SRW(Relative).The uRW was calculated using equation:

$uRW = \sqrt{(S_{RW(Relative)}\ (Level\ 1)^2 + S_{RW(Relative)}\ (Level\ 2)^2)} / 2$

RMSbias and uncertainty of nominal values (uCref) were calculated from EQC data according to the following equations:

$RMSbias = \sqrt{\Sigma\ bias\ (EQC)^2} / N$

$uCref = \Sigma(CV(EQC) / \sqrt{N_{Lab}})^2 / N$

(Σbias(EQC): Sum of squares of EQC bias values, CV(EQC): CV% of each EQC, N_{Lab}: Number of participating laboratories in each EQC using the same method and same instrument, N: Number of EQCs).

The standard uncertainty, combined uncertainty, and expanded uncertainty were calculated according to the following equations:

$Standard\ uncertainty = \sqrt{RMSbias^2 + uCref^2}$

$Combined\ uncertainty = \sqrt{uRW^2 + ubias^2}$

Expanded uncertainty = k x u (At 95% confidence interval, k=2 was taken).

The expanded uncertainty values were compared with TEa% values of international organizations (CLIA, WLSH, NYS and AAB) and MAU values in the EFLM Biological Variation database [6, 7].

Results

The MU values of the parameters are shown in Table 2 and Table 3 according to ISO/TS 20914 and Nordtest guidelines, respectively. In our study, the MU values of PT, APTT, D-dimer, and fibrinogen tests according to ISO/TS 20914 guidelines were 10.42%, 3.49%, 4.81%, and 19.10%, respectively (Table 2). The MU values of PT, APTT, D-dimer, and fibrinogen tests calculated according to the Nordtest guideline were 10.44%, 12.64%, 17.94%, and 21.69%, respectively (Table 3).

According to the ISO/TS 20914 guideline, it was observed that all coagulation tests met the TEa% analytical targets. According to the Nordtest guideline, all tests except fibrinogen met the TEa% analytical targets. When evaluated according to the MAU criterion, it was determined that only the D-dimer MU value met the targeted quality specification according to both guidelines.

Discussion

Laboratory results should never be considered absolute values because they are affected by various sources of un-

Table 1. Calibrator uncertainty (uCAL) data was obtained from the manufacturer

Parameter	Product	Reagent	Reference material	Expanded uncertainty of assigned value(s) [%]	uCAL
PT	PT-multi calibrator	Thromborel S	WHO rTF /16, fresh normal plasma samples	L1:2.8 L2:2.9 L3:3.6 L4:6.2 L5:8.1	$\sqrt{(2.8^2+2.9^2+3.6^2+6.2^2+8.1^2)/5} = 5.16$
D-dimer	INNOVANCE D-dimer calibrator	INNOVANCE D-dimer	Master standard	2.4	2.40
Fibrinogen	Fibrinogen calibrator kit	Multifibren U	Internal protein standard	L1:11.1 L2:5.2 L3:10.6 L4:10.6 L5:9.8 L6:8.7	$\sqrt{(11.1^2+5.2^2+10.6^2+10.6^2+9.8^2+8.7^2)/6} = 9.55$

Since the APTT test does not have a calibrator, it is not included in the table. PT: Prothrombin time; APTT: Activated partial thromboplastin time.

Table 2. Measurement uncertainty values calculated according to ISO/TS 20914 guideline

	IQC material	n	Mean	SD	uRW	uCAL	Combined uncertainty	Expanded uncertainty	Mean bias (%)	Desirable bias (%)	TEa%	MAU
PT (sec)	Level 1	355	12.1	0.5	0.73	5.16	5.21	10.42	-0.3	±7.5	±15 (CLIA, WSLH, NYS, AAB)	2.6
	Level 2	505	20.1	0.9								
APTT (sec)	Level 1	212	26.0	0.9	1.75	*	1.75	3.49	3.7	±7.5	±15 (CLIA, WSLH, NYS, AAB)	2.8
	Level 2	564	47.5	2.3								
D-dimer (mg/L)	Level 1	404	0.36	0.03	0.14	2.40	2.40	4.81	2.4	±15	±30 (AAB)	25.2
	Level 2	231	2.73	0.19								
Fibrinogen (g/L)	Level 1	100	2.72	0.19	0.15	9.55	9.55	19.10	-0.3	±10	±20 (CLIA, WSLH, NYS, AAB)	10.2
	Level 2	100	0.84	0.10								

*: Since the APTT test does not have a calibrator, the uCAL value was not added. ISO/TS: International organization for standardization / technical specification; IQC: Internal quality control; n: Average number of laboratories using the same method and the same device in the EQC program; SD: Standard deviation; uCAL: Calibrator uncertainty; TEa: Total allowable error; MAU: Measurement uncertainty; PT: Prothrombin time; APTT: Activated partial thromboplastin time; CLIA: Clinical Laboratory Improvement Amendments; WLSH: Wisconsin State Laboratory of Hygiene; NYS: Wadsworth Center of the New York State Department of Health; AAB: American Association of Bioanalysts.

Table 3. Measurement uncertainty values calculated according to Nordtest guideline

Test	IQC level	N	Mean	SD	$S_{RW\ (Relative)}$	uRW	RMS bias	uCref	Standard uncertainty	Combined uncertainty	Expanded uncertainty	TEa%	MAU
PT (sec)	Level 1	355	12.1	0.5	3.8	4.27	3.00	0.18	3.00	5.22	10.44	±15 (CLIA, WSLH, NYS, AAB)	2.6
	Level 2	505	20.1	0.9	4.7								
APTT (sec)	Level 1	212	26.0	0.9	3.6	4.30	4.63	0.11	4.63	6.32	12.64	±15 (CLIA, WSLH, NYS, AAB)	2.8
	Level 2	564	47.5	2.3	4.9								
D-dimer (mg/L)	Level 1	404	0.36	0.03	8.6	7.78	4.36	0.93	4.46	8.97	17.94	±30 (AAB)	25.2
	Level 2	231	2.73	0.19	6.87								
Fibrinogen (g/L)	Level 1	100	2.72	0.19	7.0	9.95	4.30	0.46	4.32	10.85	21.69	±20 (CLIA, WSLH, NYS, AAB)	10.2
	Level 2	100	0.84	0.10	12.2								

IQC: Internal quality control; N: Numbers of internal quality control data; SD: Standard deviation; uRW: Standard uncertainty component for the within-laboratory reproducibility; RMS: Root mean square; uCref: Uncertainty of nominal values; TEa: Total allowable error, MAU: Maximum expanded allowable measurement uncertainty; PT: Prothrombin time; APTT: Activated partial thromboplastin time; CLIA: Clinical Laboratory Improvement Amendments; WLSH: Wisconsin State Laboratory of Hygiene; NYS: Wadsworth Center of the New York State Department of Health; AAB: American Association of Bioanalysts.

certainty in preanalytical, analytical, and postanalytical processes. MU is crucial for physicians to compare test results accurately and reliably with reference ranges, decision limits, or the patient's previous results. Today, many accreditation programs and international organizations state that MU should be calculated and reported [2, 8–10].

In our study, it was determined that all coagulation tests met TEa% analytical targets according to the ISO/TS 20914 guideline, and PT, APTT, and D-dimer tests met TEa% analytical targets according to the Nordtest guideline. The MU value of the fibrinogen test was found to be borderline higher than the TEa% criterion. When evaluated according to the MAU criterion, the D-dimer MU met the targeted quality specification according to both guidelines, but PT, APTT, and fibrinogen MU values exceeded the allowed targets. It was determined that there was a significant difference between the MU and MAU values of PT and fibrinogen tests in particular. The calibrator uncertainty values of PT and fibrinogen being higher than the analytical target make it challenging to achieve the desired analytical performance. For evaluation according to MAU criteria, *in vitro* diagnostic (IVD) system manufacturers must first optimize their calibrators and improve MU performance.

In a study by Lapić et al. [11], the MU values obtained using the D-dimer HS 500 calibrator on Sysmex CS-5100 and Atellica COAG 360 devices were 12.6% and 15.6%, respectively, and 12.0% and 10.0% when the INNOVANCE D-dimer calibrator was used. Therefore, it was reported that it met the targeted TEa criterion (28.04%). The MU calculated using the INNOVANCE D-dimer calibrator on the ACL TOP 550 device was 28.1% and was found to be borderline higher than the targeted TEa criterion. The results show that MU is significantly affected by changes in instruments and calibrators.

In the study by Qin et al. [10], where MU was evaluated using only external quality assessment program data (Beijing Center for Clinical Laboratory proficiency testing/external quality assessment, Beijing, China), MU values of PT, APTT, and fibrinogen tests on the Sysmex device were found to be 13.6, 15.0, and 11.7, respectively. The calculated MU values of all three parameters were below the CLIA TEa criteria. When evaluated according to Ricos TEa criteria, although the MU value of fibrinogen met the targeted criterion, the MU of PT and APTT tests was higher than the target.

In the study by Lim et al. [12], the MU value of fibrinogen on the ACL TOP 750 CTS device was reported to be 9.9% when certified reference materials with a target value of 270 mg/L were used. It was suggested that when the fibrinogen test was calculated using EQC material at target values of 306.6 mg/L, 120.1 mg/L, and 83.8 mg/L, MU values were determined as 12.2%, 17.0%, and 21.3%, respectively. For the APTT test, MU values calculated using EQC material at target values of 27.9 sec, 58.0 sec, and 79.5 sec were found to be 13.5%, 15.1%, and 9.0%, respectively.

In the study by Matar et al. [13], where MU was investigated only with external quality assessment program data (ProBioQual, France, member of EQALM), MU values of fibrinogen and APTT tests calculated according to the group average of all participat-

ing laboratories were 13.4% and 18.1%, respectively. According to the peer group average (laboratories using the same test method, the same reagents, and the same device), MU values were 11.1% and 6.4%, respectively. When MU values calculated for both groups were evaluated according to Ricos TEa criteria, fibrinogen met the analytical target, while APTT did not.

The literature investigating MU in coagulation parameters is limited, and no study has evaluated MU for all four parameters in our study simultaneously. The strengths of our study are that it is the first to investigate MU in coagulation parameters in our country, calculate MU according to ISO/TS 20914 and Nordtest guidelines, and evaluate MU according to MAU analytical targets in addition to TEa%.

One limitation of our study is that preanalytical and postanalytical sources of uncertainty were not evaluated. Another is that calculations were based only on five months of data due to IQC lot changes.

Since different results can be obtained by using different MU calculation models for the same analyte in clinical laboratories, and there are multiple allowable analytical performance specification options for comparing MU values, it is essential to standardize MU calculation methods and performance targets in laboratories. The calculation needs to be revisited in cases of operator, equipment, calibrator, and reagent changes, as these may affect MU. Furthermore, to achieve more reliable MU values, we believe that in addition to analytical uncertainty, studies should include all measurable sources of uncertainty attributable to preanalytical and postanalytical steps that may affect test results.

In conclusion, our study demonstrates the importance of standardizing the MU calculation model in laboratories and carefully managing MU sources such as calibrator uncertainty to improve the analytical performance of coagulation tests. Reducing assay uncertainty through improved calibrators and methodological standardization should be a priority for both laboratories and IVD manufacturers. Standardizing the calculation of MU and aligning it with laboratory-specific performance criteria is critical to ensure the reliability of coagulation tests.

Ethics Committee Approval: The study was approved by The Bakircay University Cigli Training and Research Hospital Non-interventional Clinical Research Ethics Committee (No: 1648, Date: 03/07/2024).

Conflict of Interest: The authors declare that there is no conflict of interest.

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Research Article

Diagnostic accuracy of the combination of fecal calprotectin and occult blood tests in inflammatory bowel disease

Kubranur Unal[1], Ali Karatas[2], Ozlem Gulbahar[1], Cansu Ozbas[3]

[1]Depertmant of Medical Biochemistry, Gazi University Faculty of Medicine, Ankara, Türkiye
[2]Department of Gastroenterology, Gazi University Faculty of Medicine, Ankara, Türkiye
[3]Department of Public Health, Gazi University Faculty of Medicine, Ankara, Türkiye

Abstract

Objectives: This study aimed to assess the diagnostic accuracy of the fecal occult blood test (FOBT), fecal calprotectin (FC), and the combination of these markers in patients with suspected inflammatory bowel disease (IBD). Additionally, FC levels were compared between patients monitored for IBD and those newly diagnosed with IBD.

Methods: Conducted at Gazi University Application and Research Hospital, this retrospective study reviewed demographic, clinical, colonoscopy reports, and laboratory data (FC and FOBT) of IBD patients. The final analysis included 153 patients with suspected IBD to evaluate the diagnostic accuracy of FOBT, FC, and their combination. FC was analyzed using the Quantum Blue® fCAL extended test. The ROC curve was drawn to determine the diagnostic ability of FC, and the area under the curve (AUC) was calculated. Sensitivity, specificity, and predictive values were determined for FC and FOBT.

Results: The AUC was determined as 0.827 (95% CI:0.742–0.913) for FC ($p<0.001$). FC showed a sensitivity of 85.7%, specificity of 62.4%, positive predictive value (PPV) of 30.6%, and negative predictive value (NPV) of 95.8%. FOBT had a sensitivity of 81.3%, specificity of 78.1%, PPV of 30.2%, and NPV of 97.3%. The combination of FOBT and FC, with positivity in at least one of the tests, had a sensitivity of 93.8%, specificity of 63.5%, PPV of 23.1%, and NPV of 98.9%. The combined use of FOBT and FC demonstrated higher diagnostic accuracy than either test alone.

Conclusion: The combination of FOBT and FC provides superior diagnostic accuracy for identifying suspected IBD patients compared to each test alone. This combined approach could serve as a cost-effective strategy to avoid unnecessary invasive procedures.

Keywords: Diagnostic accuracy, fecal calprotectin, fecal occult blood test, inflammatory bowel diseases

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Abdominal pain, changes in bowel habits, rectal bleeding, and iron-deficiency anemia are indicative of gastrointestinal system (GIS) disorders. However, most patients presenting with these symptoms do not have any pathological condition [1]. Nevertheless, to accurately diagnose patients and avoid missing serious GIS pathologies such as cancer, some of these patients undergo further investigations [2].

Although colonoscopy is one of the methods most commonly used for many GIS disorders, it is an expensive and invasive method. Using simpler, cheaper, and non-invasive tests is an increasingly popular approach to distinguish between GIS disorders [3]. The fecal occult blood test (FOBT) is the most essential primary screening test for colorectal cancer. However, the prevalence of GIS bleeding is high in patients with inflammatory bowel disease (IBD). In these patients, FOBT positivity may be a late sign of inflammatory tissue damage [4]. On the other hand, the fecal calprotectin (FC) test, a relatively new tool in clinical laboratories, assists in diagnosing GIS disorders [2]. FC is a crucial biomarker for differentiating between inflammatory and non-inflammatory GIS disorders and evaluating intestinal mucosal inflammation in human stool samples. Increased FC concentrations in the intestinal

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Address for correspondence: Kubranur Unal, MD. Depertmant of Medical Biochemistry, Gazi University Faculty of Medicine, Ankara, Türkiye
Phone: +90 538 671 12 75 **E-mail:** kubranurunal@gazi.edu.tr **ORCID:** 0000-0001-7940-4590
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lumen indicate the migration of neutrophils to the mucosa and the severity of inflammation [5]. The concentration of FC in the stool is higher than in the plasma of patients with IBD. FC is widely used to diagnose IBD, monitor patients with IBD, and predict relapse in IBD patients [6].

IBD is a chronic inflammatory disease that can affect any part of the GIS tract and has a course of remission and flare-ups. Studies on disease activity and treatment monitoring in IBD show that biomarkers such as FC, C-reactive protein (CRP), albumin, and white blood cell counts are commonly used [7]. However, it has been observed that the FC test is more consistent with colonoscopy results than the other tests [8].

Literature review indicates that different studies have been conducted on the effectiveness of biomarker use in IBD diagnosis. The studies show that combining FC and FOBT tests is a valuable strategy for determining the need for a colonoscopic examination [9, 10]. However, other studies have suggested that combining these two tests does not provide additional information for evaluating GIS pathologies [11, 12].

Examining the effects of the combined use of these tests could provide essential insights into the diagnosis, follow-up, and exclusion of IBD. The combination could also serve as a predictor prior to diagnostic colonoscopy in patients with IBD symptoms. Therefore, this study aimed to investigate the diagnostic accuracy of FOBT, FC, and the combination of these markers (FOBT and FC) in patients with suspected IBD. Furthermore, this study aimed to compare FC levels between patients monitored for IBD and those newly diagnosed with IBD.

Materials and Methods

Study design and population

The study was approved by the Gazi University Clinical Research Ethics Committee (decision number: 847) on November 21, 2022. Since there was no direct patient participation, patient consent was not deemed necessary by the Ethics Committee. This study was conducted in accordance with the principles of the Helsinki Declaration.

The medical records of patients admitted to the gastroenterology outpatient clinic of Gazi University Medical Faculty Hospital between June 2021 and November 2022 were retrospectively reviewed. FOBT and FC tests were performed during the follow-up period in patients previously diagnosed with IBD, including ulcerative colitis and Crohn's disease, to assess disease severity. Tests were also performed on patients with suspected IBD who had symptoms such as persistent diarrhea, abdominal pain, rectal bleeding/bloody stool, weight loss, and fatigue, to diagnose the disease.

Colon examinations were performed when necessary. Diagnosis of IBD was confirmed by colonoscopy and by histologists. Patients' demographic and clinical information, colonoscopy reports, and laboratory data (FC and FOBT) were retrieved from the Laboratory Information Operation System (L.I.O.S.) and electronic patient records were retrospectively reviewed.

A total of 440 patients met the inclusion criteria; patients with IBD were followed up with a diagnosis of IBD, and patients with suspected IBD were treated at the outpatient clinic to confirm the diagnosis.

Exclusion criteria included patients under 18, pregnant women, and those missing colon examination results (colonoscopy or radiological imaging). Additionally, FC test requests from clinics other than gastroenterology were excluded from the study.

Patients with a confirmed final diagnosis via colon examination and whose specialist physician requested an FC test from the outpatient clinic during this process were included in the study.

Laboratory methods

FOBT and FC analyses of the stool samples were performed at the Gazi University Hospital Medical Biochemistry Laboratory.

Immunochemical-based FOBT is commonly used to detect human hemoglobin in feces. There are two types of FOBT available: qualitative (based on immunochromatography, yielding a positive or negative result) or quantitative (based on latex agglutination immunoturbidimetry, yielding a fecal hemoglobin concentration-dependent result) [13]. This study analyzed FOBT using the Toyo cassette test (Türklab, Turkiye), a qualitative immunochromatographic method. Any sample reported by the analytical system as a positive result above 10 ng hHb/mL was considered as a "detectable FHb." The specificity and sensitivity of FOBT were 99.9% and 97%, respectively.

FC was analyzed using the Quantum Blue® fCAL extended test (Buhlmann Laboratories AG, Schonenbuch, Switzerland).

BÜHLMANN Quantum Blue® fCAL is an *in vitro* diagnostic test for quantitatively determining FC extended to human stool specimens. The test was designed to measure FC antigens selectively using a sandwich immunoassay. The FC concentration was measured using a semi-quantitative lateral flow assay (Quantum Blue Reader®, Bühlmann Laboratories, Switzerland). Different manufacturers and laboratories may use varying cut-off values, such as 50 µg/g [9]. However, we adhered to the manufacturer's cut-off values for the BÜHLMANN Quantum Blue® fCAL assay: normal ≤80 µg/g, borderline 80–160 µg/g, and abnormal ≥160 µg/g [14]. The specificity and sensitivity of FC were 91.9% and 64.9%, respectively.

For patients with suspected IBD, FC concentrations of <80 µg/g feces were considered normal, and IBD was ruled out. In these patients, the risk of IBD is less than 1%. Patients with low FC levels are unlikely to require invasive procedures to determine the cause of inflammation. FC concentrations between 80 and 160 µg/g are considered borderline, also called gray-zone levels. Mid-range FC levels do not directly indicate active inflammation and require immediate follow-up and invasive testing; however, inflammation cannot be ruled out. Re-evaluation of FC levels after 4–6 weeks is recommended at gray-zone levels to assess the inflammatory status. FC values of >160 µg/g indicate neutrophil infiltration in the GIS tract, which may indicate the presence of active inflammatory disease. These concentrations require more precise interven-

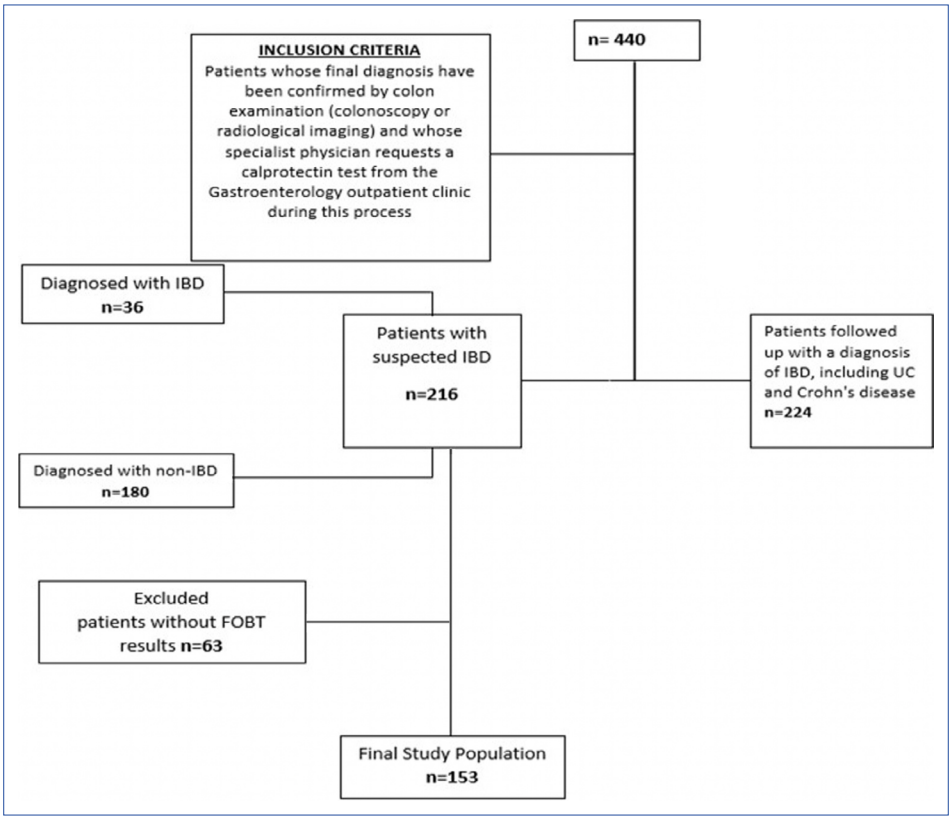

Figure 1. Study flow diagram of inclusion and exclusion.

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; FOBT: Fecal occult blood test.

tions and deeper clinical workup. Further investigations, such as colonoscopy or radiological imaging procedures, are suggested to achieve an overall clinical diagnosis.

Statistical analysis

When calculating the sample size, relevant literature and reference studies were taken into consideration. The Power Statistics Program was used for sample size calculation, with relevant literature as a reference. The research data were evaluated using the SPSS 28.0 statistical package. The Shapiro–Wilk test was used to determine whether the data were normally distributed. Descriptive statistics are presented as median (IQR 25–75), frequency distribution, and percentage. The chi-square test was used for categorical variables. The Kruskal–Wallis and Mann–Whitney U tests were employed, as the data for continuous variables did not meet parametric test conditions (normal distribution and homogeneity of variances). To assess the diagnostic utility of FC in diagnosing IBD, the area under the curve (AUC) and threshold values were determined using receiver operating characteristic (ROC) curve analysis. Sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) were calculated for FC and FOBT. Statistical significance was accepted at $p<0.05$.

Results

A total of 440 patients, whose final diagnosis was confirmed by colon examination (colonoscopy or radiological imaging) and whose specialist physician requested FC testing from the gastroenterology outpatient clinic, were included in this study.

Of the 440 patients, 224 were followed up with a diagnosis of IBD, including ulcerative colitis (UC) and Crohn's disease, while 216 patients were suspected of having IBD. After further examinations, 36 of the 216 patients were diagnosed with IBD, while the remaining 180 patients received non-IBD diagnoses, such as cancer, polyp, and aneurysm. A total of 153 of the 216 patients were included in the final analysis to evaluate the diagnostic accuracy of FOBT, FC, and their combination for diagnosing patients with suspected IBD. 63 patients without FOBT results were excluded from the study; the study flow diagram for inclusion and exclusion is shown in Figure 1.

In this study, a similar proportion of both genders participated, and there were no significant differences in gender and age between groups. The groups in the study were classified as follows: Group I = Patients followed up with IBD, Group II = Diagnosed with IBD, Group III = Diagnosed with non-IBD. The FC median value of patients followed up with IBD (Group I) was 198 µg/g (IQR 25–75: 57–809), while the FC median value of patients diagnosed with IBD (Group II) was 708 µg/g (IQR 25–75: 114–1000). The FC median value of patients with non-IBD (Group III) was 36 µg/g (IQR 25–75: 30–178). There was a significant difference in FC levels between the groups ($p<0.001$). The demographic data and FC levels in the groups are shown in Table 1.

Table 1. Demographic data and FC levels (µg/g) changes in the studied groups.

	Group I (n=224)	Group II (n=36)	Group III (n=180)	p
Age, median (IQR 25–75)	41 (31–55)	39 (27–51)	44 (28.5–58)	0.345
Gender, n (%)				
Female	114 (48.7)	20 (8.5)	100 (42.7)	0.606
Male	110 (53.4)	15 (7.3)	81 (39.3)	
FC (µg/g), median (IQR 25–75)	198 (57–809)	708 (114–1000)	36 (30–178)	**<0.001**

FC: Fecal calprotectin; IQR: Interquartile range.

FC levels (µg/g) were significantly higher in Group I compared to Group II and Group III (p=0.013, p<0.001, respectively). FC levels were statistically significantly lower in Group III than in the other two groups. The distribution of FC levels (µg/g) across groups is shown in Figure 2.

Based on the ROC curve analysis, the AUC was determined as 0.827 (95% CI: 0.742–0.913) for FC (p<0.001). The ROC analysis for FC is visually represented in Figure 3.

For detecting IBD, FC returned a sensitivity of 85.7%, specificity of 62.4%, PPV of 30.6%, and NPV of 95.8%. FOBT had a sensitivity of 81.3%, specificity of 78.1%, PPV of 30.2%, and NPV of 97.3%. The combination of FOBT and FC, with positivity in at least one of the two tests, was associated with a sensitivity of 93.8%, specificity of 63.5%, PPV of 23.1%, and NPV of 98.9%. Diagnostic accuracy performance (sensitivity (%), specificity (%), NPV (%), and PPV (%)) for FOBT, FC, and the combination of tests for detecting IBD are presented in Table 2. Cut-off values for FOBT and FC were determined as "positive" and 80 µg/g, respectively, for this study.

Discussion

IBD is a chronic, progressive, and highly heterogeneous disease that affects both adults and children, with severity and symptoms ranging from mild to severe. Delays in diagnosing IBD are common, correlating with adverse outcomes and potentially leading to significant morbidity and complications, including fissures, fistulas, systemic inflammation, and cancer [15]. The initial goal in diagnosing the disease is to evaluate disease activity and relieve symptoms, while long-term objectives aim to prevent disease progression and reduce complications. Early diagnosis of IBD and prompt treatment are essential for improving outcomes and maximizing health [16]. There is no gold standard diagnostic method for IBD; instead, clinical assessment, endoscopic examination, histopathological analysis, and laboratory tests are utilized. FC levels emerge as a valuable biomarker, strongly correlating with endoscopic activation [17]. The Food and Drug Administration has approved FC as a biomarker of intestinal inflammation, with potential clinical applications as a diag-

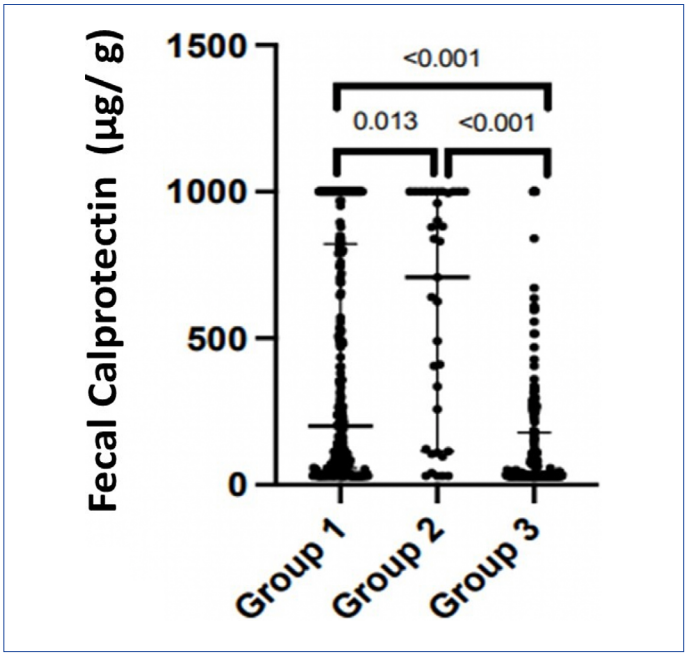

Figure 2. Distribution of FC levels in groups.
FC: Fecal calprotectin.

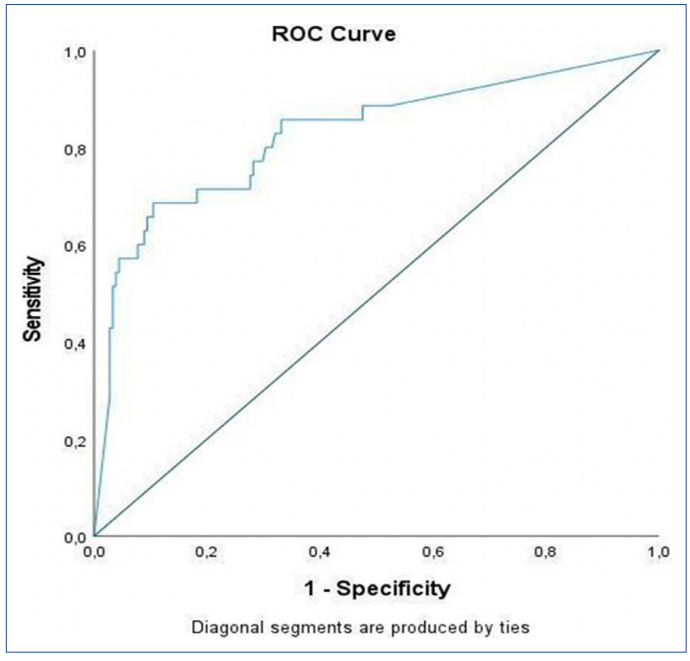

Figure 3. ROC analysis for FC.
ROC: Receiver operating characteristic; FC: Fecal calprotectin.

Table 2. Diagnostic accuracy performance of FOBT, FC and the combination of both these markers

	FC	FOBT	FOBT and/or FC
Total number of cases	216	153	153
True positives (n)	30	13	15
True negatives (n)	113	107	87
False positives (n)	68	30	50
False negatives (n)	5	3	1
NPV (%)	95.8	97.3	98.9
PPV (%)	30.6	30.2	23.1
Sensitivity (%)	85.7	81.3	93.8
Specificity (%)	62.4	78.1	63.5

FOBT: Fecal occult blood test; FC: Fecal calprotectin; NPV: Negative predictive value; PPV: Positive predictive value.

nostic and follow-up adjunct for IBD [18, 19]. Therefore, it is crucial for monitoring disease activity, assessing treatment efficacy, and detecting postoperative recurrences.

In our retrospective study, we observed a significant difference in FC levels between groups, including patients followed up with IBD and patients diagnosed with IBD and non-IBD. Furthermore, it was determined that FC levels were higher in patients diagnosed with IBD than in patients who were followed up with IBD. Studies evaluating disease progression using FC levels have been reported in the literature. In one study, FC levels were higher in patients newly diagnosed with IBD than in those under follow-up, consistent with our findings [20]. A study by Zhu et al. [21] found that FC levels in the active IBD group were significantly higher than those in the standard group, while no difference was observed between the inactive IBD and control groups. A decrease in FC levels is expected during the IBD follow-up period, corresponding with disease treatment. It should be noted that high FC levels during follow-up indicate disease relapses [22].

Diagnostic accuracy is crucial for tests and markers used to evaluate IBD [23]. This study investigates the diagnostic accuracy of FOBT, FC, and the combination of these markers in patients with suspected IBD.

Before the routine clinical use of FC in diagnosing and monitoring IBD, numerous studies have been conducted on appropriate cut-off values for IBD diagnosis and the diagnostic accuracy of FC [18, 24, 25]. Although many studies have demonstrated the diagnostic accuracy of FOBT for colorectal cancer, studies have also examined FOBT's diagnostic accuracy for IBD [10, 26].

The diagnostic accuracy of combining FOBT and FC for various GIS pathologies, including IBD, has been evaluated in previous studies [9, 27]. This study is the first retrospective investigation into the role of combining FOBT and FC specifically for predicting IBD diagnosis. Our literature review reveals limited data on the diagnostic accuracy for patients with IBD.

In our study, FC had a sensitivity of 85.7%, specificity of 62.4%, and NPV of 95.8%. When combined with FOBT, the sensitivity, specificity, and NPV were 93.8%, 63.5%, and 98.9%, respectively. Sensitivity, specificity, and NPV increased with this combination. These results indicate that combining FOBT and FC had better diagnostic accuracy than each test used alone in our study. Lué et al. [9] showed that the combination of FOBT and FC had a sensitivity of 89.7% and NPV of 100% for diagnosing IBD, which was higher than when either test was used alone, similar to our findings. A study by Mowat et al. [11] found that combining FOBT and FC achieved a sensitivity of 99.3% and NPV of 97.1% for diagnosing IBD when using an FC cut-off value of 50 µg/g. Although different FC cut-off values were used in that study, the combination outperformed both tests used alone, in line with our findings.

Högberg et al. [28] demonstrated that the combination of FOBT and FC had a sensitivity of 90% and an NPV of 99.6% when using a cut-off value of 100 µg/g for FC. Additionally, when the cut-off value was 20 µg/g, they found that the combination of both tests did not provide additional diagnostic information for IBD. Our findings differ from the conclusions reached in other studies, where the combination of both tests did not appear to provide additional information. In a study by Widlak et al. [12], the tests demonstrated a sensitivity of 86% and an NPV of 100% for diagnosing IBD, both when assessed individually and in combination. When these studies are examined, the results are broadly similar, showing that the combination offers better diagnostic accuracy than the tests used alone. However, sensitivity and NPV percentages vary, potentially due to population characteristics, whether the FOBT is qualitative or quantitative, and the chosen cut-off values.

Our study observed that the combined utilization of FOBT and FC demonstrated superior diagnostic accuracy in identifying patients with suspected IBD compared to individual tests alone. This finding suggests that implementing a combination of FOBT and FC before colon examination (colonoscopy or radiological imaging) is a cost-effective strategy to mitigate unnecessary procedures and reduce potentially associated complications.

Limitations

This study has some limitations, including the short data collection period and the fact that not all patients had both FOBT and FC results. In our study, we used data from 216 patients to assess the diagnostic accuracy of FC in suspected IBD, with FC results available in our hospital registry. However, not all of these patients had FOBT results. To ensure data consistency and completeness in our analysis, we excluded patients without an FOBT result when evaluating the diagnostic accuracy of the combination. Additionally, using quantitative methods in evaluating FOBT prevented us from obtaining AUC values for both FOBT alone and in combination with FC. Despite this, our approach allows for a comprehensive evaluation of the diagnostic utility of the combined tests in suspected cases of IBD and adds valuable information to clinical practice.

Conclusion

In conclusion, the sensitivity and NPV of the FC and FOBT combination are notably high compared to using them alone. We believe that this combination can be a valuable strategy for identifying patients with suspected IBD and for accurately identifying truly negative cases. These findings suggest that FC testing combined with FOBT could help clinicians identify patients who may benefit from further diagnostic evaluations for IBD, ultimately facilitating timely and appropriate management strategies. Well-designed studies are needed to confirm whether a positive FOBT and/or elevated FC test is a reliable predictor of IBD and therefore an indication for colonoscopy. For this reason, we designed this study. However, larger-scale studies are needed to confirm these findings, and research on the effectiveness of this testing approach for different types of IBD and varying disease activity levels is required.

Ethics Committee Approval: The study was approved by The Gazi University Clinical Research Ethics Committee (No: 847, Date: 21/11/2022).

Authorship Contributions: Concept – K.U.; Design – K.U., A.K., C.O.; Supervision – K.U.; Funding – K.U.; Materials – K.U., A.K.; Data collection &/or processing – K.U., A.K., C.O.; Analysis and/or interpretation – K.U.; Literature search – K.U.; Writing – K.U., C.O.; Critical review – K.U., A.K., O.G.

Conflict of Interest: The authors declare that there is no conflict of interest.

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Technical Report

Evaluation of the hemolysis threshold for the measurement of serum lipase on Roche Cobas systems

Claudio Ilardo[1], Batricia Al Muhanna[2], Chèhine Lamarti[2]

[1]Laboratory of Inovie Labosud, Montpellier, France
[2]UFR Pharmaceutical and Biological Sciences, Montpellier, France

Abstract

Objectives: Following the release of an informational bulletin, Roche Diagnostics adopted a more restrictive hemolysis index (100 HI) for the release of serum lipase results on all Cobas systems. This study aimed to evaluate the interference threshold for serum lipase hemolysis on Cobas C501/311/701/Integra 400 systems using a total allowable error set by the Royal College of Pathologists of Australasia (RCPA).

Methods: To assess the influence of hemolysis on lipase, the parameter was quantified in serum pools spiked with escalating concentrations of a hemolysis interferent. The lipase assay was performed using the colorimetric lipase method (LIPC), and the HI was determined by absorbance measurements of diluted samples in accordance with the system protocol.

Results: The Cobas Integra 400 and Cobas C311 showed the greatest interference of lipase with hemolysis (≤300 HI). The Cobas C501 and C701 demonstrated less sensitivity to hemolysis (≤1300 HI).

Conclusion: The results of this study demonstrate that interference limits may vary between different Roche systems, even when the same reagent is used. Our study indicated that the lipase hemolysis threshold (100 HI) currently set by the manufacturer was excessively restrictive. This finding highlights the necessity of verifying manufacturers' information bulletins to provide better medical care.

Keywords: Hemolysis, interference, lipase, threshold

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Lipase is a glycoprotein that reduces triglycerides and diglycerides to free fatty acids and glycerol by catalyzing the hydrolysis of ester bonds [1]. Lipases are primarily present in pancreatic secretions but can be found in several tissues, including hepatic lipase in the liver, hormone-sensitive lipases in adipocytes, lipoprotein lipase in endothelial cells, and pancreatic lipase in the small intestine [2]. Clinically, a lipase test may be used to help diagnose disorders of the pancreas, most often acute pancreatitis. Other pathologies can cause an increase in blood lipase levels, such as pancreatic cancer, gallbladder issues, chronic kidney disease, intestinal problems, cancer of a salivary gland, peptic ulcer disease, primary biliary cirrhosis, and certain drugs [3]. The quantitative determination of lipase in human serum and plasma on the Roche Cobas c system is based on the enzymatic hydrolysis of 1,2-O-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester, followed by monitoring the production of resorufin, measured photometrically between 570 and 583 nm. The intensity of the red color developed is directly proportional to lipase activity. Hemolysis involves the release of hemoglobin into the extracellular compartment [4]. Oxyhemoglobin and deoxyhemoglobin have maximum absorption at 415 nm, with a detection range between 320 and 450 nm and 540 and 589 nm, respectively [5]. Thus,

Address for correspondence: Claudio Ilardo, MD. Laboratory of Inovie Labosud, Montpellier, France
Phone: 0 663898904 **E-mail:** calogero.ilardo@labosud.fr **ORCID:** 0000-0002-0708-5516
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colorimetric assays using absorbance measurements within these ranges may be susceptible to spectral interference [6, 7]. According to Ishiguro et al. [8], spectral interference is linked to the additivity of the Beer-Lambert law and will be observed for parameters whose final measurement is spectrophotometric and carried out at wavelengths close to hemoglobin's main absorption wavelengths (415, 540, and 578 nm). Following an informational bulletin, Roche's quality control center evaluated the potential for interference in serum lipase results due to hemolysis. The hemolysis index (HI) threshold for Cobas C501/311/502/701/702 instruments was adjusted from 1000 to 100, while the Cobas Integra 400 plus and Cobas C111 systems were adjusted from 300 to 100 [9]. This change could result in more lipase results being rejected. The aim of this study was to evaluate the interference threshold for serum lipase hemolysis on Cobas C501/311/701/Integra 400 systems, using a total allowable error set by the RCPA as the limit [10].

Materials and Methods

Ethical approval

The laboratory investigations were conducted in accordance with the General Data Protection Regulation (EU Regulation 2016/679 and Directive 95/46/EC) and the French data protection law (Law 78–17 of 6 January 1978 and Decree 2019–536 of 29 May 2019). The reuse of human body elements and products for medical or scientific purposes other than that for which they were removed or collected is permitted (Article L.1211-2 of the Public Health Code) and does not require ethics committee review.

Instruments

The HI and lipase activities were determined using four different analytical systems (Cobas C501/311/701/Integra 400, Roche Diagnostics, Mannheim, Germany). The lipase assay was performed using the colorimetric lipase method (LIPC),

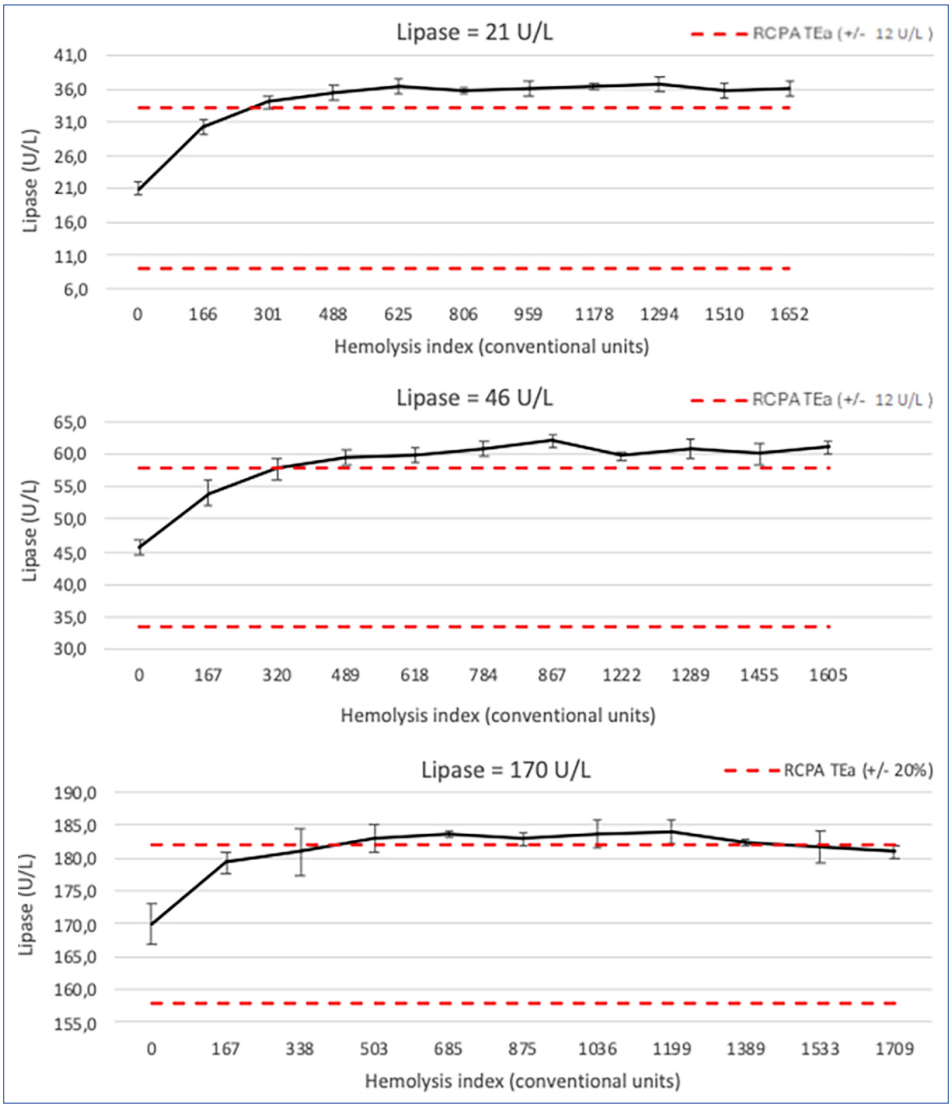

Figure 1. The lipase measured from the Cobas C311 according to the hemolysis index.
RCPA: Royal College of Pathologists of Australasia; TEa: Total error allowable.

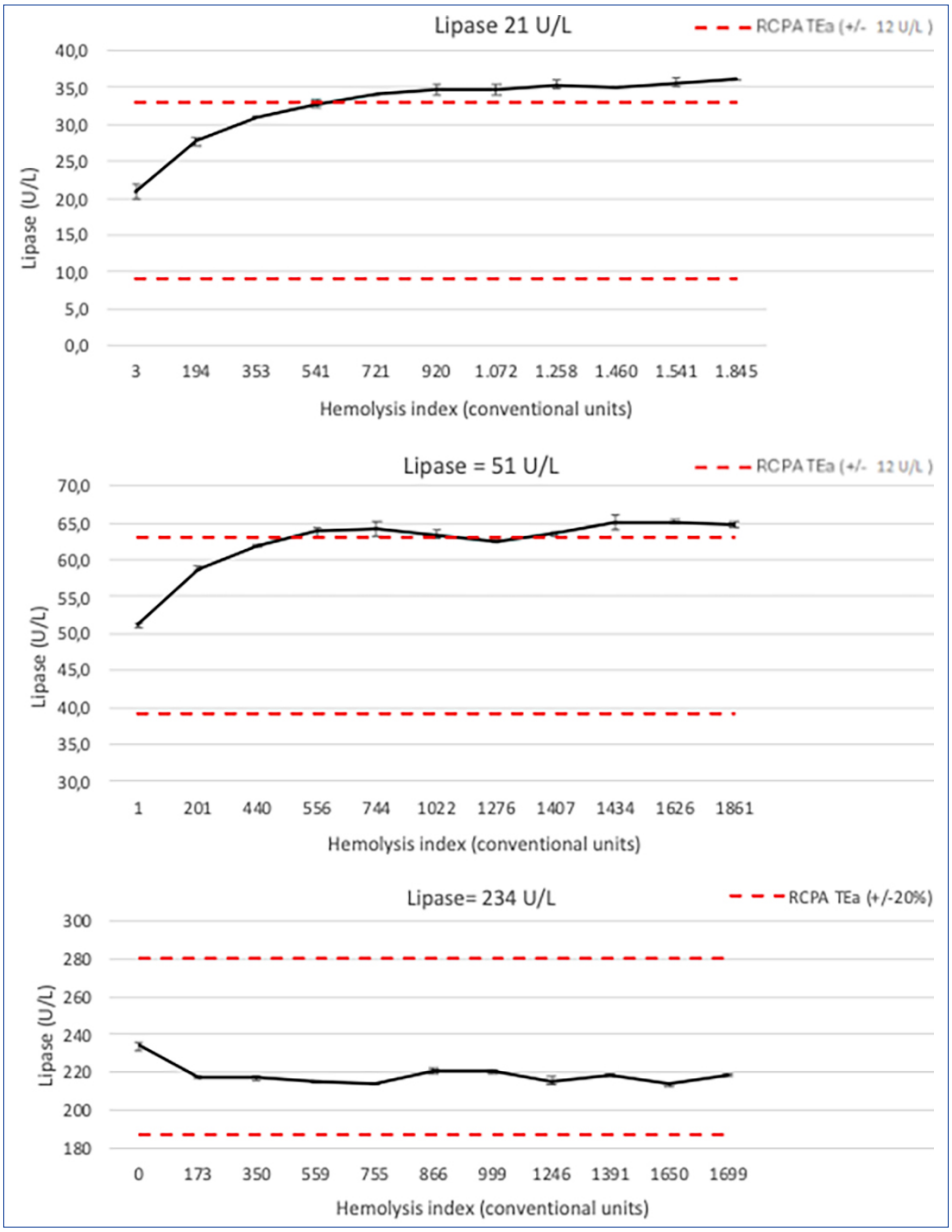

Figure 2. The lipase measured from the Cobas Integra 400 according to the hemolysis index.
RCPA: Royal College of Pathologists of Australasia; TEa: Total error allowable.

and the HI was determined by absorbance measurements of diluted samples in accordance with the system protocol. The expanded uncertainty for lipase was estimated at 15.4%, with a 95% confidence interval (CI) between 10.9% and 26.1%. Technical data sheets recommended an HI cutoff of 1000 for Cobas C501/311/701 and 300 for Cobas Integra 400, based on the requirement for a recovery of ±10% of the initial value. The HI unit is equivalent to a hemoglobin concentration of approximately 1 mg/dL, as stated by the manufacturer. Regarding the hemolysis index, the expanded uncertainty was estimated at 18.8%, with a 95% CI between 12.4% and 38.3%.

The Cobas Integra employs dual measurement at 589/659 nm, while the Cobas C311, C501, and C701 perform dual measurement at 570/700 nm.

Preparing the hemolysate and serum pools spiked

Serum pools with an HI of less than 10 were prepared to obtain three concentration ranges of lipase: approximately 22 U/L (normal), 50 U/L (close to the clinical threshold), and 180 U/L (high). To assess the influence of hemolysis on lipase, the parameter was quantified in serum pools spiked with escalating concentrations of a hemolysis interferent. The discrepancy between the result and the non-spiked baseline value was evaluated against the RCPA total error allowable (TEa). The hemolysate was produced from whole blood samples collected in lithium-heparin blood tubes in accordance with CLSI recommendations by osmotic shock [11]. A blood sample from a healthy subject was spun at 2000 g for 10 minutes. The red blood cells were obtained

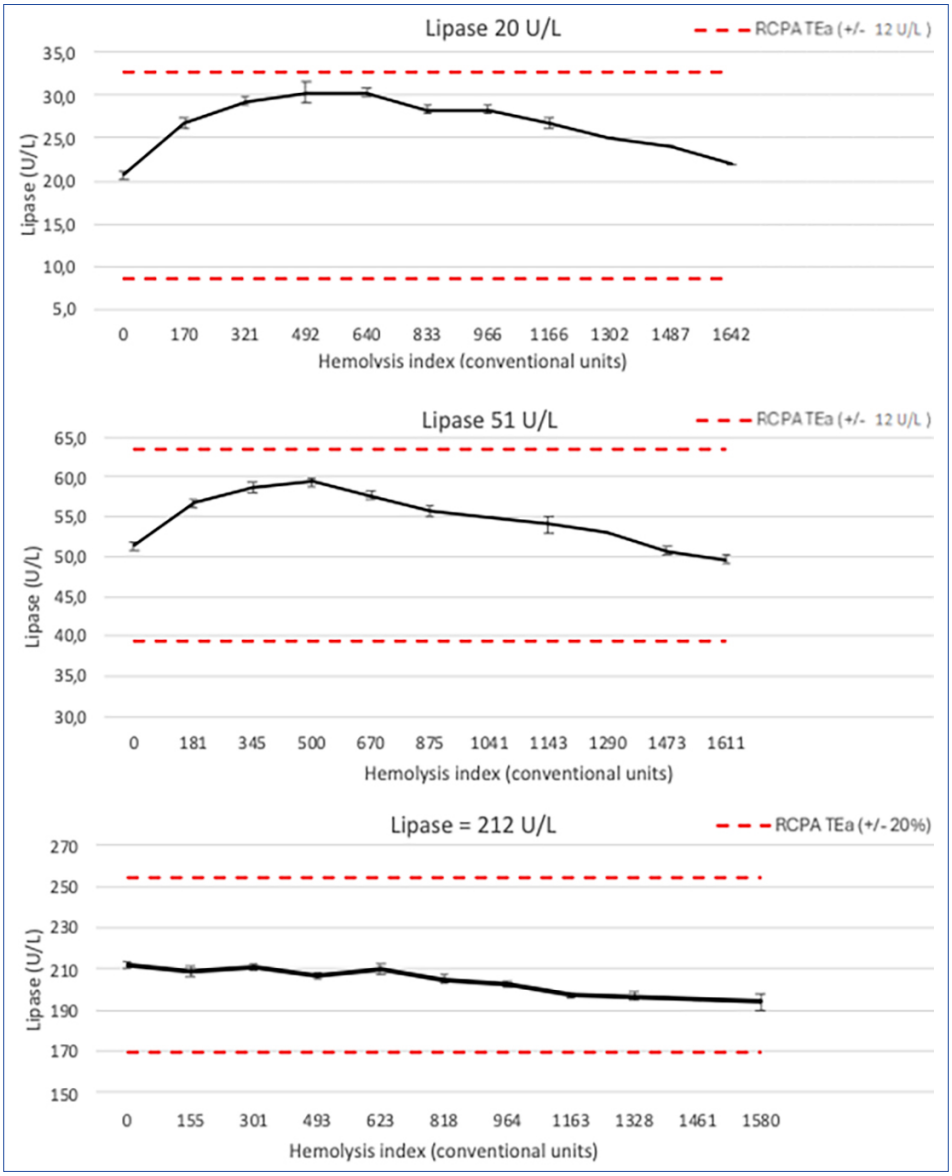

Figure 3. The lipase measured from the Cobas C501 according to the hemolysis index.
RCPA: Royal College of Pathologists of Australasia; TEa: Total error allowable.

after the plasma and buffy coat were removed, followed by three washes with physiological serum. The red blood cells were lysed by adding distilled water in a 1:1 ratio and centrifuged at 4000 g for 10 minutes to remove cellular debris. The hemoglobin content of the hemolysate was determined using the Siemens RP500® analyzer. The hemolysate was stored at -20°C until required.

Data analysis

For each system, the impact of hemolysis on lipase was measured in pools of serum spiked with increasing concentrations of hemolysis interferent. The result was assessed against the RCPA TEa. The relative deviation is shown on the graphs, with the acceptability criteria (RCPA TEa) represented by horizontal dotted lines (+/- 12 U/L for lipase values below 60 U/L and +/- 20% for lipase values above 60 U/L).

Results

Cobas C311

For this instrument, regardless of the lipase concentration, positive interference occurred from a hemolysis index of 300. The observed overestimation did not exceed 15 U/L of the true value, irrespective of the hemolysis level (Fig. 1).

Cobas Integra 400

For this system, lipase activity measurement was positively affected by hemolysis but only for normal or borderline positive lipase activity. At the specified concentration levels, overestimation did not exceed 15 U/L of the true value, regardless of hemolysis degree. In contrast, for high lipase activities (approximately 240 U/L), a hemolysis index exceeding 170 HI resulted in a 6% reduction in the measured activity (Fig. 2).

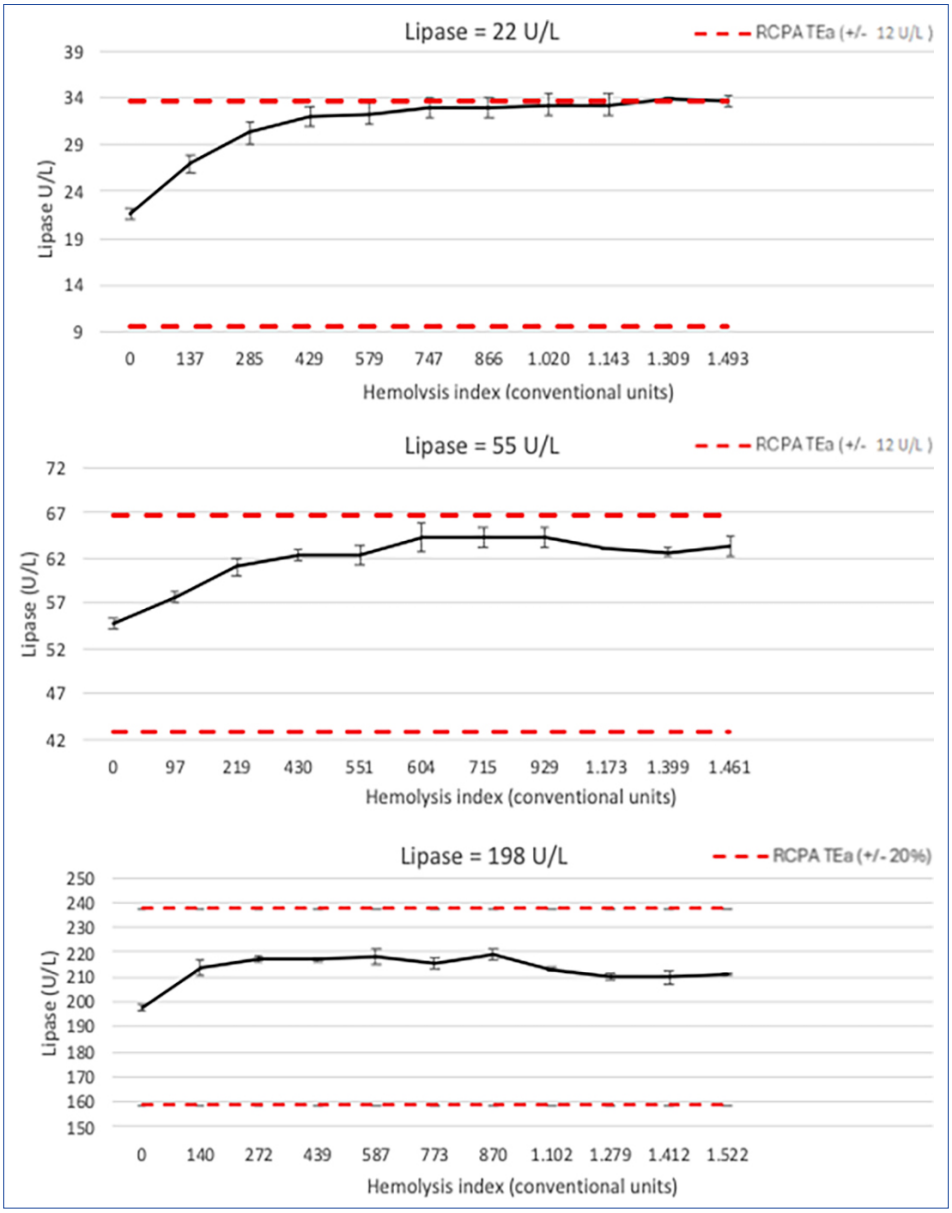

Figure 4. The lipase measured from the Cobas C701 according to the hemolysis index.
RCPA: Royal College of Pathologists of Australasia; TEa: Total error allowable.

Cobas C501

For this system, normal or borderline positive lipase activity was positively affected by hemolysis up to 500 HI (without exceeding the RCPA TEa). Thereafter, a decline was observed until values returned to approximate those of the non-spiked serum. For high lipase activities (approximately 212 U/L), a hemolysis index exceeding 1000 HI resulted in a 10% reduction in measured activity (Fig. 3).

Cobas C701

For normal lipase activity, measurement was positively influenced by hemolysis up to 1143 HI. For borderline high lipase activity, hemolysis caused a moderate increase in lipase (without exceeding the RCPA TEa) (Fig. 4).

Discussion

Despite the identicality of lipase assay methods, different interactions with hemolysis may be observed depending on the system used. The Cobas Integra 400 (previous generation) and Cobas C311 (latest generation) showed the greatest interference of lipase with hemolysis (from 300 HI). The Cobas C501 and C701 (intermediate generation systems) demonstrated less sensitivity to hemolysis when measuring lipase, with no significant interference up to 1000 HI. Differences in sensitivity to hemolysis among the Roche systems are unclear. Although measurement wavelengths differ slightly (589/659 nm for Cobas Integra 400), this difference does not account for hemolysis sensitivity, as the Cobas Pure, using identical measurement wavelengths to the Cobas C311 and C701 (570/700 nm), exhibited similar sensitivity.

Spectral interference with lipase was not proportional to the extent of hemolysis and did not occur throughout the entire measurement range. This phenomenon is characteristic of spectral interference [12]. Interference was more pronounced for normal values and reduced or negligible for higher values, enabling reliable results with minimal risk of over- or under-estimation.

Data sheet hemolysis interference limits differed only for Cobas C311 (1000 versus 300), justifying a decrease in the hemolysis index alert threshold. For other systems, our findings aligned with data sheets, indicating no need to adjust the alert threshold.

A statistical analysis of the hemolysis indices of 1,260 samples on our platform revealed that 98.6% of samples showed hemolysis of less than 100 HI, 1.3% between 100 and 300 HI, and 0.08% above 300 HI. A rigorous application of the Roche bulletin could lead to the rejection of approximately ten lipase results per day, which would be unacceptable for clinical staff.

Study limitations

1. The method of cell lysis by the addition of water may differ from *in vivo* or *in vitro* hemolysis observed in samples received by the laboratory. Delgado et al. [13] demonstrated that the methodology used to prepare the hemolysate can contribute to data variability.
2. The hemolysis threshold may vary depending on the quality specifications chosen.

Conclusion

This study demonstrates that interference limits vary between different Roche systems. Results showed that the lipase hemolysis threshold (100 HI) proposed by Roche was unduly restrictive. By raising the threshold above the new Roche limit, sample rejection can be reduced without compromising result quality.

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Review

Investigation of a number of rare deletional mutations in the alpha globin gene cluster

Majid Arash[1], **Mehdi Gholami Bahnemiri**[2]

[1]Faculty of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran
[2]Department of Laboratory Sciences, Sari Branch, Islamic Azad University, Sari, Iran

Abstract

Alpha thalassemia is one of the most common genetic diseases in the world. This disease is prevalent in various parts of the world, such as India, the Middle East, Africa, and many other countries. Several clinical conditions can result from mutations. In the condition where only one of the alpha globin genes is expressed, hemoglobin H disease (Hb H) occurs. Alpha thalassemia trait and silent carrier are milder forms of the disease, caused by the deletion of one and two alpha globin genes, respectively. Several mutations result in the deletion of alpha-globin. Seven common deletional mutations include -$\alpha^{4.2}$, -$\alpha^{3.7}$, -$(\alpha)^{20.5}$, --^{MED}, --^{SEA}, --^{Fil}, and --^{THAI}. The deletional mutations -$\alpha^{4.2}$ and -$\alpha^{3.7}$ remove only one of the alpha globin genes, while others remove both α1 and α2 globin genes from the gene cluster. Nowadays, laboratories identify these mutations using the Gap PCR method and other advanced methods. In addition to these mutations, some deletional mutations are found only in certain families or certain regions.

Keywords: Alpha thalassemia, deletional mutation, globin, hydrops fetalis

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Alpha thalassemia is a genetic disease with autosomal recessive inheritance, characterized by a reduction or lack of production of alpha globin chains. These alpha globin chains are components of fetal and adult hemoglobin. Unlike beta thalassemia, which is caused by non-deletion mutations, most known types of alpha thalassemia involve the deletion of one or two alpha globin genes on chromosome 13.3p16 [1, 2]. Both non-deletional and deletional mutations cause alpha thalassemia. Many non-deletional mutations of the alpha globin gene result from nucleotide substitutions. The most common deletional mutations are monogenic deletions of -$\alpha^{3.7}$ and -$\alpha^{4.2}$ in Asia and bigenic deletions --^{SEA} and --^{FIL} in Southeast Asia, -$(\alpha)^{20.5}$ and --^{MED} in Mediterranean regions, and --^{THAI} in Taiwan [3, 4]. Several other deletional mutations have been identified in recent years. In this article, we review eight of these deletional mutations. These deletions have a high prevalence and are seen in different proportions in different parts of the world. However, some deletional mutations are seen only in cases or in much smaller numbers.

Alpha Thalassemia

The alpha globin cluster at its locus includes duplicated alpha genes, an embryonic α-like gene (ζ2), three pseudogenes (Ψζ1, Ψα2, Ψα1), and a gene with an undetermined function (θ1). These genes and pseudogenes are arranged in the order 1ζ2-Ψζ1-Ψα2-Ψα1-α2-α1-θ1 [5–7]. Alpha globin is an essential subunit of human hemoglobin from the sixth fetal week to adulthood. Fetal hemoglobin (α2γ2) and adult hemoglobin (α2β2) are produced by the combination of two chains of alpha globin with two chains of gamma and beta globin, respectively [8, 9]. Based on how many of the alpha globin chain genes are deleted, the clinical symptoms of alpha thalassemia vary from mild anemia to severe symptoms. If one, two, three,

Address for correspondence: Majid Arash, MD. Faculty of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran
Phone: 989900994024 **E-mail:** majidarashbioc@gmail.com **ORCID:** 0009-0006-9590-437X

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or four of the alpha globin genes are deleted, alpha thalassemia trait, silent carrier state, hemoglobin H disease, and Hydrops fetalis syndrome occur, respectively. The frequency of alpha thalassemia has been studied in different parts of the world, with varying frequencies in different regions of countries such as Iran, Morocco, China, and Syria [10–13].

Alpha Thalassemia Phenotypes

Alpha thalassemia phenotypes are divided into four categories: Alpha thalassemia silent carrier, Alpha thalassemia trait, Hemoglobin H disease (Hb H), and Hydrops fetalis.

Table 1 shows the characteristics of alpha thalassemia types.

Classification of Alpha Thalassemia Based on the Number of Deleted Genes

Different types of deletional mutations with different frequencies have been investigated in various parts of the world [14–19]. Some of these mutations cause the deletion of one of the two alpha globin genes, while others cause the deletion of both genes [14–21].

Single Gene Deletions

The most common deletional mutations in alpha thalassemia delete one of the genes of the alpha globin chain, which leads to a mild alpha thalassemia phenotype (-α/αα). Reciprocal recombination, which occurs between highly similar regions called Z-boxes, leads to the deletion of 3.7 kb, which only includes one alpha gene ($-\alpha^{3.7}$). In contrast, recombination between the mismatched X-boxes results in the deletion of 4.2 kb ($-\alpha^{4.2}$). These recombinations lead to the creation of chromosomes containing three alpha globin genes [22, 23].

Double Gene Deletions

Several α0 defects that delete both alpha globin genes have been observed in different populations and are named according to their geographic region. These deletions include Southeast Asian ($--^{SEA}$), Mediterranean ($--^{MED}$), Filipino ($--^{FIL}$), Thai ($--^{THAI}$), and 20.5 kb type ($-(\alpha)^{20.5}$) [24, 25]. Partial or complete deletion of both alpha globin genes in the cis state leads to the absence of alpha globin chain synthesis in the body. Homozygotes with these deletions have hydrops fetalis syndrome. Many deletions also remove ζ and α globin genes. Individuals in the heterozygous state appear to develop normally, but survival is unlikely for individuals in the homozygous state even in the early months or days of pregnancy, as neither α2γ2 nor ζ2γ2 hemoglobin can be produced. It is worth mentioning that rare deletions causing α0 thalassemia delete the regulatory region 40–50 kb upstream of the alpha globin gene cluster, leaving the alpha globin chain genes intact.

Detailed analysis of the deletional mutation $-(\alpha)^{20.5}$ reveals the importance of providing a more precise description of the 5' end of this deletion from the alpha globin genes. This is especially crucial because different phenotypes are associated with defects in the alpha globin gene cluster. The first cases of alpha-α0 thalassemia deletion, which deleted the entire alpha globin gene set, described a rare condition characterized by Hb H disease and congenital mental retardation [26]. While the $--^{SEA}$ deletional mutation is the most common cis deletion in alpha globin chains in Southeast Asia, several other deletions have also been reported in other scientific papers. $--^{THAI}$ and $--^{FIL}$ deletional mutations are common among some Southeast Asian populations. These common deletional mutations, which were first described by Fischel Ghodsian and colleagues, cover about 30–38 kilobases and delete the zeta2 globin gene as well as alpha globin genes located on chromosome 16 [27].

New Deletional Mutations

$-\alpha^{6.9}$ deletion

This deletion mutation, first observed in China, causes the deletion of the α2 gene but leaves the α1 gene intact (NG-000006.1.29785–36746 del 6962bp). The researchers who found this mutation initially identified alpha thalassemia by examining the phenotype of the subjects. Then, using the Multiplex Ligation-dependent Probe Amplification (MLPA) method, they evaluated the new mutation, and through DNA sequencing and bioinformatics analysis, confirmed the mutation's existence [28]. If this deletional mutation is combined with a single deletion, it causes alpha thalassemia trait, and if it is combined with a double deletional mutation, it causes Hb H disease.

$--^{27.2}$ deletion

In a study, researchers in China discovered this new mutation after examining a young woman in pre-pregnancy screening who had hypochromic and microcytic hematological features [29]. This mutation causes the deletion of both alpha globin genes located on chromosome 16, and if it is combined with another double deletional mutation, it causes hydrops fetalis.

Table 1. Characteristics of alpha thalassemia types

Name of disease	Number of deleted genes	Genetic condition of the disease	Severity of clinical symptoms
Silent carrier	One	-α/αα	Usually mild or no symptoms
Trait	Two	--/αα	
-α/-α	Usually mild anemia		
Hb H	Three	--/-α	mild to severe
Hydrops fetalis	Four	--/--	Severe symptoms

$(\alpha\alpha)^{ZRX}$ deletion and $--^{336}$ deletion

These deletions, which cause the deletion of both alpha globin chains, were observed in two Chinese families. In the patient with the $--^{336}$ deletion, a spontaneous mutation was identified, as neither parent had this mutation. Both carriers had symptoms of microcytic hypochromia, a characteristic of people with α^0-thalassemia [30].

$--^{GB}$ deletion

This deletion mutation, first observed in Malaysia, results in the deletion of both genes of the alpha globin chain (α^0-thalassemia). Most individuals with this mutation are of Malay descent. The main risk of this mutation, when accompanied by a single or double deletion, is Hb H disease or hydrops fetalis, respectively [31].

$(\alpha\alpha)^{FJ}$ deletion

This deletion mutation, which caused the deletion of 91.5 kilobases in the alpha globin gene cluster in a family from China, deletes both α1 and α2 genes. In areas with a high frequency of alpha thalassemia, this mutation should be investigated [32].

$-\alpha^{6.3}$ deletion and $-\alpha^{27.6}$ deletion

These mutations, identified in two Chinese patients, delete one of the alpha globin chains (α+-thalassemia). Like single deletion mutations such as $-\alpha^{3.7}$ and $-\alpha^{4.2}$, they are accompanied by double deletion mutations, causing Hb H disease [33].

$--^{CR}$ deletion

This deletion mutation, found in Thailand, deletes 44.6 kilobases of the alpha globin gene cluster. There is limited information on the effects of this mutation, especially in combination with other types of thalassemia. Hypochromic and microcytic blood phenotypes have been observed in carriers [34].

$--^{14.9}$ deletion

This mutation, discovered in China, deletes both alpha globin chains. Affected individuals had abnormal blood parameters, and after screening for 23 types of common mutations, this specific mutation was identified [35].

107 kilobase deletion

In a patient suffering from mild anemia, screening for thalassemia was performed by Gap-PCR and PCR-reverse dot blot methods. Following additional investigations, this deletional mutation was identified [36].

Common Diagnostic Methods in Identifying Deletional Mutations in Alpha Thalassemia Disease

For a long time, methods such as loop-mediated isothermal amplification, Multiplex Ligation-dependent Probe Amplification (MLPA), and single-tube multiplex polymerase chain reaction (PCR) have been used worldwide in the diagnosis of alpha thalassemia. However, new methods with greater speed and accuracy are also needed, as they can identify many genetic mutations in a short period of time [37]. In addition to traditional methods that have been used for years by diagnostic centers, new methods have also been introduced in recent years [38–40].

Conclusion

Considering the high prevalence of alpha thalassemia worldwide, as well as the complications of this disease, particularly in cases of Hb H disease and hydrops fetalis, it is advisable to screen individuals suspected of having this condition. Following diagnosis, genetic counseling should be provided to inform patients about the risks in pregnancy and prevent the birth of infants with severe alpha thalassemia. In areas where rare mutations are present, these mutations should also be studied.

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Opinion Paper

Navigating the 2024 revised guidelines for Undergraduate Competency Based Medical Education (CBME) curriculum: Newer insights and implications for biochemistry education

Krishna Mohan Surapaneni[1,2]

[1]Department of Biochemistry, Panimalar Medical College Hospital & Research Institute, Chennai, India
[2]Department of Medical Education, Panimalar Medical College Hospital & Research Institute, Chennai, India

Abstract

The recent release of the 2024 revised guidelines for the Competency Based Medical Education (CBME) curriculum by the National Medical Commission (NMC) marks a pivotal moment in the evolution of medical education in India. Building upon the foundation established in 2019, this revised curriculum introduces critical advancements designed to align medical training with contemporary global standards. These updates not only enhance the educational experience but also ensure that future medical professionals are equipped with the knowledge, skills, and competencies necessary to thrive in modern healthcare environments. This article focuses on the significant changes within the biochemistry curriculum, highlighting its importance and the shift towards integrating clinical relevance, innovative teaching methodologies, and robust assessment strategies. Educators are encouraged to prioritize tailoring their teaching approaches according to these expected standards. The article also provides strategies for incorporating these changes into teaching methodologies, offering educators evidence-informed guidance.

Keywords: Assessment strategies, biochemistry curriculum, clinical relevance, Competency Based Medical Education (CBME), teaching methods

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The National Medical Commission (NMC) of India recently announced the release of the 2024 revised guidelines for the undergraduate Competency Based Medical Education (CBME) curriculum on September 12, 2024 [1]. Building on the success of the CBME framework introduced in 2019, this revised version is expected to include significant enhancements, with modified or additional components designed to better equip medical students with the necessary skills, knowledge, and competencies required for contemporary medical practice. These enhancements aim to globalize the medical education system in India in line with the latest advancements and international educational standards.

In biochemistry, this new upgrade offers several new insights and implications for educators. Understanding these modifications is crucial as they directly impact how biochemistry is taught and delivered to learners. This article explores the changes made to the biochemistry curriculum in terms of competencies, teaching-learning methods, and assessment strategies. By doing so, it offers valuable guidance on how educators can adapt their methods to ensure that students are not only meeting but excelling in the revised curriculum's expectations.

Discussion

In the earlier version of the CBME curriculum, the focus was predominantly on biochemical pathways and metabolites with associated disorders, which provided students with a solid foundation in understanding the intricate processes of life at the molecular level. However, the new curriculum transcends

Address for correspondence: Krishna Mohan Surapaneni, PhD., MHPE. Department of Biochemistry, Panimalar Medical College Hospital & Research Institute, Chennai, India; Department of Medical Education, Panimalar Medical College Hospital & Research Institute, Chennai, India
Phone: 919789099989 **E-mail:** krishnamohan.surapaneni@gmail.com **ORCID:** 0000-0002-5204-5708
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this foundational knowledge by emphasizing the clinical implications of these pathways, particularly in the context of specific disorders related to the metabolism of essential macromolecules like carbohydrates, lipids, proteins, and nucleic acids, as well as their management. This shift is crucial as it bridges the gap between theoretical biochemistry and its practical application in diagnosing and managing metabolic disorders, making the learning experience more clinically relevant and impactful [2, 3]. This not only reinforces students' foundational knowledge but also teaches them how to apply it in a clinical setting.

The curriculum's inclusion of modern molecular techniques, such as CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9), marks a progressive step towards integrating cutting-edge science with clinical practice. These tools, once the domain of specialized research, are now being brought into the educational fold, reflecting their growing importance in diagnosing and treating genetic disorders [4]. This prepares students not just to understand these techniques but to apply them in real-world clinical scenarios, keeping their education at the forefront of medical innovation.

One of the most forward-thinking additions to the curriculum is the emphasis on Artificial Intelligence (AI) in clinical biochemistry laboratory practices. AI is revolutionizing healthcare, particularly in diagnostic laboratories, where it enhances accuracy, efficiency, and personalized patient care [5]. By incorporating AI into the curriculum, educators are ensuring that students are not only aware of these advancements but are also prepared to harness them in their future careers. This competency is crucial as AI becomes increasingly integral to laboratory medicine, offering opportunities for more sophisticated analyses and improved patient outcomes [6].

Educators can create interactive sessions where students work with AI-powered diagnostic tools in simulated clinical biochemistry labs. Students can experience firsthand how these advancements improve diagnostic accuracy and efficiency, preparing them for future roles in healthcare settings where AI is increasingly utilized.

Notable changes have been introduced in the practical biochemistry curriculum, with a stronger emphasis on case scenario-based interpretation of analytes being tested. This revision encourages students to not only perform biochemical experiments but also interpret results within clinical contexts, fostering critical thinking and practical application. The inclusion of additional demonstrations, such as uric acid estimation, and an increase in the number of certifiable skills from five previously to eleven in the revised guidelines, further enriches the hands-on learning experience, ensuring that students are proficient in a wider range of essential techniques.

The curriculum also places greater emphasis on quality control and the identification of analytical errors, highlighting the importance of accuracy in laboratory work. New components, such as the identification and interpretation of Levey-Jennings charts, further contribute to preparing medical graduates with a strong foundation in analytical precision and error management in clinical biochemistry.

Additionally, a new component requires students to actively observe, interpret, and discuss baseline, diagnostic, prognostic, and discharge investigations in clinical biochemistry. This hands-on approach enhances their ability to connect laboratory results with clinical outcomes, fostering a deeper understanding of the role biochemistry plays throughout patient care.

The revised guidelines for the CBME biochemistry curriculum have introduced a range of innovative teaching and learning methods designed to enhance student engagement and deepen understanding. While foundational methods such as Large Group Teaching (LGT), Small Group Teaching (SGT), DOAP (Demonstration-Observation-Assistance-Performance), Self-Directed Learning (SDL), demonstrations, and case studies continue to play a crucial role, the curriculum now integrates several new approaches to further enrich the educational experience.

Among these new methods, the flipped classroom model stands out. This model shifts the focus from passive to active learning, requiring educators to carefully curate pre-class materials and plan interactive, in-class activities that encourage deeper student engagement and critical thinking [7]. Role play has been introduced to help students simulate real-life clinical scenarios, fostering critical thinking and communication skills in a controlled environment. Home assignments now play a more significant role, promoting self-directed learning and ensuring that students engage with the material continuously outside the classroom [8]. These enhancements further highlight how educators have to restructure their approaches to teaching in a way that is more relevant, inclusive, and impactful. Educators will need to plan these experiences carefully to align with theoretical lessons, ensuring a well-rounded education.

The curriculum also places greater emphasis on experiential learning through lab visits, providing students with direct exposure to the working environment of a clinical biochemistry laboratory. Most notably, Early Clinical Exposure (ECE) has been integrated into the curriculum through Small Group Teaching (SGT) during bedside or ward visits and interactions with the medical record department in biochemistry. This new element fosters early exposure to real patient cases, helping students connect theoretical knowledge with practical application, offering a comprehensive understanding of biochemistry and its critical role in diagnosis [9], treatment, and patient care. This will require collaboration between educators and clinical departments, strengthening the link between classroom learning and patient care. Together, these enhancements foster a more interactive, reflective, and clinically relevant learning experience directing educators to shift from classroom-based teaching in biochemistry.

Assessment and Teaching

Assessment and teaching are always complementary to each other. Learners should be assessed using the same methods in which they have been taught [10]. The recent revisions to the

biochemistry curriculum represent a significant shift in the assessment paradigm, moving towards a more holistic, relevant, and outcome-based evaluation model. The revised guidelines of the curriculum now place a stronger emphasis on Objective Structured Practical Examinations (OSPE), which have become a central component of practical skill assessment. OSPEs are designed to objectively evaluate students' ability to perform specific tasks in a controlled, standardized environment, ensuring that the assessment of practical skills is both rigorous and fair [11].

In addition to the integration of OSPE, the new curriculum has expanded its assessment framework to include direct observation and case study interpretation. Direct observation allows educators to assess students' procedural skills and decision-making abilities in real time, providing valuable insights into their clinical proficiency and areas for improvement. Case study interpretation requires students to analyze complex clinical scenarios, fostering a deeper understanding of the biochemical principles underlying patient care. These methods collectively ensure that students are evaluated not only on their knowledge but also on their critical thinking and application skills, aligning the assessment process with the practical demands of medical practice [12].

Furthermore, the introduction of logbooks and reflective exercises in biochemistry further enhances the assessment process by promoting a culture of formative evaluation with effective feedback, continuous self-assessment, and lifelong learning [13, 14]. Overall, these comprehensive assessment methods, particularly the emphasis on OSPE, ensure that the curriculum not only tests students' knowledge but also prepares them for the practical realities of clinical biochemistry, making the assessment process more robust and aligned with the principles of Competency Based Medical Education (CBME).

Conclusion

The 2024 revised guidelines for the CBME curriculum in biochemistry signify a transformative shift in medical education, focusing on the integration of clinical relevance, innovative teaching methodologies, and comprehensive assessment strategies. By moving beyond traditional approaches, this curriculum equips students with the critical skills and knowledge required to excel in modern medical practice. The emphasis on practical application, early clinical exposure, and cutting-edge technologies such as AI and molecular techniques reflects the evolving demands of the healthcare landscape. Moreover, the holistic assessment framework ensures that students are not only knowledgeable but also adept at translating their learning into real-world clinical scenarios.

This curriculum challenges educators to focus on practical applications, foster critical thinking, and utilize hands-on assessments like OSPEs. By tailoring their teaching to these new standards, educators will play a vital role in preparing students to excel in the complexities of contemporary medical practice.

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Letter to the Editor

Impact of preanalytical storage on the accuracy of CD3, CD4, CD8 testing results using the BD FACSLyric™ clinical flow cytometry system

Majid Arash

Department of Clinical Biochemistry, Kermanshah University of Medical Sciences Faculty of Medicine, Kermanshah, Iran

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Dear Editor,

I found a valuable article titled "Impact of preanalytical storage on the accuracy of CD3, CD4, CD8 testing results using the BD FACSLyric™ Clinical Flow Cytometry System" in "International Journal of Medical Biochemistry [1]. I need to thank the research team of this article for conducting such a research. But it seems necessary to mention some points about this research. CD3, CD4 and CD8 can be investigated in various situations such as inflammation, cancer, and immunological disorders [2]. As mentioned in this article, not all laboratories have the hardware facilities to measure these markers and they must be stored in various forms such as freezers or storage at room temperature until they reach the laboratory with the equipment to start them. In this research, the samples were examined at room temperatures at 24 and 72 hours after sampling. Sometimes, due to the long distance, more time is needed for the sample to reach a more equipped laboratory. This time may be more than 72 hours. It is suggested that in future studies by this group of researchers or other researchers, longer times should be considered for the investigation of these markers in order to examine their stability in these time periods. It would also be better that part of the samples be kept at the refrigerator temperatures at the same time so that their stability at room temperatures and refrigerator temperatures can be compared at the same time.

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Address for correspondence: Majid Arash, MD. Department of Clinical Biochemistry, Kermanshah University of Medical Sciences Faculty of Medicine, Kermanshah, Iran
Phone: 989900994024 **E-mail:** majidarashbioc@gmail.com **ORCID:** 0009-0006-9590-437X

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