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ORIGINALS

Prevalence and risk factors of vitamin D deficiency among a diverse cohort of Indian collegiate athletes
Saju A,, et al.

Genomic and structural analysis of genes involved in epigenetic regulations of diffuse large B cell lymphoma by computational approaches
Akcesme B, et al.

Patient based real time quality control using average of normal approach
Shaikh S, et al.

The importance of capillary protein electrophoresis in the early diagnosis and follow-up of monoclonal gammopathies
Dogan N, et al.

Immature granulocyte, immature granulocyte-lymphocyte ratio, and other hematological inflammatory parameters in Alzheimer disease
Yegin D, et al.

Assessment of relationship between triglyceride/HDL-C ratio and incident type 2 diabetes mellitus risk
Katar M, et al.

Study the genetic variations of GRIAs genes to investigate association to methamphetamine addiction
Al-Attar MM, et al.

Assessing the pathogenicity of missense single nucleotide polymorphisms in the human FUCA1 gene using multiple bioinformatics tools
Fahim I, et al.

Machine learning-assisted prediction of positive urine cultures using urinalysis and hemogram data: A retrospective cohort study
Demirci F, et al.

The serum NF-κB and adiponectin levels in patients with acute pancreatitis
Mısırlıoglu NF, et al.

TECHNICAL REPORT

Pre- and post-centrifugation stability of total and free prostate specific antigen samples at room temperature storage conditions
Ilardo C, et al.

LETTER TO THE EDITOR

Advancing metabonomics in understanding immunological mechanisms of recurrent spontaneous abortion
Akbay A, et al.

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CONTENTS

VOLUME VIII ISSUE 3 YEAR 2025

ORIGINAL ARTICLES

Prevalence and risk factors of vitamin D deficiency among a diverse cohort of Indian collegiate athletes
Saju A, Rao VM, Dharanirajan K, Ravindran GC, Alwar T, Sivaraman A 159

Genomic and structural analysis of genes involved in epigenetic regulations of diffuse large B cell lymphoma by computational approaches
Akcesme B, Sezer A 165

Patient based real time quality control using average of normal approach
Shaikh S, Jain S, Baku S 178

The importance of capillary protein electrophoresis in the early diagnosis and follow-up of monoclonal gammopathies
Dogan N, Gumus A, Ayer M 185

Immature granulocyte, immature granulocyte-lymphocyte ratio, and other hematological inflammatory parameters in Alzheimer disease
Yegin D, Korkmaz S 192

Assessment of relationship between triglyceride/HDL-C ratio and incident type 2 diabetes mellitus risk
Katar M, Demir O 199

Study the genetic variations of GRIAs genes to investigate association to methamphetamine addiction
Al-Attar MM, Al-Awadi SJ 205

Assessing the pathogenicity of missense single nucleotide polymorphisms in the human *FUCA1* gene using multiple bioinformatics tools
Fahim I, Idris H, Alyousef RA, Joujeh D 212

Machine learning-assisted prediction of positive urine cultures using urinalysis and hemogram data: A retrospective cohort study
Demirci F, Arikan Y, Akbulut I, Topcu DI 222

The serum NF-κB and adiponectin levels in patients with acute pancreatitis
Mısırlıoglu NF, Orucoglu GG, Himmetoglu S, Aydın SN, Uzun H 233

TECHNICAL REPORT

Pre- and post-centrifugation stability of total and free prostate specific antigen samples at room temperature storage conditions
Ilardo C, Hammer-Dedet F, Coulon G 242

LETTER TO THE EDITOR

Advancing metabonomics in understanding immunological mechanisms of recurrent spontaneous abortion
Akbay A, Baba B 249

INTERNATIONAL JOURNAL OF
MEDICAL BIOCHEMISTRY

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Research Article

Prevalence and risk factors of vitamin D deficiency among a diverse cohort of Indian collegiate athletes

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Abstract

Objectives: Vitamin D is essential for optimal musculoskeletal function and athletic performance. While the prevalence of serum 25(OH)D levels below 20.00 ng/mL exceeds 70% in the general population of South Asian countries, research on vitamin D status in athletes from Asian countries, including India, remains limited. This study examines the extent of low serum cholecalciferol levels among Indian athletes and investigates the impact of gender and sport type (indoor vs. outdoor) on serum vitamin D levels.

Methods: Upon receiving consent, 331 athletes (male=243, female=88) who competed in various sporting events were recruited. A serum assay was undertaken to measure vitamin D, calcium, and parathyroid hormone. Serum 25(OH)D levels were categorized using the guidelines provided by the Endocrine Society Clinical Practices Guidelines. Statistical analysis was done using IBM SPSS 20.

Results: The study reported an average serum vitamin D level of 17.76±6.93 ng/mL. Only 8.2% of athletes had sufficient serum 25(OH)D, while 54.4% were deficient. The mean serum 25(OH)D level was significantly lower in female athletes compared to male athletes (15.72±5.92 ng/mL vs. 20.72±7.73 ng/mL, $p<0.001$), with females having a 4.32 times higher risk of vitamin D deficiency. Additionally, indoor athletes had lower mean serum 25(OH)D levels than outdoor athletes (49.24% vs. 74.63%, $p<0.001$), increasing their risk of deficiency by 3.03 times.

Conclusion: The rate of low serum cholecalciferol levels is high among athletes. Females and athletes partaking in indoor sports were at a higher risk for having lower vitamin D. Biannual serum assessments of those at risk for developing deficiency could help to better assess the situation.

Keywords: Athlete, cholecalciferol, deficiency, India, prevalence, vitamin D

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Lower vitamin D levels have been shown to hamper athletes' performance and increase their risk of muscle injuries, stress fractures, and upper respiratory tract infections. Research indicates a beneficial association between vitamin D levels with jump performance and muscle strength [1–4]. Analysis of serum 25-hydroxyvitamin D is a reliable investigation for measuring vitamin D levels but consensus regarding adequate serum 25(OH) D levels remains uncertain for the general population due to various medical societies' different clinical and health approaches. Endocrine Society Clinical Practice Guidelines suggest that serum 25 (OH) D levels lower than 20 ng/mL as deficiency and insufficiency as levels between 20 and 29.99 ng/mL, while National Academy of Medicine (NAM) (previously known as Institute of Medicine) recommends a lower cut-off of >20 ng/ml as sufficient and <12 ng/mL as deficient [5, 6].

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The lower cut-off of >20.00 ng/ml of serum 25(OH)D has been recommended as sufficiency in a recent consensus statement for a clinical perspective for the public health to avoid over diagnosing and overtreatment [7]. Whereas, research on Indian population recommended an even lower cut-off of 13.5 ng/mL as the lower cut-off of normal range [8]. However, an accord has not been reached for what is a sufficient serum level for vitamin D among athletic population who might have higher demand and nutrient utilization, [9] but a few reports suggest that serum 25(OH) D above 40.00 ng/mL is optimal for maintaining health and optimizing performance in athletes [10].

A pooled prevalence study which included 7,947,359 participants showed that 76.6% had vitamin D levels <30ng/mL [11]. Reports indicate that among the global athletic population, 44–67% of athletes have inadequate serum 25(OH)D [12]. These high rates were linked to living in higher latitudes (>40º North or South), winter-spring months, lower-middle income countries, female gender, and the Eastern Mediterranean region [11]. In outdoor and indoor athletes, a slightly higher serum cholecalciferol was noted among outdoor athletes, but it could vary based on confounding factors such as season, latitude and race [13].

The magnitude of vitamin D inadequacy among the Indian adult population has been reported to be higher than 50%. A larger magnitude of vitamin D deficiency amid Indians could be attributed to darker skin tone, urbanization, increased use of sunscreens, cultural and religious restrictions that reduce skin surface exposure to sunlight, and genetic variations leading to lower serum 25(OH)D [14–16]. While multiple studies have been published regarding vitamin D status among healthy, pregnant, and lactating children in India, very few studies have focused on low vitamin D and the risk factors of among athletes. This study attempts to identify and classify the serum vitamin D levels among Indian athletes. It also aims to investigate the impact of gender and types of sports (indoor vs. outdoor) on vitamin D levels.

Materials and Methods

Athletes who resided in Tamil Nadu (13.0843° N, 80.2705° E), which is the south-eastern state of India, were recruited for the study after obtaining the Institutional Ethical Clearance. The study was approved by Sri Ramachandra Institute of Higher Education and Research Ethics Committee (No: IEC/22/APR/171/35, Date: 14/06/2022). The athletes were briefed about the importance of cholecalciferol and provided an overview of the research methodology. After receiving the participants' written informed consent, the data collection was completed for 331 athletes between August 2022 and November 2023, following the principles of the Declaration of Helsinki.

Both female (n=88) and male (n=243) athletes aged 18 to 25 years who actively participated in competitive events in team or individual sports and indoor or outdoor sports at national and international levels and trained for a mean total duration 20 hours per week were recruited. This duration was established to ensure that the athletes were regularly training in their specific sport. Athletes who took vitamin D supplements in the six months leading to the study recruitment period and those who had any comorbidities, such as skin disease affecting melanin content and liver or renal disease that affected the metabolism and absorption of vitamin D, were not included in the study.

Demographic details such as age, gender, type of sport, and total training duration were collected. To ensure standardized data collection, a level 2 International Society for the Adavancement of Kinanthropometry (ISAK) certified kinanthropometrist performed all anthropometric measurements, such as weight, height, girth, and skinfold thickness, following the ISAK guidelines [17]. A trained phlebotomist drew blood to analyze serum vitamin D, parathyroid hormone (PTH), and calcium levels.

Recommendations by the Endocrine Society Clinical Practices Guidelines and/or the National Academy of Medicine (NAM) are used globally (Table 1) [5, 6]. The results of this study are reported based on the serum cut-offs suggested by the Endocrine Society Clinical Practice Guidelines. The normal cut-offs for parathyroid hormone were 12–88pg/mL, and for calcium, was 8.8–10.6 mg/dL.

Statistical analysis

The prevalence rate of 83.7% low serum cholecalciferol (insufficient and deficient vitamin D levels) among athletes from a previous study conducted in India [18] was used to determine a minimum sample size of 285, considering a 99% confidence interval and 10% relative precision. Data analysis was completed using IBM SPSS 20 (SPSS Inc, Chicago, USA). Variables were calculated as mean±SD or percentage. The association between gender and type of sport and vitamin D status was identified using a chi-square test. Odds Ratio was performed to identify the group at higher risk for low vitamin D levels. P-value <0.05 was interpreted as statistically significant.

Results

Three hundred and thirty-one athletes were recruited for the study, who were further divided based on gender and type of sports for correlation. Of the 331 athletes (Mage=19.68±1.78 years), 73.4% were male (Mage=19.74±1.73 years), and 26.6% were female (Mage=19.30±1.56 years) athletes. When categorized based on sport, 79.46% of athletes participated in

Table 1. Vitamin D status categorisation

Status	NAM guidelines		Endocrine society	
	ng/mL	nmol/L	ng/mL	nmol/L
Deficient	<12	<30	<20	<50
Insufficient	12–20	30–50	20.01–29.99	50.01–74.99
Sufficient	>20	>50	>30	>75

NAM: National academy of medicine.

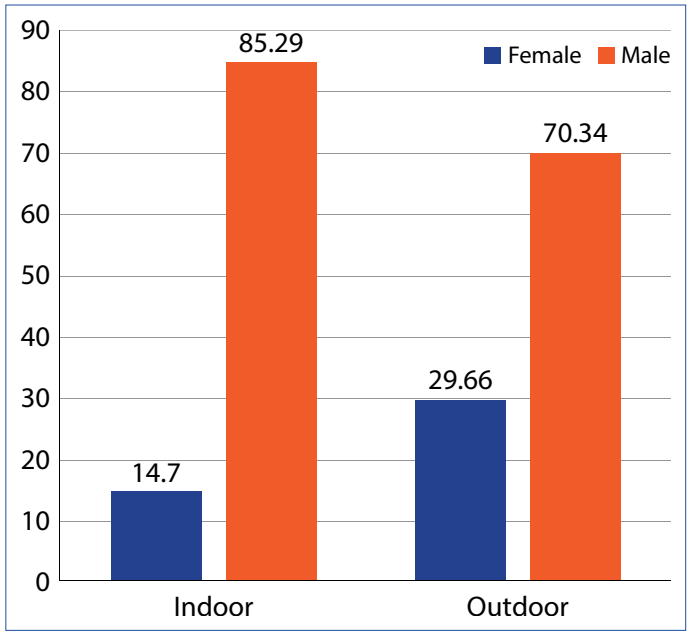

Figure 1. Distribution of gender across indoor and outdoor sports.

outdoor sports such as Athletics, Basketball, Cricket, Football, Hockey, Kabbadi, Kho-kho, Rowing, Tennis, Throwball, and Volleyball. In comparison, 20.54% played indoor sports such as Badminton, Combat sports, Shooting, and Swimming.

The distribution of athletes based on gender across the type of sports is provided in Figure 1.

The female athletes were found to train for significantly longer hours than their male counterparts (29.41±8.05 vs 25.90±7.88, p<0.001). Biochemical details of the athletes based on gender and type of sports are given in Table 2.

The mean serum cholecalciferol levels were 17.76±6.93 ng/mL, which is below the indication for deficiency set by Endocrine Society Clinical Practice Guidelines (<20.00 ng/ml). The mean serum calcium and PTH levels were 9.67±0.38 mg/dL and 25.76±14.99 pg/mL, respectively, both values were within the sufficient range.

In this study the use of categorisation by Endocrine Society Clinical Practices Guidelines indicated that only 8.2% had sufficient levels while 91.8% had low serum cholecalciferol (Fig. 2).

Across genders, female athletes had significantly lower mean vitamin D levels (15.72±5.92 ng/ml vs 20.72±7.73 ng/ml, p<0.001) than male athletes. Deficiency rates among female

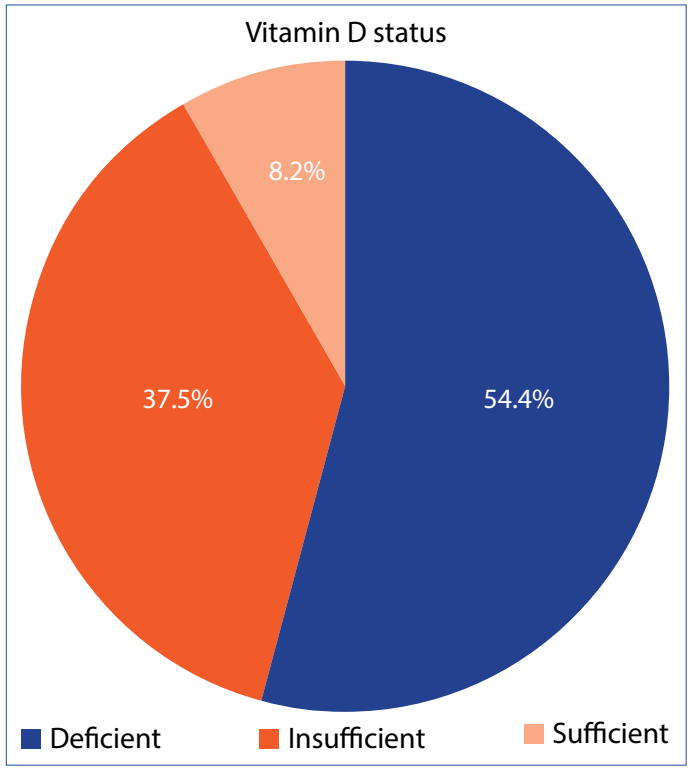

Figure 2. Vitamin D status among study population.

athletes were higher in comparison to male counterparts (78.41% vs 45.68%, p<0.001) (Table 3). The odds ratio was 4.32, and the 95% CI was 2.45 to 7.61 indicating that females were at a higher risk for developing lowed vitamin D levels. The extensiveness of vitamin D deficiency was significantly lower amid outdoor athletes in comparison to indoor athletes (49.24% vs 74.63%, p<0.001) (Table 3). The odds ratio was found to be 3.03, and the 95% CI was 1.66 to 5.53. A similar sample size among both the groups would help to confirm this effect.

Discussion

Vitamin D is an essential fat-soluble nutrient that affects the athletic population's general health and sports performance [1]. While serum 25(OH)D has been considered the best measure, there is inconsistency in the serum cutoffs used to categorize vitamin D levels among general population [9]. Low serum vitamin D was highly prevalent among the athletes who participated in the study. The results from a meta-analysis on prevalence among basketball players [19] showed

Table 2. Biochemical details among gender and types of sport

Parameter (Mean±SD or %)	Female (n=88)	Male (n=243)	p	Indoor (n=68)	Outdoor (n=263)	p
Vitamin D (ng/mL)	15.72±5.92	20.72±7.73	<0.001^	15.04±6.12	20.42±7.63	<0.001^
Calcium (mg/dL)	9.59±0.43	9.69±0.35	0.059	9.77±0.32	9.64±0.40	0.006*
PTH (pg/mL)	24.97±16.22	26.06±14.54	0.559	21.62±13.49	25.75±15.03	0.029*

Significant at p-value <0.05* and <0.001^. SD: Standard deviation; PTH: Parathyroid hormone.

Table 3. Vitamin D status of collegiate athletes

	Sufficient (%)	Insufficient (%)	Deficient (%)	p
Gender				
Male	10.7	43.6	45.7	<0.001*
Female	1.1	20.5	78.4	
Type of sport				
Indoor	1.5	23.9	74.6	<0.001*
Outdoor	9.8	40.9	49.2	

Significant at p-value <0.001*.

that 77% of the athletes had vitamin D inadequacy, while another meta-analysis reported that the rates were as low as 30% among elite adult athletes versus 39% among adolescent athletes [20]. Serum assessment on Indian athletes reported a higher rate of serum vitamin D deficiency [16, 18]. Inadequate vitamin D levels seem prevalent across different sports, though reports of prevalence rates could vary based on the cut-off criteria used by the concerned organization. The lack of a global consensus on the cut-off for athletes could result in an over or underestimation of deficiency rate, and lead to difficulty in summarizing the prevalence rates of vitamin D inadequacy among athletes.

Women have been found to have higher chances of vitamin D deficiency than men among the general Indian population and this has been attributed to the limited outdoor movement and traditional clothing among females [14]. Similarly, our study identified a higher rate of low serum vitamin D levels among female athletes than male athletes. Only 1.14% of females reported adequacy compared to 10.70% of male athletes, which indicated a 4.32 times higher risk for females in developing vitamin D deficiency. However, among Turkish [21], and German elite athletes, [22] no significant difference has been noted between genders regarding serum vitamin D, and similar results were noted in a pooled analysis of elite athletes [20] The impact of gender on vitamin D levels has been poorly studied among athletes, most studies are conducted on male athletes, making it difficult to conclude the effect of gender. The uneven distribution among the genders in the study could have led to the results that we obtained.

The evaluation between indoor and outdoor sports in our study reports a remarkably higher rate of deficiency among athletes participating in indoor sports than outdoor sports. Indoor athletes' mean serum vitamin D levels were significantly lower than outdoor athletes (15.04±6.12 vs 20.42±7.63, p<0.001). These results indicate that the prevalence of low vitamin D levels is higher among indoor athletes in comparison to outdoor athletes, indicating a 3.03 times greater risk among indoor athletes. Similar results were identified among elite male Japanese collegiate athletes [23], Caucasian adolescent athletes [24], and elite German athletes [22] who noted a positive link between vitamin D levels and outdoor sports participation. Similarly, during the winter months, athletes who trained for outdoor sports were found to have improved mean 25(OH)D, while severe deficiency was prominent among indoor athletes [25] This could be due to sunlight exposure during outdoor physical activity, which can increase vitamin D synthesis in human skin. [26] Contrasting results were found while assessing elite and sub-elite Indian athletes, where indoor athletes were found to have higher mean vitamin D levels than outdoor athletes (20.19±2.55ng/mL vs 10.49±2.03ng/mL) [16]. However, the study conducted among Turkish athletes did not show a significant correlation between the type of sports and serum cholecalciferol levels [21].

Since the extensiveness of vitamin D insufficiency and deficiency depends on the serum cut-off levels, the authors recommend a global or regional consensus on defining and categorizing vitamin D categories in athletes as the first step towards tackling the issue of low serum cholecalciferol. Since athletes undergo high-intensity training and are prone to musculoskeletal injuries, they could benefit from periodical screening, and supplementation in high-risk groups can prevent deficiencies [3, 7]. Providing nutrition education that focuses on dietary sources, safe sunlight exposure habits, the inclusion of fortified food, and careful supplement intake could help reduce the prevalence of low serum vitamin D. Sunlight exposure is an economical and beneficial way to improve vitamin D levels, [27] therefore safe sunlight exposure practices should be endorsed among athletes.

The present study used valid assessment techniques and robust methodology but is not without limitations. However, details regarding regular exposure to sun/UVB light and intake of vitamin D sources and other confounding factors that could affect serum 25(OH)D levels were not collected. Such information could have given a more comprehensive picture of the risk factors. A larger sample size and similar distribution of athletes from both genders and type of sport would provide a better understanding of the risk ratio. Future studies could also look at the prevalence of serum vitamin D levels among different disciplines of sport, the effect of vitamin D on athletic performance and general health among Asian and Indian athletes and the impact of cholecalciferol supplementation on their performance.

Conclusion

The concerningly high rate of serum vitamin D deficiency and insufficiency among athletes indicates that policies should be modified to ensure frequent assessment of their vitamin D levels. Female athletes and those participating in indoor sports can be considered more prone to having inadequate serum vitamin D status and should undergo regular assessment. Future research could focus on the identification of optimal levels in athletes which ensures optimal musculoskeletal health and improve performance. Research could also look at the impact of vitamin D on athletic performance and if supplementation in those with lower serum levels could help by providing an ergogenic boost.

Ethics Committee Approval: The study was approved by the Sri Ramachandra Institute of Higher Education and Research Ethics Committee (no: IEC/22/APR/171/35, date: 14/06/2022).

Informed Consent: Informed consent was obtained from all participants.

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Research Article

Genomic and structural analysis of genes involved in epigenetic regulations of diffuse large B cell lymphoma by computational approaches

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Abstract

Objectives: Despite the advances and huge efforts in determination of efficient therapy options for diffuse large B cell lymphoma (DLCBL), various issues, including resistance and toxicity, are among the main concerns. Additional contributor to the poor prognosis is genomic complexity of DLBCL, where high number of mutations are associated with the DLBCL pathogenesis. Epigenetic regulations expand the complexity of gene expression process by various mechanisms, including histone deacetylation and DNA methylation, without altering the DNA sequence.

Methods: Present study focused on analysis of genes involved in epigenetic regulations in DLBCL by using computational approaches, including sequence and structure-based predictions. C-bioportal database was used to find mutations, which are further analyzed by structural bioinformatic tools, including PredictSNP, SNPs&GO, AlphaMissense and DynaMut servers.

Results: Our results showed that mutations R1446H/C and Y1503F/D of CREBBP, L415P, H1451Y, Y1467D of EP300 and Y641N/F of EZH2 proteins are among most common mutations in DLBCL, with the mutations mostly being putative drivers and having a negative impact on the sequence and structure of the proteins.

Conclusion: The understanding of correlation of identified mutations in our study and DLBCL pathogenesis could contribute to the enhanced prediction, diagnosis and treatment of DLBCL. Identification of critical epigenetic mutations might enhance the efficiency and development of epigenetic drugs, further leading to better and more effective targeted therapies. However, further *in-vitro* and *in-vivo* investigations are required to verify our findings based on computational methods.

Keywords: Diffuse large B-cell lymphoma, epigenetic regulators, mutation analysis, structural bioinformatics

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Although recent advances in cancer treatment contributed to more efficient treatment options, the number of deaths caused by cancer is increasing. Recent report of the European Union (EU) discovers that the new cancer cases and cancer related deaths in EU states increased more than 2.3% and 2.4%, respectively, over the last two years, while the prognosis for 2040 is that there will be increase of 21% in new cancer cases compared to 2020 [1, 2].

Diffuse large B-cell lymphoma (DLBCL) is characterized as mature B-cell neoplasm and one of the most common lymphoma subtypes [3], where neoplastic cells are large in size and organized in a diffuse pattern when compared to the

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non-cancerous tissue [3]. While median age for DLBCL diagnosis is between 60 and 70 years, younger patients can be diagnosed as well [4]. Enlargement of lymph nodes and highly aggressive growth of tumor mass in bone marrow, liver and other organs are some of the characteristic representations of DLBCL [3, 5]. During the cell development stages, malignant B-cell proliferation triggers DLBCL formation, which is classified into two main subgroups based on the cell of origin: Activated B-cell (ABC) DLBCL and germinal center B-cell (GCB) DLBCL [6]. Despite the advances and huge efforts in determination of efficient therapy options for DLCBL treatment, various issues, including resistance and toxicity, are among the main concerns. Additional contributor to the poor prognosis is genomic complexity of DLBCL, where high number of mutations are associated with the DLBCL pathogenesis [7]. Treatments of DLBCL commonly include groups of drugs such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) and R-CHOP (with addition of rituximab antibody) immunochemotherapy [8]. Additionally, chimeric antigen receptor-modified (CAR) T-cell therapy shows great potential for DLBCL treatment, especially for patients with resistance and relapsed DLBCL. This therapy is based on the genetic modification of the patient's own T-cells to target the cancer [9].

Epigenetic regulations expand the complexity of gene expression process by various epigenetic mechanisms, including histone deacetylation and DNA methylation, without altering the DNA sequence [10, 11]. Numerous groups of genes, including DNA methyltransferases (DNMTs) [12], ten eleven translocation genes (TET) [13], histone deacetylases (HDACs) [14], histone acetyltransferases (HATs) [15], polycomb group associated genes [16], switch/sucrose non-fermentable (SWI/SNF) genes [17], lysine methyltransferases (KMTs) [18], and lysine demethylases (KDMs) [19] are essential for epigenetic regulations. Mutations of these genes are leading to epigenetic dysregulations and alterations, further affecting gene expression and increasing the risk of cancer development. Various cancer types, including breast [20], prostate [21] and colorectal [22] cancer, acute myeloid leukemia [23], follicular lymphoma [24] and DLBCL [25] are associated with the mutations of epigenetic regulator genes. In particular, frequent mutations in the epigenetic regulatory genes CREBBP, KMT2D, EZH2 and TET2 were revealed upon genetic profiling of DLBCL patients, promoting the malignant transformation of B cells through altered epigenetic regulation [26]. Promising steps are being taken in relation to epigenetic therapy for DLBCL, where tazemetostat inhibitor of epigenetic regulator EZH2, might offer favorable treatment option for patients with EZH2 mutations. However, this approach still has certain risks, including selectivity of targets and oncogenic activation [27].

Additionally, important role in cancer research belongs to the computational analysis, where genomic data is analyzed by using various computational methods in order to correlate mutations with the cancer progression and pathogenesis. Computational analysis might be used to provide additional information and help in prognosis, early detection, as well as targeted therapy for cancer treatments [28]. Moreover, identification of critical epigenetic mutations might enhance the efficiency and development of epigenetic drugs, further leading to better and more effective targeted therapies [29].

The effort devoted to a better understanding of the factors and trigger points associated with cancer progression and development can have pivotal role in reducing the trend of new cancer cases. The scope of this study is to analyze genes categorized as epigenetic regulators involved in DLBCL pathogenesis, their mutations, along with the effects on protein sequence and structure by using multiple databases.

Materials and Methods

Gene selection

Based on the literature analysis, genes selected for this study are mainly involved in epigenetic regulations and are potentially able to contribute to development of different cancer types. 69 genes belonging to the following groups: DNA methyltransferases, HDACs, HATs, ten-element translocation genes, polycomb group associated genes, SWI/SNF complex genes, KMTs, KDMs and sirtuins were included. C-bioportal database [30] was used for DLBCL studies, and the database with the largest sample size (1001 samples, Duke, cell 2017) was selected, from which genes involved in epigenetic regulations with certain mutations in DLBCL patients were selected.

Mutation analysis

Mutations in selected genes were further filtered based on the mutation type by using c-bioportal database [30]. Research focused on the mutations that are known to be putative drivers, including missense, truncating (nonsense, nonstop, frameshift deletion, frameshift insertion or splice site), inframe (deletion or insertion), splice and fusion mutations. Mutations that are categorized as mutations of unknown significance are excluded from the analysis. Following that, genes with often reported mutations in DLBCL patients were selected.

Sequence-based prediction

Impact of the mutations on the protein function was evaluated by using sequence-based computational tools, including PredictSNP [31], SNPs&GO [32], and AlphaMissense [33]; where PredictSNP is a consensus of six constituent tools, including MAPP, PhD-SNP, PolyPhen-1, PolyPhen-2, SIFT and SNAP for disease-related mutation prediction obtained from the Uniprot and Protein Mutant Database; SNPs&GO is a server for single point protein mutation prediction based on annotation of functional protein; and AlphaMissense is a tool used as a predictor of the pathogenicity of missense variants. Sequences of CREBBP (Q92793), EP300 (Q09472) and EZH2 (Q15910) were used for the analysis from the Uniprot database [34].

Structure-based prediction

Impact of the mutations on the protein stability was evaluated by using structure-based computational tool DynaMut [35], which is using normal mode analysis to evaluate changes

Table 4. Structure-based prediction results based on the DynaMut tool

Gene	Mutation	ΔΔG (kcal/mol)	$\Delta\Delta S_{Vib}$ (kcal.mol^{-1}.K^{-1})
CREBBP	R1446H	-1.135 (-)	0.244 (–)
	R1446C	-0.904 (-)	0.279 (–)
	Y1503F	-2.475 (-)	0.918 (–)
	Y1503D	-2.475 (-)	0.918 (–)
EP300	L415P	-1.341 (-)	0.409 (–)
	H1451Y	9.706 (+)	-0.167 (++)
	Y1467D	-2.399 (-)	0.882 (–)
EZH2	Y641N	0.578 (+)	0.053 (–)
	Y641F	0.578 (+)	0.053 (–)

ΔΔG change in the Gibbs free energy; $\Delta\Delta S_{Vib}$ change in vibrational entropy energy between wild-type and mutant; (+): Stabilizing; (-): Destabilizing; (++): Increased molecule flexibility; (--): Decreased molecule flexibility.

in protein stability. Protein Data Bank in Europe Knowledge Base (PDBe-KB) database [36] and Protein Data Bank in Europe (PDBe) database [37] were both used to select the PDB protein structures, chains containing the mutation positions, along with analysis of interaction interfaces.

Visualization of interactions in wild-type and mutant structures

Selected proteins were visualized by using BIOVIA Discovery Studio 2021. After selection of mutation position, interaction types, including hydrogen, electrostatic and hydrophobic bonds, along with the unfavorable bumps were selected from the interaction monitor. Additionally, molecular scope was set to any atom-to-atom interaction. Interactions with the surrounding residues were analyzed and evaluated in both, wild-type and mutant structures.

This study was conducted in accordance with the ethical principles of the Helsinki Declaration. As no human participants or patient data were directly involved, specific ethical approval was not required. All data used in this study are publicly available online at https://www.cbioportal.org/ [30].

Results

Based on the literature analysis, 69 genes that belong to the various groups of epigenetic regulators were selected (Appendix 1). By using the c-bioportal database, 14 genes, including DNMT3A, CREBBP, EP300, TAF1, EZH2, ARID1A, ARID1B, ARID5b, SETD1B, SETD2, SETD5, KMT2C and KMT2D are further filtered and selected with the reported mutations in DLBCL patients (Table 1).

Next, among above mentioned genes, genes with the highest number of reported mutations in various DLBCL patients from c-biportal database were selected, including CREBBP, EP300 and EZH2, with four (R1446H, R1446C, Y1503F, Y1503D), three (L415P, H1451Y, Y1467D) and two (Y641N, Y641F) often reported missense mutations in DLBCL patients, respectively. R1446H/C and Y1503F/D missense mutations of CREBBP were reported in 18 patients, L415P, H1451Y, and Y1467D missense mutations of EP300 were reported in 9 patients, while Y641N/F missense mutations of EZH2 were reported in 57 patients.

PredictSNP computational tool, together with the consensus tools, predicted that nearly all selected mutations are deleterious with the high expected accuracy percentages, except for Y1503F mutation with the expected accuracy of 58% for PhD-SNP and L415P mutation with the expected accuracy of 50% for SNAP tool (Table 2). Additionally, according to SNPs&GO computational tool, all mutations are categorized as disease related polymorphisms with the highest reliability index (RI) values being reported for EP300 gene, being 8, 10 and 9 for L415P, H1451Y and Y1467D mutations respectively, while the

Table 1. List of selected genes with the mutation percentages from C-bioportal database, 1001 DLBCL samples

No	Gene	Group	Mutation (%)	Source
1	DNMT3A	DNA methyltransferase	4.6	[38]
2	CREBBP	Histone acetyltransferase	11.5	[15]
3	EP300	Histone acetyltransferase	5.7	[15]
4	TAF1	Histone acetyltransferase	3.4	[39]
5	TET2	Ten element translocation	6.2	[40]
6	EZH2	Polycomb group	6.1	[41]
7	ARID1A	SWI/SNF complex	9.6	[42]
8	ARID1B	SWI/SNF complex	8.3	[42]
9	ARID5B	SWI/SNF complex	3.2	[43]
10	SETD1B	Lysine methyltransferase	8.3	[44]
11	SETD2	Lysine methyltransferase	6.2	[44]
12	SETD5	Lysine methyltransferase	3.1	[45]
13	KMT2C	Lysine methyltransferase	5.6	[46]
14	KMT2D	Lysine methyltransferase	24.7	[46]

DLBCL: Diffuse large B-cell lymphoma; SWI/SNF: Switch/sucrose non-fermentable.

Table 2. Sequence-based prediction results based on the PredictSNP and its consensus tools

Gene	Mutation	Predict SNP	MAPP	PhD-SNP	PolyPhen-1	PolypPhen-2	SIFT	SNAP
CREBBP	R1446H	87% (-)	51% (-)	88% (-)	74% (-)	81% (-)	79% (-)	85% (-)
	R1446C	87% (-)	76% (-)	88% (-)	74% (-)	81% (-)	79% (-)	85% (-)
	Y1503F	76% (-)	62% (-)	58% (+)	74% (-)	81% (-)	79% (-)	62% (-)
	Y1503D	87% (-)	77% (-)	82% (-)	74% (-)	81% (-)	79% (-)	81% (-)
EP300	L415P	76% (-)	57% (-)	82% (-)	59% (-)	81% (-)	79% (-)	50% (+)
	H1451Y	87% (-)	57% (-)	88% (-)	74% (-)	81% (-)	79% (-)	89% (-)
	Y1467D	87% (-)	86% (-)	86% (-)	74% (-)	81% (-)	79% (-)	89% (-)
EZH2	Y641N	87% (-)	82% (-)	77% (-)	59% (-)	60% (-)	53% (-)	85% (-)
	Y641F	87% (-)	86% (-)	82% (-)	74% (-)	65% (-)	79% (-)	89% (-)

%: Expected accuracy; (+): Neutral; (-): Deleterious.

Table 3. Sequence-based prediction results based on the SNPs&GO computational tool and AI generated AlphaMissense server

Gene	Mutation	SNPs&GO		AlphaMissense	
		Effect	RI	Class	PS
CREBBP	R1446H	Disease related polymorphism	7	Likely pathogenic	0.995
	R1446C	Disease related polymorphism	7	Likely pathogenic	0.994
	Y1503F	Disease related polymorphism	4	Likely pathogenic	0.907
	Y1503D	Disease related polymorphism	5	Likely pathogenic	0.999
EP300	L415P	Disease related polymorphism	8	Likely pathogenic	0.999
	H1451Y	Disease related polymorphism	10	Likely pathogenic	0.985
	Y1467D	Disease related polymorphism	9	Likely pathogenic	0.999
EZH2	Y641N	Disease related polymorphism	3	Likely pathogenic	0.999
	Y641F	Disease related polymorphism	3	Likely pathogenic	0.965

RI: Reliability index.

lowest RI reported is 3 for both, Y641N/F mutations for EZH2 (Table 3). Moreover, all selected mutations are showed to be likely pathogenic based on the AlphaMissense tool with pathogenicity values higher than 0.9 (Table 3).

Pathogenicity Score: Pathogenicity values range from 0 to 1 representing the likelihood of pathogenicity as follows: 0.0 – 0.33 Likely benign; 0.34 – 0.66 ambiguous; 0.67 – 1.0 likely pathogenic.

Prior to structure-based prediction, protein structures, along with the chains containing the position of mutations were selected by using PDBe-KB and PDBe. For CREBBP mutations, R1446H/C and Y1503F/D, chain K of 8HAN (PDB ID) protein structure was selected, for EP300 L415P mutation, chain A of 7QGS (PDB ID) protein structure and for EP300 H1451Y and Y1467D mutations chain A of 5KJ2 (PDB ID) protein structures were selected. For EZH2 Y641N/F mutations, chain K of 6C23 (PDB ID) protein structure was selected. Structure-based prediction analysis revealed that most of the selected mutations are either destabilizing or/and they are increasing the molecule flexibility. R1446H/C and Y1503F/D mutations of CREBBP and L415P and Y1467D mutation of EP300 are both, destabilizing and increasing the molecule flexibility. On the other hand, only H1451Y mutation of EP300 decreases the molecule flexibility and stabilizes the structure (Table 4 and Fig. 1).

Furthermore, differences in the interactions in wild-type and mutant structures were observed. CREBBP wild-type Arg1446 has four conventional hydrogen bond interactions with Tyr1450, Val1449, Arg1443 and His1438 and two Pi-Alkyl interactions with Tyr1450 and Phe1440. CREBBP mutant His1446 has three conventional hydrogen bond interactions with Tyr1450, Vak1449 and Arg1443 and one Pi-Pi stacked interaction with Phe1440, while Cys1446 mutant of the same protein has three conventional hydrogen bond interactions with Tyr1450, Val1449 and Arg1443, Pi-Alkyl interaction with Phe1440 and Pi-donor hydrogen bond interaction with Tyr1450 (Fig. 2a-c). CREBBP wild-type Tyr1503 and mutant Phe1503 have four conventional hydrogen bond interactions with Leu1499, Gln1500, Met1506, and Leu1507, Pi-Alkyl interactions with Leu1499 and Ala1474, Pi-Pi T-shaped interactions with Trp1502 and Pi-Sigma bond interactions with Ile1471, while Asp1503 mutant of the same gene has same conventional hydrogen bond interactions without other interactions for the particular mutation position (Fig. 2d-f).

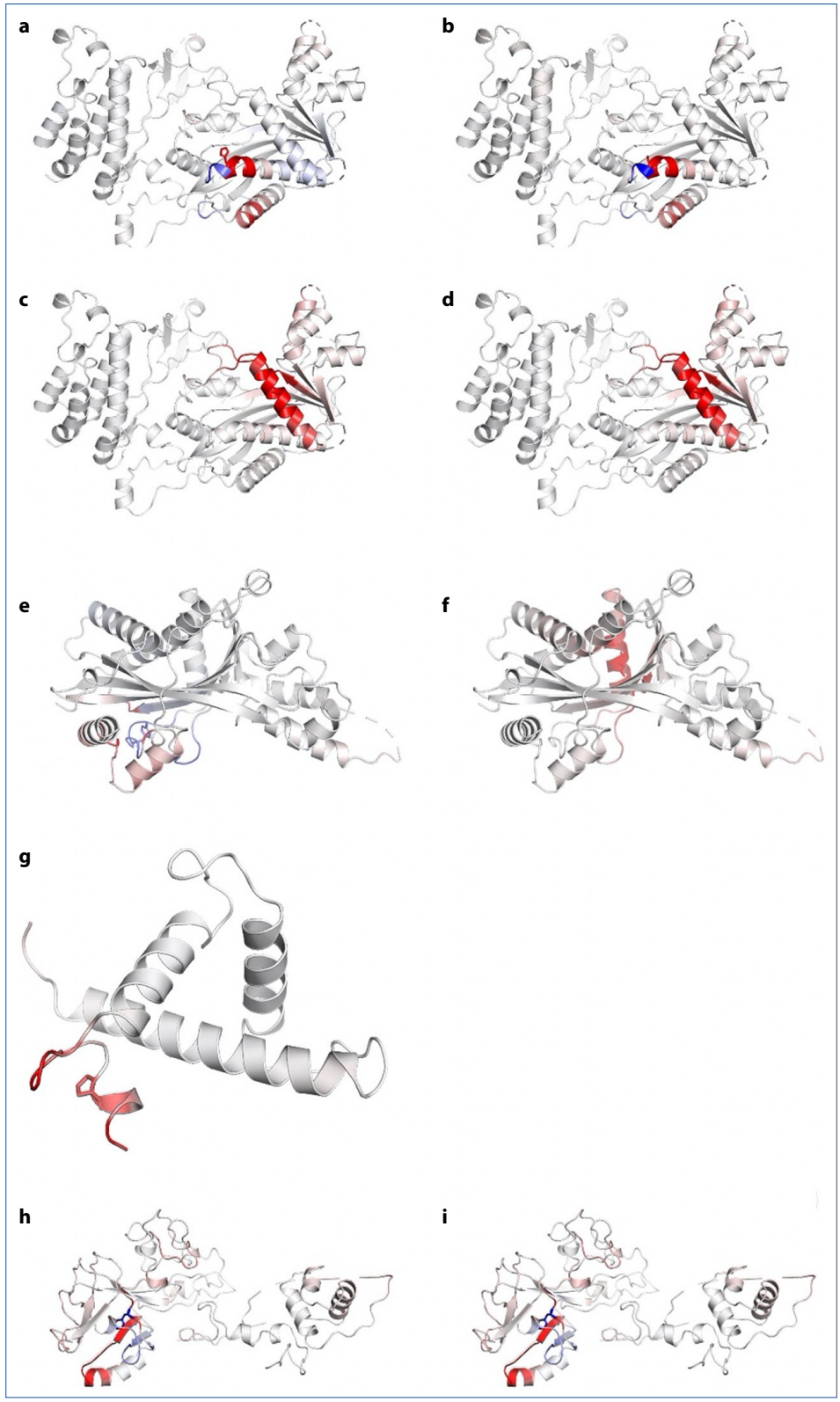

Figure 1. Flexibility visualization of mutations on CREBBP (a-d), EP300 (e-g) and EZH2 (h, i). (a) R1446H; (b) R1446C; (c) Y1503F; (d) Y1503D; (e) H1451Y; (f) Y1467D; (g) L415P; (h) Y641N; (i) Y641F.

Differences are also observed between wild-type and mutant structures on the positions 415, 1451 and 1467 of EP300. Leu415 wild-type structure has two conventional hydrogen bond interactions with Cys411 and Asn419, while Pro415 mutant structure has only one conventional hydrogen bond interaction with Asn419 (Fig. 3a, b). His1451 wild-type structure of

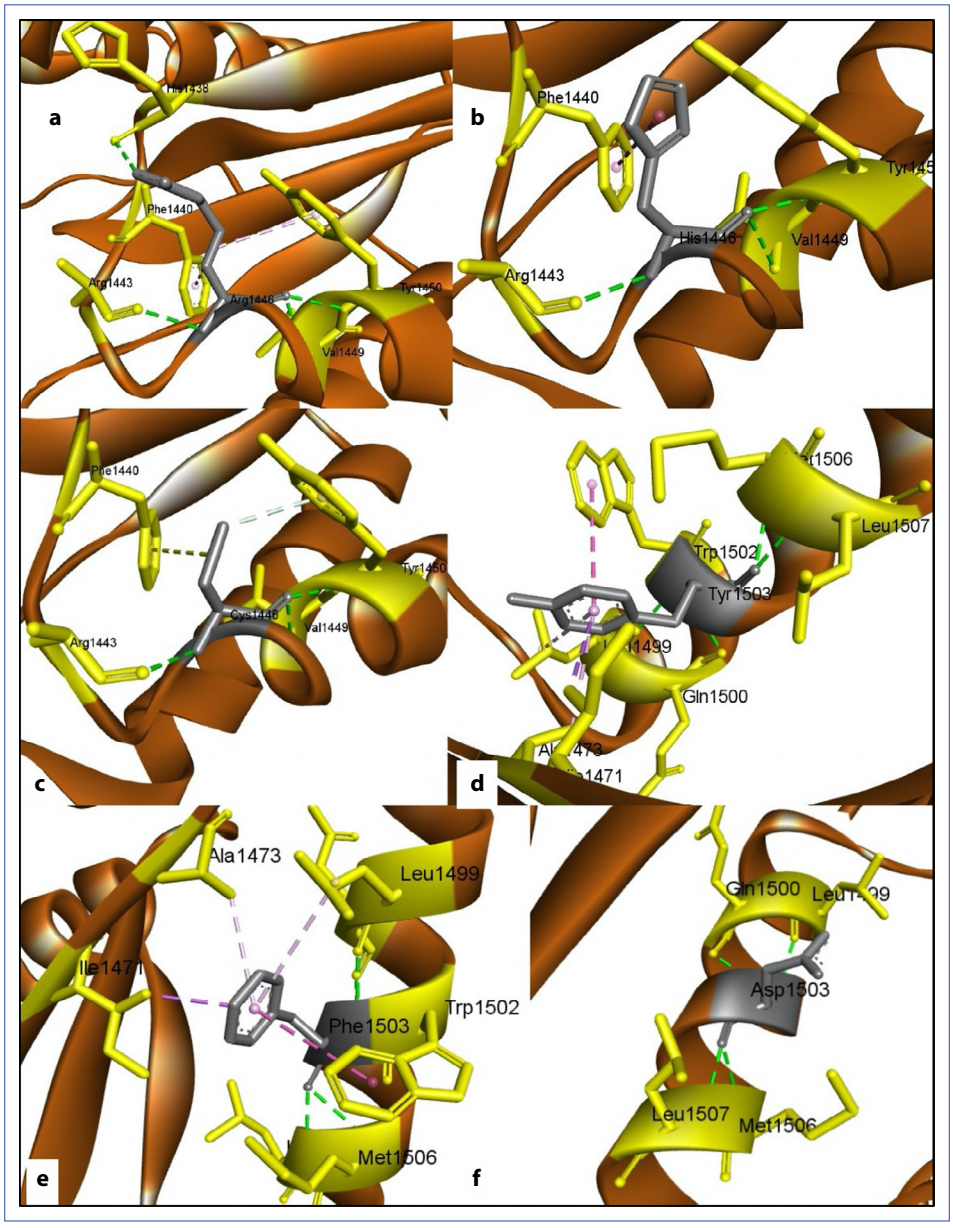

Figure 2. Visualization of interaction in wild-type and mutant structures on 1446 and 1503 positions of CREBBP protein. Brown – protein chain; yellow – interacting residues; grey – mutation position; conventional hydrogen bond interactions – green; Pi bond interactions – pink; (a) Arg1446 wild-type; (b) His1446 mutant; (c) Cys1446 mutant; (d) Tyr1503 wild-type; (e) Phe1503 mutant; (f) Asp1503 mutant (Created by BIOVIA Discovery Studio 2021).

the same protein has one carbon hydrogen bond interaction while Tyr1451 mutant has no interactions (Fig. 3c, d). Wild-type Tyr1467 of EP300 has four conventional hydrogen bond interactions with Leu1463, Gln1464, Met1470 and Leu147, three Pi-Alkyl interactions with Ile1435, Ala1437 and Leu1463 and one Pi-Pi T-shaped interaction with Trp1466 while Asp1467 mutant has same conventional hydrogen bond interactions without any Pi bond interactions (Fig. 3e, f).

Wild-type Tyr641 of EZH2 has two conventional hydrogen bond interactions with Phe665 and Ile708 and one Pi-Alkyl interaction with Arg685. Asn641 mutant has one conventional hydrogen bond interaction with Ile708 while Phe641 mutant has same conventional hydrogen bond interaction in addition to Arg685 Pi-Alkyl and Phe667 Pi-Pi stacked interaction (Fig. 4).

Discussion

Epigenetic regulators are potent proteins involved in various mechanisms related to gene expression regulations. Any alterations in their structure carries a high risk for tumor development [47]. Although there are numerous epigenetic regulators, focus of this study are epigenetic regulatory genes reported in DLCBL patients. Our results showed that CREBBP, EP300

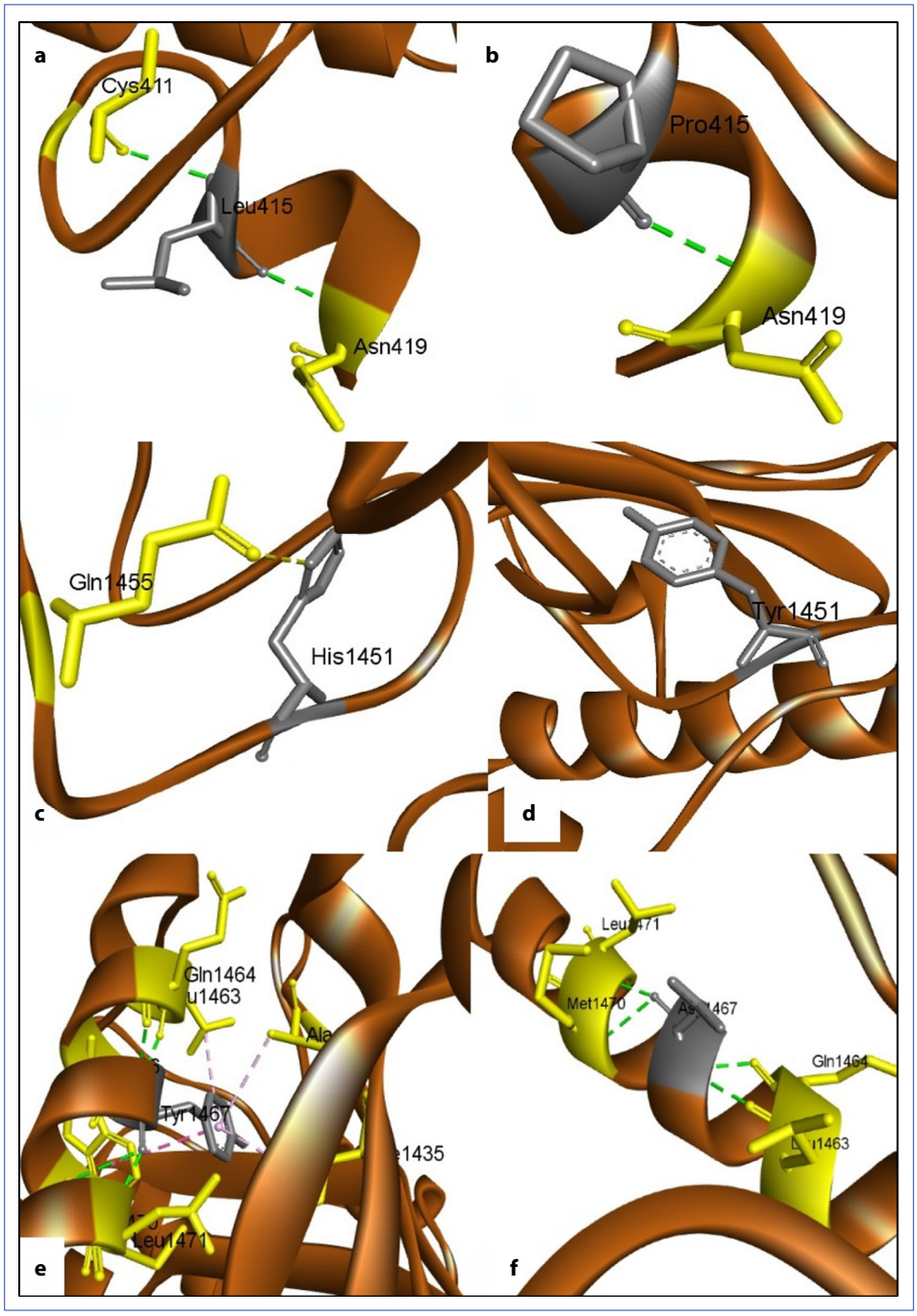

Figure 3. Visualization of interaction in wild-type and mutant structures on 415, 1451 and 1467 positions of EP300 protein. Brown – protein chain; yellow – interacting residues; grey – mutation position; conventional hydrogen bond interactions – green; Pi bond interactions – pink; (a) Leu415 wild-type; (b) Pro415 mutant; (c) His1451 wild-type; (d) Tyr1451 mutant; (e) Tyr1467 wild-type; (f) Asp1467 mutant (Created by BIOVIA Discovery Studio 2021).

and EZH2 are among most mutated epigenetic regulators in DLBCL. CREBBP and EP300 function as the histone acetyltransferases, while EZH2 has a role in polycomb repressive complex [48]. Various reports underline the importance of these genes in hematological malignancies, especially in DLBCL [49, 50]. CREBBP has crucial role in different processes, including regulation of gene expression, along with the involvement in cell growth, development and differentiation [50]. Similarly, EP300 has a pivotal role in cell proliferation, apoptosis and differentiation regulation through interactions with various transcription factors, including p53 and NF-κB [50]. Moreover, EZH2 acts as a transcriptional repressor, where EZH2 dysregulation is mainly associated with negative outcomes when it comes to the cancer progression and resistance [51].

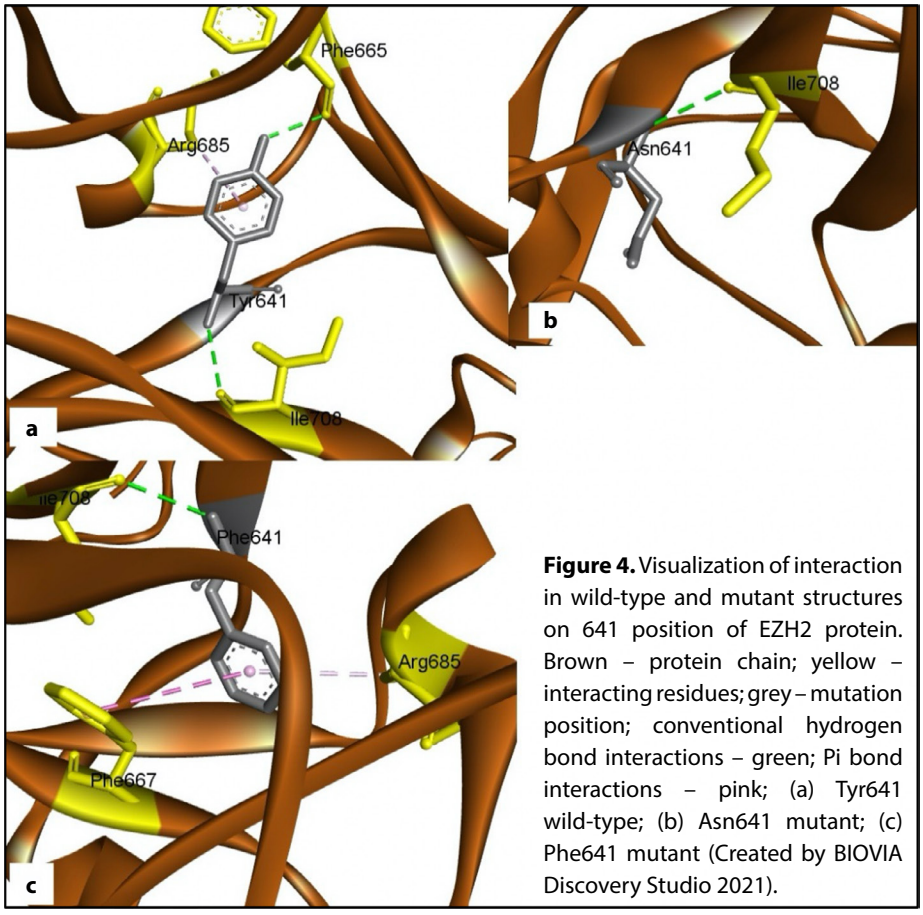

Figure 4. Visualization of interaction in wild-type and mutant structures on 641 position of EZH2 protein. Brown – protein chain; yellow – interacting residues; grey – mutation position; conventional hydrogen bond interactions – green; Pi bond interactions – pink; (a) Tyr641 wild-type; (b) Asn641 mutant; (c) Phe641 mutant (Created by BIOVIA Discovery Studio 2021).

Our results suggest that selected mutations, including R1446H/C and Y1503F/D for CREBBP, L415P, H1451Y and Y1467D for EP300, and Y641 N/F for EZH2 proteins have mostly negative impact on the sequence and structure of the proteins, being either deleterious, likely pathogenic, destabilizing and/or enhancing the molecule flexibility, along with the impact on the change of interactions between the residues.

Although the bonds in wild-type and mutant structures are not directly interfering with the interaction interfaces indicated in PDBe-KB database, they are in close proximity to mutation positions. In relation to that, selected mutations could possibly affect these interactions. Additionally, some of the mutations are positioned on ligand binding sites, which could impact the binding affinity and drug resistance [52]. Our results showed that most common CREBBP mutations are R1446 and Y1503. These mutations are also reported in relapse acute lymphoblastic leukemia as well [53]. Previous studies indicated the negative correlation between the R1446 and Y1503 mutations of CREBBP and cancer progression in DLBCL and B-cell lymphomas respectively [54]. Pasqualucci et al. [55], showed that the commonly shared pathogenic mechanisms of non-Hodgkin B-cell lymphoma are CREBBP and EP300 mutations. However, additional analysis is required to achieve a more comprehensive understanding.

Another common mutation selected in current study is H1451 of EP300. Duex et al. [56], discussed that H1451 residue mutations of EP300 lead to inactivation of HAT. This is in accordance with the findings of current study, as the most often reported mutation of EP300 gene is on H1451 in DLBCL patients. HAT domain has role in gene regulation by neutralizing the positive charge of histones, weakening their interaction with DNA and making chromatin more accessible for transcription. As the mutational hotspots occur in the HAT domain, it might alter the cellular regulations and development, leading to negative outcomes in relation to carcinogenesis. As Huang et al. [57] reported that CREBBP/EP300 gene mutations increased the rate of tumor progression in DLBCL with the lower progression-free survival and lover overall survival rate when compared to the patients without CREBBP/EP300 mutations. It is obvious that both CREBBP/EP300 mutations have a crucial effect on DLBCL pathogenesis.

Another commonly identified mutation is Y641 of EZH2. This particular mutation possesses the high importance, as the overexpression of EZH2 is closely related to tumor suppressor gene silencing [58]. It has been reported that Y641 and A677 heterozygous point mutations in EZH2 occur in 10–24% of all non-Hodgkin lymphoma cases [59]. In addition, Y641 mutation of EZH2 is associated with the immunodeficiency in lymphoid malignancies [60]. Morin et al. [61], also identified Y641 mutation of EZH2 and its correlation with the GCB subtype of DLBCL. According to their study, this particular mutation is related to the pathogenesis of GCB lymphomas.

Recent studies suggest that targeting the epigenetic regulators in various cancer types, including DLBCL, might poses beneficial effects regarding the treatment [27, 62, 63]. The understanding of correlation of analyzed mutation positions and DLBCL pathogenesis could contribute to the enhanced prediction, diagnosis, and treatment of DLBCL.

Conclusion

Any alterations in epigenetic mechanisms carry a high risk for cancer development, and understanding the factors and trigger points associated with cancer progression can play a pivotal role in reducing the incidence of new cancer cases. Since epigenetic regulators are reported to impact DLBCL progression, we focused our research on identifying and analyzing potential proteins and genes involved in the epigenetic regulation of DLBCL. Using various computational tools, we identified and analyzed the effects of commonly mutated positions in the CREBBP, EP300, and EZH2 proteins. Computational analysis, especially with recent advances in artificial intelligence and machine learning methods, plays an important role in identifying specific mutations and their correlation with cancer. However, it also has limitations, including the lack of experimental data, which necessitates additional *in vitro* and *in vivo* research. To the best of our knowledge, this is the first in silico study analyzing sequence and structure-based effects of R1446H/C, Y1503F/D, L415P, H1451Y, Y1467D and Y641N/F mutations.

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Appendix 1. 68 genes selected for this study mainly involved in epigenetic regulations in different cancers

Gene	Name	Percentage	Group
DNMT1			DNA Methyltransferase
DNMT3A	DNA Methyltrasferase 3 Alpha	4.6	
DNMT3B			
DNMT3L			
HDAC1			HDAC
HDAC2			
HDAC3			
HDAC8			
HDAC4			
HDAC5			
HDAC7			
HDAC9			
HDAC6			
HDAC10			
HDAC11			
CREBBP	cAMP-responsible element binding protein	11.5	HAT
EP300	Histone acetyltransferase p300	5.7	
TAF1	Transcription initiation factor TFIID subunit 1	3.4	
TET1			TET
TET2	Ten element translocation 2	6.2	
TET3			
EZH2	Enhencer of zeste homolog 2	6.1	Polycomb group proteins
ARID1A	AT-rich interactive domain 1A gene	9.6	SWI/SNF complex
ARID1B	AT-rich interactive domain 1B gene	8.3	
PBRM1			
SMARCA4			
SMARCB1			
ARID1			
ARID2			
ARID3			
ARID4			
ARID5 (B)	AT-rich interactive domain 5B	3.2	
JARID1			
JARID2			
SETD1A			KMTs
SETD1B	Histone lysine methyltransferase 1B	8.3	
SETD2	Histone lysine methyltransferase 2	6.2	
SETD5	Histone lysine methyltransferase 5	3.1	
KMT2A			
KMT2C	Lysine methyltransferase 2C	5.6	
KMT2D	Lysine methyltransferase 2D	24.7	
KMT3A			
KMT3B			
KMT6A			
KMT8			
KMT2A			
KMT1C			
KMT1E			
KMT2E			
KMT3C			

Appendix 1. Cont.

Gene	Name	Percentage	Group
KMT3E			
KDM1A			KDMs
KDM3A			
KDM5A			
KDM2B			
KDM4B			
KDM5B			
KDM6B			
KDM3B			
KDM5C			
KDM6A			
SIRT1			SIRTUINS
SIRT2			
SIRT3			
SIRT4			
SIRT5			
SIRT6			
SIRT7			

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Research Article

Patient based real time quality control using average of normal approach

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Abstract

Objectives: The limitation of internal quality control (IQC) based on daily running of commercially available QC material is that it is non-commutable and errors cannot be detected in between the scheduled runs. This study was carried out with the objective of finding the utility of patient based real time quality control (PBRTQC) in overcoming these limitations.

Methods: This observational descriptive study was carried out in the clinical chemistry laboratory of a tertiary care hospital between July 2023 to December 2023. PBRTQC was initiated in the laboratory by using Average of Normal (AoN) approach for serum sodium and potassium. Patients' sample-based reference mean (RP_M) and standard deviation (RP_{SD}) were calculated from the previous six months' data using reference intervals as truncation limits. For the next 2000 samples, the mean was calculated for each block of 20 samples ($\bar{x}$) and designated as $\bar{x}1$, $\bar{x}2$, $\bar{x}3$... These block means were plotted on the LJ chart and alarms were raised on the violation of predefined control rules. These alarms were investigated and necessary corrective measures were implied in the laboratory.

Results: $RP_M \pm RP_{SD}$ for sodium was 139.23±3.72 mEq/L and potassium was 4.26±0.45 mEq/L. The scheduled IQC was within range during the study. Alarms were raised for $\bar{x}13$, $\bar{x}28$, $\bar{x}29$, $\bar{x}30$, $\bar{x}35$, $\bar{x}36$, $\bar{x}55$, $\bar{x}74$ and $\bar{x}96$. The workup of these alarms revealed instrument calibration error in most of the cases. However, analysis of $\bar{x}35$ and $\bar{x}36$ revealed delayed transport, improper temperature maintenance and partial hemolysis. All responsible personnel were given training regarding sample transport procedure. Using real time monitoring, we were able to detect errors which would have otherwise gone unnoticed by conventional IQC.

Conclusion: PBRTQC permits stringent quality control in analytical as well as pre-analytical phase of testing procedure, even during the intervals between scheduled IQC runs. Successful implementation of PBRTQC will provide additional confidence in reporting laboratory results.

Keywords: Average of normal, quality control, real-time QC, serum potassium, serum sodium, reference mean (RP_M), reference mean standard deviation (RP_{SD})

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Conventional internal quality control (IQC) analysis strategies employed by laboratories involve periodic testing of QC sera. However, this strategy can detect only analytical errors at the scheduled QC run and there is no way to detect errors in the intervals between the run time. Further, preanalytical factors which affect patient's results are not reflected during IQC analysis. So, periodic IQC analysis delays error detection and is not useful for real time monitoring of results [1, 2].

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To augment existing QC strategies, various laboratories have tried different techniques, including inter-laboratory comparison, proficiency testing, patient based real time quality control (PBRTQC), six sigma metrics, etc. PBRTQC was introduced as a technique of quality control early in the 1960s [3]. The earliest method was called 'Average of Normal' strategy (AoN) [4]. It was largely based on a patient-centric approach and used the results generated by the laboratory over a defined number of patients' results to calculate the central tendency (mean/median) of an analyte. Subsequent patient results are then monitored in real time for any unusual deviation from this central tendency. This real time analysis will immediately detect any error which shift the patients' results in one direction. This could be analytical errors like calibration failure, instrument malfunction, etc., or even pre-analytical errors like hemolysis, delayed sample transport, sample processing errors, etc. So, PBRTQC strengthens and complements traditional IQC and so is a useful tool for quality assurance [5, 6].

With time, many strategies for PBRTQC were developed, like moving average, Bull's algorithm, moving SD, moving percentiles, exponentially weighted moving averages, etc. [7]. However, PBRTQC programs are still not widely implemented in clinical chemistry laboratories because of complex procedures and calculations, lack of any standard protocol for implementation, limited software support and a deficit of trained personnel to analyze and interpret the vast stream of data.

As a step towards continual improvement, we planned to initiate PBRTQC program in our clinical chemistry laboratory for critically important parameters like serum sodium and serum potassium using AoN approach.

Materials and Methods

This observational descriptive study was carried out in Clinical Chemistry Laboratory of a tertiary care hospital between July 2023 and December 2023. We planned to initiate PBRTQC program by AoN approach for serum sodium and serum potassium. All samples for electrolyte analysis are tested on Microlab 'MICROLYTE ANALYZER' in our laboratory. The instrument undergoes scheduled calibration every twelve hours. We validate this calibration by analyzing IQC sample. So, we perform two levels of Internal Quality Control (IQC) testing every 12 hours, in line with the Clinical Laboratory Standards Institute (CLSI) guidelines, which recommend running at least two levels of QC once daily. Further, we follow Westgard's rule of 1^{3S} and 2_{2S} for rejecting the QC run [8]. 1_{3S} rule rejects the run when a single control measurement exceeds the mean plus 3SD or the mean minus 3SD control limit. 2_{2s} rule rejects the run when two consecutive control measurements exceed the same mean plus 2SD or the same mean minus 2SD control limit [8].

To initiate AoN approach, we selected serum electrolytes based on two criteria. The first criterion was the daily number of performed tests. Serum electrolytes were representatives of high-frequency tests in our laboratory. Secondly, electrolytes have low biological variation (less than 1%) and by applying narrow truncation limits, we can get normal distribution of data [9]. We calculated a reference mean for serum sodium and serum potassium from previous six months' data (January to June 2023) of patients' results (n=8990). These results included all inpatient and outpatient departments representative of the general hospital population. The results were anonymized and exported from the laboratory information system(LIS) to excel sheets. The reference intervals of serum sodium (135–145 mEq/L) and serum potassium (3.5–5.5 mEq/L) were used as truncation limits to exclude 528 values from 8990 values. The remaining values (n=8462) were used for computing reference mean, i.e., patients' sample-based reference mean (RP_M) and reference standard deviation (RP_{SD}).

The subsequent samples were tested in blocks of 20 samples each. The samples were tested in blocks of 20, based on the recommendation by Li et al. [10], who proposed using a block size between 20 and 50. This block size facilitates quick error detection while minimizing the risk of false rejections. We excluded any value outside of $RP_M \pm 3\ RP_{SD}$ from these blocks. On applying Shapiro Wilk test, we found the blocks had normal distribution of data. A block mean ($\bar{x}$) for 20 samples was calculated and designated as $\bar{x}1$, $\bar{x}2$, $\bar{x}3$ ... $\bar{x}n$. We then plotted Levey Jennings (LJ) chart using RP_M and action/control limits were applied at ±2% and ±3% of RP_M. These narrower action limits were chosen rather than RP_{SD} as action limit as electrolytes have low biological variation (less than 1%) and it would increase sensitivity of analysis [9]. Any patient value crossing these action limit would alert the analyst.

All block means were being continuously plotted and analyzed for violation of following rules:

Rule I: One of the block means crosses ±3% action limit or

Rule II: Three consecutive block means crossing ±2% action limit.

The block means which violated any of the above rules were investigated to identify whether it is an analytical error or pre-analytical error. The workup of alarm raised was done according to the algorithm as shown in Figure 1. The error was rectified and necessary corrective measures were implemented in the laboratory.

Results

The RP_M derived from 8462 patient samples for serum sodium was 139.23 mEq/L and for serum potassium was 4.26 mEq/L. The action limits calculated from 2% of RP_M and 3% of RP_M are shown in Table 1. The subsequent patient results were being continuously divided into blocks of 20 values. The block means for 100 such blocks is shown in Table 2. These block means were being continuously plotted on LJ chart having RP_M and action limits (Figs. 2, 3).

During monitoring the 100 block means, we found that most of the block means were within ±1SD for both serum sodium and serum potassium. The PBQRTC alarm was raised seven times during our study. The routine twelve hourly IQC was

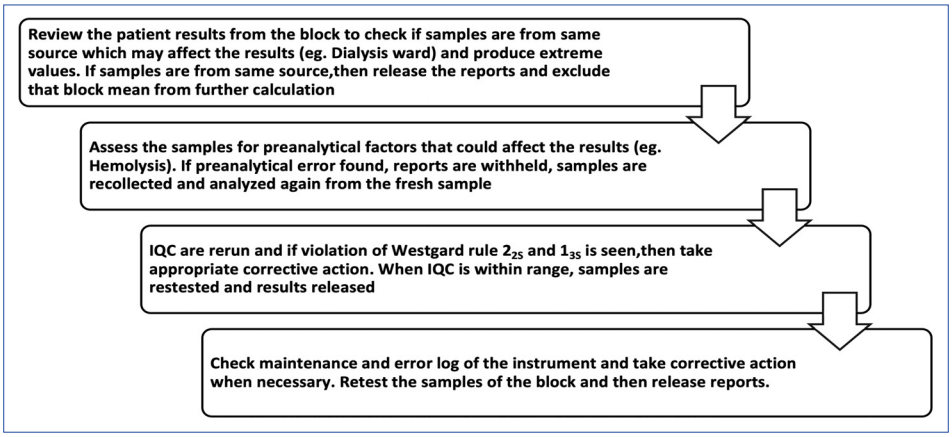

Figure 1. Figure shows sequential steps in the workup of alarm.

IQC: Internal quality control.

Table 1. Table shows the patients based reference mean (RPM), reference standard deviation (RPSD), 2% and 3% action limits for 8462 samples

Parameter	Serum sodium (mEq/L)	Serum potassium (mEq/L)
RPM	139.23	4.26
RPSD	3.72	0.45
RPM + 2% of RPM	142.01	4.34
RPM - 2% of RPM	136.45	4.18
RPM + 3% of RPM	143.40	4.38
RPM - 3% of RPM	135.09	4.14

within range during the study. The analysis and corrective action of the alarms raised are explained below:

- Rule I was violated by x̄28, x̄29, x̄30 for both serum sodium and potassium. For sodium, these three block means were below the 2% action limit, whereas for potassium, these block means were above the 2% action limit. The workup for these alarms is shown in Table 3. We found that there was no analytical error, as the IQC was retested and found to be within range and no instrument malfunction had occurred during the analysis. We suspected these samples to be hemolyzed but no apparent hemolysis was visible on inspection. On investigating the records for the time of

Table 2. Table shows block means (x̄) for serum sodium (Sod.) and serum potassium (Pot.). Each block represents an average of twenty samples

Block no.	Block mean sod. mEq/L	Block mean pot. mEq/L	Block no.	Block mean sod. mEq/L	Block mean pot. mEq/L	Block no.	Block mean sod. mEq/L	Block mean pot. mEq/L	Block no.	Block mean sod. mEq/L	Block mean pot. mEq/L	Block no.	Block mean sod. mEq/L	Block mean pot. mEq/L
1	138.70	4.21	21	137.20	4.21	41	140.23	4.32	61	140.05	4.19	81	139.31	4.20
2	141.10	4.28	22	136.42	4.24	42	140.76	4.27	62	137.70	4.25	82	140.94	4.19
3	135.78	4.21	23	138.66	4.18	43	140.73	4.29	63	138.55	4.16	83	139.20	4.24
4	136.16	4.24	24	139.78	4.19	44	139.57	4.25	64	138.15	4.2	84	142.05	4.3
5	137.78	4.27	25	138.64	4.21	45	137.16	4.29	65	139.50	4.25	85	142.16	4.22
6	139.30	4.25	26	138.30	4.22	46	136.52	4.22	66	136.80	4.21	86	138.15	4.24
7	139.00	4.21	27	137.17	4.29	47	135.95	4.24	67	135.65	4.31	87	141.00	4.3
8	137.00	4.27	28	135.20	4.15	48	136.75	4.29	68	138.52	4.24	88	141.10	4.36
9	141.20	4.28	29	135.60	4.16	49	137.20	4.29	69	139.20	4.27	89	137.00	4.25
10	138.88	4.34	30	135.30	4.15	50	139.10	4.23	70	136.40	4.25	90	138.85	4.29
11	136.60	4.23	31	138.17	4.32	51	140.33	4.32	71	139.15	4.31	91	137.31	4.32
12	137.25	4.2	32	138.00	4.22	52	140.23	4.29	72	139.15	4.22	92	137.75	4.2
13	134.95	3.9	33	136.00	4.23	53	136.63	4.19	73	136.26	4.31	93	138.27	4.3
14	140.20	4.27	34	138.00	4.25	54	138.94	4.21	74	134.80	4.04	94	142.00	4.23
15	137.42	4.27	35	131.85	5.12	55	134.75	3.95	75	136.36	4.22	95	139.50	4.21
16	139.73	4.24	36	132.35	4.93	56	139.55	4.22	76	138.41	4.3	96	134.65	4.03
17	137.55	4.2	37	138.35	4.25	57	138.42	4.28	77	136.94	4.33	97	136.52	4.32
18	139.23	4.2	38	139.83	4.27	58	140.70	4.24	78	141.05	4.22	98	142.35	4.26
19	138.82	4.24	39	140.38	4.17	59	137.30	4.26	79	136.47	4.29	99	140.50	4.26
20	139.75	4.25	40	137.70	4.21	60	138.27	4.24	80	138.95	4.21	100	138.42	4.28

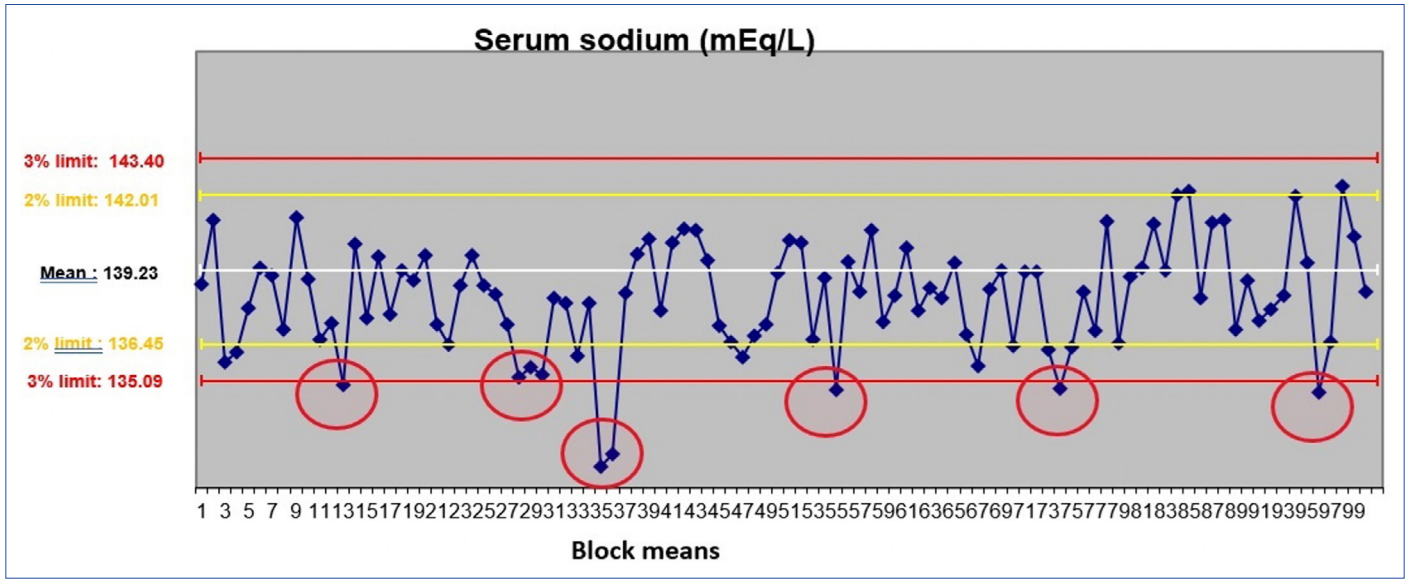

Figure 2. Shows LJ chart for serum sodium; White line indicates RP_M:139.23 mEq/L; Yellow line indicates 2% action limit:142.01 mEq/L and 136.45 mEq/L; Red line indicates 3% action limit:143.04 mEq/L and 135.09 mEq/L; Circle indicates alarm raised.

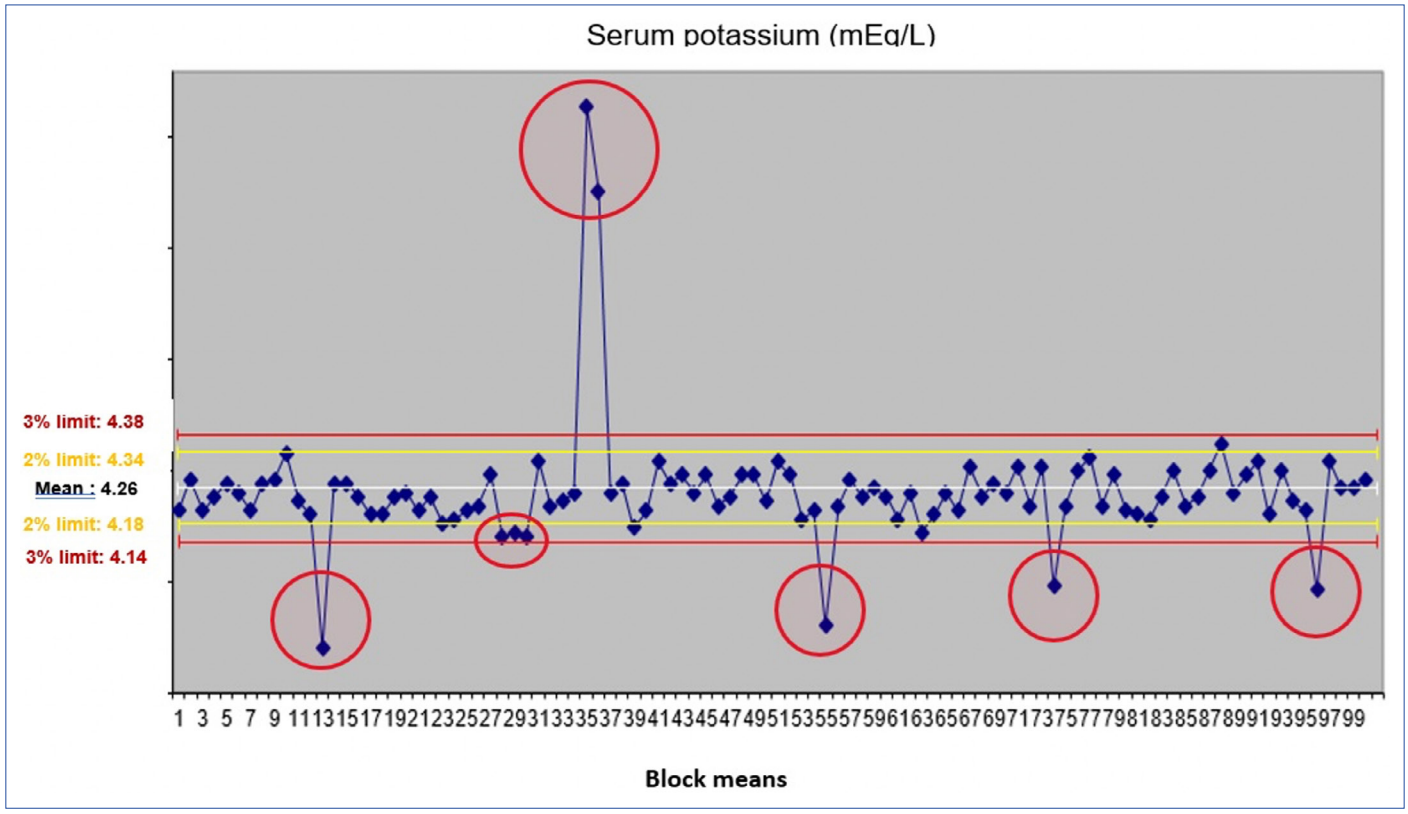

Figure 3. Shows LJ chart for serum potassium; White line indicates RP_M:4.26 mEq/L; Yellow line indicates 2% action limit:4.34 mEq/L and 4.18 mEq/L; Red line indicates 3% action limit:4.38 mEq/L and 4.14 mEq/L; Circle indicates alarm raised.

sample collection, transport and sample receipt in the laboratory. It was found that the transport of these samples from OPD was delayed. Transport boxes were inspected and it was found samples were transported without proper temperature maintenance. These could be the possible causes of partial hemolysis of these samples and hence outliers were seen on PBRTQC charts. These preanalytical errors could be detected by only PBRTQC monitoring and traditional IQC alone could have missed it. As a corrective action, all OPD personnel were trained regarding proper storage and transport of samples and they were instructed to recollect and retest all the affected samples.

Table 3. Table shows workup and corrective action for alarms raised

		PBRTQC alarm workup				
Block mean	**Rule violated**	**Review of patients results**	**Observed preanalytical problem**	**IQC testing (2 levels)**	**Maintenance and error log review**	**Corrective action performed**
x̄13	Block mean crossing ±3% action limit	Samples not from same source	No	Out of range	No error found	Recalibration done and patient samples retested and new results issued
x̄35	Block mean crossing ±3% action limit	Samples not from same source	No	Out of range	No error found	Recalibration done and patient samples retested and new results issued
x̄36	Block mean crossing ±3% action limit	Samples not from same source	No	Out of range	No error found	Recalibration done and patient samples retested and new results issued
x̄55	Block mean crossing ±3% action limit	Samples not from same source	No	Out of range	No error found	Recalibration done and patient samples retested and new results issued
x̄74	Block mean crossing ±3% action limit	Samples not from same source	No	Out of range	No error found	Recalibration done and patient samples retested and new results issued
x̄96	Block mean crossing ±3% action limit	Samples not from same source	No	Out of range	No error found	Recalibration done and patient samples retested and new results issued
x̄28, x̄29, x̄30	Three consecutive block means crossing ±2% action limit	Samples are not from same source	Delayed transportation could have caused partial hemolysis	Within range	No error found	Recalibration done and patient samples retested and new results issued

PBRTQC: Patient based real time quality control; IQC: Internal quality control; x̄: Block mean.

- Rule II was violated by x̄13, x̄35, x̄36, x̄55, x̄74, x̄96 as these block means crossed ±3% action limit. The workup of these alarms is shown in Table 3. It revealed that IQC was out of range during that time of testing these samples. So, as a corrective action, the instrument was calibrated and then IQC was retested which came within range. For these blocks, patient reports were released only after retesting and a new block mean was obtained. So, we found that even though the analyzer went out of calibration at some point of time, but the conventional IQC done at scheduled intervals failed to detect it.

Continuing the PBRTQC monitoring

For continuous monitoring, we revised the RP_M value after obtaining 100 block means within $RP_M \pm 3\ RP_{SD}$. The revised value (RP_{M1}) is calculated using average of previous RP_M and 100 block means. This revised RP_M is then used for further real time monitoring of patients' results.

Discussion

Successful implementation of PBRTQC programs provide an exciting opportunity for clinical laboratories to strengthen their existing quality control procedures. This paper presents the steps in establishment of the most fundamental AoN strategy for real time monitoring of patient results in a tertiary care hospital.

AoN strategy was the earliest version for PBRTQC, described by Hoffman et al. [4] in 1965. In this approach, selected patient's result that falls within the reference range is used in the calculation of a stable mean and 95% confidence interval was used as control limits [11]. After that, the average of selected consecutive patient results should fall within the control limits established for that population. According to Badrick T et al. [1], the control limits for PBRTQC can be defined by the user according to the quality goal desired. In this study, control limits were designed according to method of Korpman and Bull [12]. We chose serum electrolytes for initiating PBRTQC in our laboratory because they have low biological variation, thus simplifying the monitoring process [13, 14].

The procedure was initiated by calculating an RP_M and RP_{SD} from two months of data of electrolytes and using the reference interval as the truncation limits. These truncation limits would exclude values above or below a defined threshold, which could unduly shift the mean value. The use of reference range for truncation limits was proposed by Hoffman et al. [4] If narrower truncation limits are used, it would hinder error detection and exclude all results affected by bias [5]. In addition to truncation limits, other criteria can also be used to exclude certain patient groups (e.g., based on specific department, age, patient on dialysis) which would shift the mean on one side [1, 15].

After calculating RP_M and RP_{SD}, we used PBRTQC operated in batch mode. The number of results included in one batch is

called block size. A smaller block size will detect large errors earlier [1, 10]. So, we divided the subsequent samples into blocks of 20 and block means ($\bar{x}$) were calculated and plotted on LJ and analyzed for any violation of the predefined control limits. Whenever a block mean crossed the control limits, an alarm was generated in real time to ensure proper notification of personnel.

The workup of our alarms was designed according to the recommendations given by many authors [7,16–18]. These guidelines were adapted and modified according to the practical applicability for our laboratory. Since most errors occur during pre-analytical phase, it was justified to initially review patient results and samples as recommended by Badrick et al. [7] This was followed by analysis of internal quality control and reviewing maintenance logs to evaluate the alarm [17] . As we had only one electrolyte analyzer, we could not perform repeat testing of the sample on another analyzer, which should have been an integral part of alarm workup.

In our study, total of eight alarms were raised for 100 blocks analyzed (2000 samples). The frequency of alarm occurrence was in agreement with the results of other authors [16–20]. The workup of these alarms was manageable by the existing laboratory staff and the frequency of alarms did not cause alarm fatigue [21]. Our laboratory runs IQC every twelve hours but we noted IQC violations in most cases when PBRTQC alarm was raised. This indicated that PBRTQC is helpful in detecting errors even between the IQC runs. We performed recalibration of equipment in most of these violations. We need to further examine if more frequent recalibration of the electrolyte analyzer could reduce the frequency of occurrence of such alarms [22].

Seven out of eight alarms raised in this study were due to analytical errors, which were corrected by instrument recalibration and retesting of patient samples. Hence, PBRTQC is useful for detecting analytical errors which could not be detected by traditional IQC. This is in agreement with studies done by various authors [14, 19, 23–25].

Further, in our study one preanalytical error was detected in the form of delayed transport of samples to the laboratory. This could have caused partial hemolysis and led to erroneous results. This error couldn't be detected by traditional IQC, thus signifying the utility of PBRTQC in detecting pre-analytical errors. This use of PBRTQC has been explored by some authors, like Westgard et al. [26] who implemented PBRTQC for blood gas analysis samples and reported that 1.91% of the total errors were due to preanalytical factors, mainly due to micro-clots caused by improper mixing or improper anticoagulant. Lorde et al. [27] implemented machine learning algorithms on PBRTQC procedures for detecting pre-analytical errors like contamination of samples with intravenous fluid, delayed sample analysis and incorrect vacutainer errors [27]. However, there is limited research on the use of PBRTQC for detecting pre-analytical errors, which may stem from the extensive workup needed, adding on to the already complex and burdensome process of implementing PBRTQC.

Overall, our results demonstrated that the introduction of PQRTQC in laboratory should be done in a phased manner and supported by LIS to ease the implementation and evaluation of alarms. Commencing PBRTQC in a laboratory could have its own hurdles, but in the long run, it will provide additional confidence in reporting laboratory results. We also agree that PBRTQC cannot replace the existing internal quality control programs, but it can supplement and strengthen it.

The major limitation of our study is the fact that we performed PBRTQC on only serum sodium and potassium, who have low biological variation and hence, the best bias detection capabilities. However, the Clinical and Laboratory Standards Institute guidelines suggest that selection of tests for implementation of PBRTQC should be based on a risk-based quality control assessment for all parameters [28]. In our study, most of the calculations were done from Microsoft Excel and LIS support. However, as software support continues to improve, complex PBRTQC procedures will become easier to implement and more accessible for use in clinical chemistry laboratories.

Conclusion

This paper demonstrates that it is possible to effectively implement a simple PBRTQC procedure in a laboratory, even with limited software support. Each laboratory must assess the available PBRTQC strategies and tailor the techniques to meet their specific needs. Future research should concentrate on integrating the PBRTQC procedure outlined here with existing traditional control tools to develop a laboratory quality control plan grounded on risk assessment.

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Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Research Article

The importance of capillary protein electrophoresis in the early diagnosis and follow-up of monoclonal gammopathies

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Abstract

Objectives: Serum protein electrophoresis is a low-cost and low-sensitivity technique used for screening monoclonal gammopathies with capillary protein electrophoresis. Immunofixation electrophoresis, on the other hand, is a highly sensitive technique used in the diagnosis, treatment, and follow-up of monoclonal gammopathies in patients with peak or abnormal patterns in serum protein electrophoresis scans; however, it is also more expensive. In monoclonal gammopathies, excessive production of immunoglobulins, known as monoclonal bands, is observed as sharp bands in immunofixation electrophoresis. In this study, we aimed to highlight the contribution of serum protein electrophoresis and immunofixation electrophoresis in detecting monoclonal bands in laboratory reports and their role in the early diagnosis and follow-up of patients.

Methods: In this study, 781 serum protein electrophoresis and 144 immunofixation electrophoresis analysis reports were retrospectively evaluated. Of the 52 patients with deterioration in their SPE, 10 patients with SUD detected in their IFE were included in the study. The serum samples of the patients were analyzed using the Minicap Flex Piercing and Hydrasys 2 Scan Focusing analysis devices (Sebia, France).

Results: Among serum protein electrophoresis patterns, pathology (peak or distortion) was detected in 196 cases. Of these, peak was detected in 144 cases in SPE, while distortion was observed in 52 cases. For the evaluation of the disturbed electrophoretic patterns, immunofixation electrophoresis was recommended in the reports. Following clinical evaluation by the treating physician, immunofixation electrophoresis was performed for 26 patients. Monoclonal band was observed in 10 (38.5%) of these patients who underwent IFE study.

Conclusion: In our retrospective study, it was found that the frequency of monoclonal bands was high in patients with abnormal serum protein electrophoresis patterns where immunofixation electrophoresis was recommended. This underscores the importance of serum protein electrophoresis as a screening tool in the early diagnosis and follow-up of monoclonal gammopathies.

Keywords: Electrophoresis, monoclonal gammopathy, serum protein electrophoresis

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Immunoglobulin (Ig) is a glycoprotein composed of two identical heavy chains (α, γ, μ, δ, or ε) and two identical light chains (κ or λ), with a molecular weight of 55 kDa for the heavy chains (composed of 440 amino acids) and 23 kDa for the light chains (composed of 220 amino acids). The polypeptide chains are held together by covalent and non-covalent bonds, stabilized by disulfide linkages. There are five different heavy chain isotypes (IgG, IgA, IgM, IgD, and IgE) and two different light chain isotypes (κ and λ) [1]. Approximately 60% of immunoglobulins are of the κ chain type, and 40% are of the λ chain type. There is no functional difference between the κ and λ chains. In the initial response to an antigen, B lymphocytes secrete IgM and IgD. These lymphocytes later differentiate into plasma cells, which secrete larger amounts of immunoglobulins, mainly IgG, and also IgA and IgE, in response to the second dose of the same antigen [2].

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Monoclonal gammopathy (MG) is associated with the clonal proliferation of plasma cells or B lymphocytes due to genetic or environmental factors. MGs are frequently observed in clinical conditions such as multiple myeloma (MM), plasmacytoma, plasma cell leukemia, Waldenström macroglobulinemia (WM), light chain amyloidosis, light chain deposition diseases, primary Amyloidosis (AL amyloidosis), POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes), and smoldering myeloma (asymptomatic multiple myeloma, SMM), as well as in MG of undetermined significance (MGUS) with low tumor burden, whose significance has not yet been established [3]. The measurement of monoclonal immunoglobulins is important for diagnosis, treatment, and monitoring. Laboratory techniques used in the screening and identification of MG have advantages and disadvantages in terms of specificity, sensitivity, and cost [2]. These laboratory techniques include serum and urine protein electrophoresis (SPE and UPE), serum immunofixation electrophoresis (IFE) and urine immunofixation electrophoresis (UIFE), nephelometric measurement of immunoglobulin heavy chains in serum, immunochemical measurement of free immunoglobulin light chain (κ and λ) components (nephelometric and turbidimetric), quantitative measurement of free κ and λ light chains, and the serum free κ/λ ratio. SPE is used as the initial screening test [4, 5].

Electrophoresis is a method in which proteins are separated based on their physical properties. For this, cellulose acetate, agarose, or fine capillaries are used as support media. The separation occurs according to the net charge, size, and shape of the protein when an electric current is applied. Cations from the buffer create an osmotic flow toward the cathode [6]. In non-gel techniques (such as capillary electrophoresis), there is no staining or washing process. Capillary electrophoresis is widely used because it provides rapid results and high-resolution separation. Other advantages of this technique are small sample volume and a wide range of detection methods. Other advantages are high selectivity, automation, linearity, reproducibility and use with mass spectrometry. The protein fraction is measured by optical densitometry, and an electrophoretic pattern is observed [7]. SPE consists of proteins from the albumin and globulin groups. Albumin is the largest peak and is located closest to the positive electrode. The proteins in the globulin group are divided into α1, α2, β1, β2, and γ globulins. The subgroups and relative amounts of these proteins are the primary focus in the interpretation of the electrophoretic pattern. In MG, the M-protein typically appears as a long, narrow, sharp peak in the α, β, or γ globulin regions of the electrophoretic pattern. If there is a polyclonal increase in immunoglobulins, a broad band is seen in the gamma region. In hypogammaglobulinemia, a reduction and flattening of the gamma globulin region is observed. Patients whose SPE shows no visible peaks but exhibit disturbances (such as decreases or flattening) in the bands should be monitored with follow-up SPEs to track their clinical course [8].

The evaluation of peaks in SPE, the detection of smaller M-proteins, and the identification of the heavy and light chain subtypes of M-proteins are more sensitive and costly methods, such as IFE. In IFE, the patient's serum is incubated with monospecific antibodies against the Ig heavy and light chains, and the resulting antigen-antibody complexes are assessed. Initially, antibodies against IgG, A, M, κ and λ are used. If no reaction occurs, the procedure continues with antibodies against IgD and IgE [5, 8].

Sharp MBs are observed in SPE, which is a screening test due to excessive production of immunoglobulins in MG. In our study, when IFE, which has high cost and sensitivity, is performed as a reflex test in patients with deterioration in beta and gamma regions in SPE, MB can be reported at a high rate. In the light of the data we obtained, we aimed to emphasize the importance of cost-effective use of laboratory tests in the early diagnosis and follow-up of MG in SPE deterioration.

Materials and Methods

The study included adult patients aged 18 and over who applied to the internal medicine and its subspecialties clinics of our hospital between 01.07.2022 and 31.10.2022, and for whom SPE and IFE tests were requested as part of their laboratory investigations. Of the 52 patients with deterioration in their SPE, 10 patients with SUD detected in their IFE were included in the study. Approval was received from the ethics committee of Basaksehir Cam and Sakura Hospital dated 25.01.2023, with protocol code 2023-36. Data were evaluated retrospectively in accordance with the Declaration of Helsinki. In our study, the data of the patients evaluated retrospectively were extracted from LIS (Laboratory Information System). During the study period, if multiple SPE and IFE tests were performed on the same patient, only the most recent sample was considered. Exclusion criteria was: Individuals aged 0–17 (not adults), patients who underwent SPE and IFE tests in units other than internal medicine and its subspecialties, and samples deemed unsuitable for pre-advantagetical evaluation (e.g., hemolyzed, lipemic, or icteric samples). In our study, an IFE test was not added as a reflex test. Among the patients with peak and/or deterioration in SPE, the doctor requested an IFE test for those with clinical indication.

SPE was performed using the Sebia Minicap (Sebia, Issy-les-Moulineaux, France) capillary zone electrophoresis kit. In the cases where pathology was not observed in the electrophoretic pattern of the six fractions constituting the total protein, peaks or peak(s) in the beta 1, beta 2, and gamma globulin fractions, where disturbances were observed, were marked manually, and explanations were added to the reports. For those patients whose SPE reports included additional explanations, IFE was performed using the Sebia Hydrasis 2 (Sebia, Issy-les-Moulineaux, France) agarose gel electrophoresis kit.

Statistical analysis

Data obtained from the study were classified based on parameters such as age, gender, date of application, referring unit, clinical diagnosis, and electrophoresis findings.

Table 1. Distribution of SPEs according to gender, age, clinical unit and diagnosis

	Those with distortions in the explanations of SPEs		IFEs requested from those with impaired SPE		Those with MCB detected IFE of those with in SPE disorder	
	n	%	n	%	n	%
Sex						
Female	34	65.3	14	53.8	4	40
Male	18	34.6	12	46.2	6	60
Age						
30–39 years	3	5.77	2	7.69	2	20
40–49 years	8	15.4	4	15.4	1	10
50–59 years	19	36.5	7	26.9	2	20
60–69 years	8	15.4	4	15.4	1	10
70–79 years	12	23.1	7	26.9	3	30
80 years old and above	2	3.85	2	7.69	1	10
Clinical unit						
Internal Medicine	9	17.3	3	11.5	2	20
Gastroenterology	1	1.92	–	–	–	–
Hematology	19	36.5	14	53.8	7	70
Nephrology	4	7.69	2	7.69	–	–
Rheumatology	19	36.5	7	26.9	1	10
Diagnosis						
Hematological disorders	6	11.5	4	15.4	2	20
Joint pain	18	34.6	8	30.8	2	20
Kidney diseases	6	11.5	3	11.5	–	–
General examination	12	23.1	3	11.5	–	–
Monoclonal gammopathy	3	5.77	2	7.69	2	20
Multiple Myeloma	7	13.4	6	23.1	4	40

SPE: Serum protein electrophoresis; IFE: Immunofixation electrophoresis; MCB: Monoclonalband.

Results

The distribution of the patients with disturbances in the explanations of the SPEs, the IFE tests performed on those with disturbances, and the IFE samples showing MCBs according to gender, age, clinical unit, and diagnosis are shown in Table 1.

The evaluation of the cases in our study is given in Figure 1.

A total of 781 SPE and 144 IFE tests were included in the study. Of the analyzed SPE tests, 74.90% (n=585) showed no pathology, while 25.10% (n=196) showed pathology. Among the SPE tests evaluated as pathological in the study, 18.43% (n=144) showed a manually marked peak in the electrophoresis pattern, and 6.55% (n=52) exhibited a disturbance in the six fractions of total protein.

In the study, 144 SPE tests with manually marked peaks were analyzed using IFE in 64.58% (n=93) of the cases. Monoclonal bands (MCB) were observed in 66.66% (n=52) of the IFE samples tested from the patients with marked peaks in the SPE report. 52 SPE tests showing disturbances in the six fractions of total protein were analyzed with IFE in 50% (n=26) of the cases. MCB were observed in 38.5% (n=10) of the IFE samples tested from the patients who showed disturbances in the six fractions of total protein. In our study, the electrophoretic pattern of the control sample in the SPE was evaluated as normal (Fig. 2). In Figure 2, a narrow and sharp peak is observed in the first albumin band. The alpha 2 (α2) globulin band is wider and higher than the alpha 1 (α1) globulin band; the beta 1 (ß1) globulin band is higher and sharper than the beta 2 (ß2) globulin band; and the gamma globulin band has an inverted "T" shape.

In the electrophoretic patterns of the patients in our study, disturbances were reported in the beta 2 and/or gamma globulin bands. In these disturbances, the beta 2 globulin band is higher and sharper than the beta 1 globulin band, with visual defects such as depressions and peaks on the surface of the beta 2 globulin band and gamma globulin band. To visually evaluate these disturbances, the relevant bands were zoomed in on using the device. If the disturbances become clearer upon zooming, it was recommended to perform IFE for the evaluation of the disturbances in the relevant bands and/or peaks. In our study, MCB were detected by IFE in patients with disturbances in the beta 2 and/or gamma globulin bands (Fig. 3). MCBs found in the IFE of cases with pathological disturbances in the electrophoretic pattern of the SPE are shown in Figure 4.

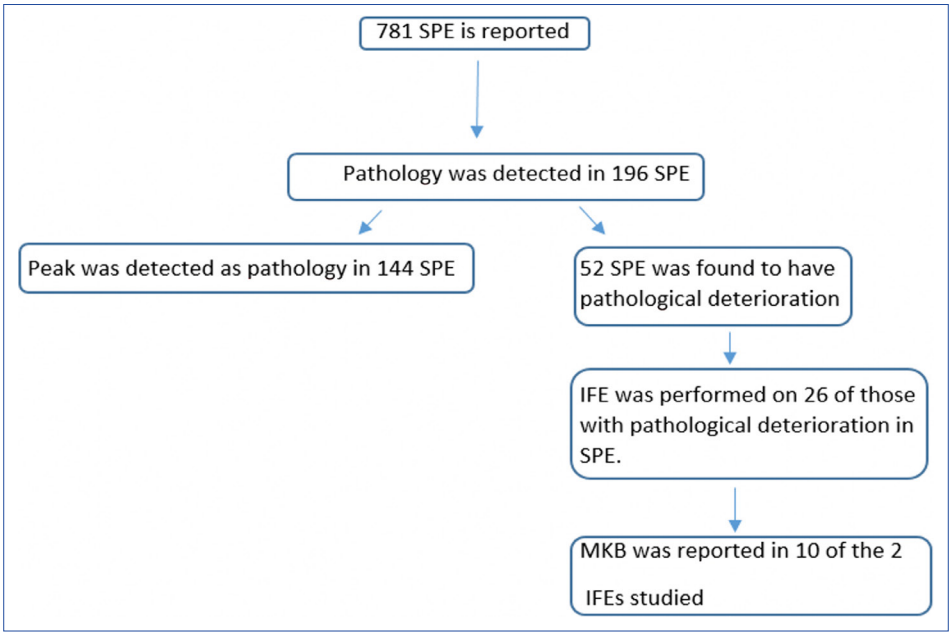

Figure 1. Evaluation scheme of the cases in our study.
SPE: Serum protein electrophoresis; IFE: Immunofixation electrophoresis; MCB: Monoclonalband.

Discussion

MG is a group of diseases characterized by the proliferation of one or more clones of differentiated plasma cells. It is characterized by the detection of MCB in the patient's serum and urine [8]. The frequency of MG in plasma cell dyscrasias is observed in 7% of all hematologic malignancies. MG observed in plasma cell dyscrasias is generally associated with MM, amyloidosis, Waldenström's macroglobulinemia (WM), or plasmacytoma. MG cases without such associations are termed as MG of undetermined significance (MGUS) [9, 10]. In our study, 38.5% (n=10) of the patients with pathological changes in the SPE had MCBs detected by IFE. In patients suspected of having MG, the lower-cost SPE was used as a screening test to evaluate any pathologies in the electrophoretic pattern. As a result of these evaluations, patients with abnormalities in the SPE were then tested with the more expensive IFE, which showed a higher frequency of MCB detection. Literature shows that the prevalence of MG is associated with increasing age and male gender. In some studies, MM is slightly more common in women than in men [11]. However, MGUS is observed at least 1.5 times more frequently in men than in women [12]. Since SPE is more commonly requested as a screening test in women, pathological findings (peaks and/or disturbances) are expected to be more frequently reported in women. In our study, disturbances in the SPE were more commonly observed in women, while MCBs detected by IFE were more frequent in men.

MG can develop at any age, but its frequency increases with age. The frequency of MCB occurrence is 1.7% in individuals over 50 years old and 3.6% in individuals over 70 years old [13]. When these findings were divided into 10-year age groups for those over 50, the frequency of MG was found to be 3.91%, 5.72%, 7.75%, 8.67%, and 12.75% for those over 90 years old [14]. MM

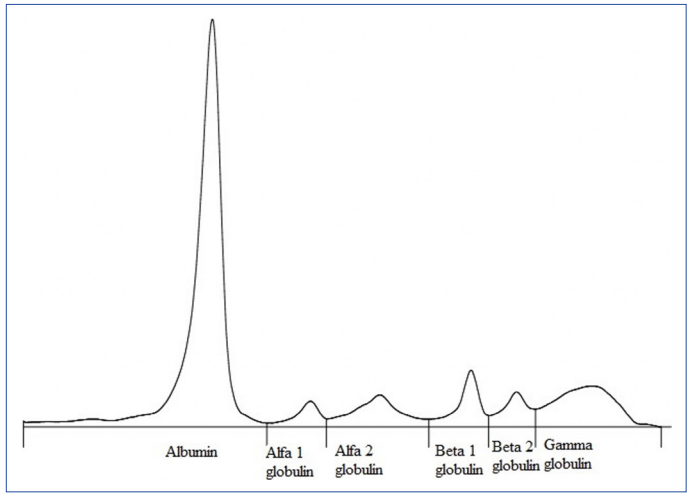

Figure 2. Visualization of normal electrophoretic pattern in control sample in SPE.

is the most common form of MG, and the age of diagnosis is typically between 66 and 70 years. MM has an asymptomatic premalignant stage referred to as MGUS, and it is observed that 1% of MGUS patients progress to MM each year. The frequency of MGUS is 1.7% in individuals over 25 years of age, 3% in those over 60, and 10% in individuals over 80 years old [15]. In our study, the age distribution of patients with disturbances in the SPE and the age distribution of patients with MCBs detected by IFE were found to be compatible. The frequency of MCB detection by IFE was higher in patients over 40 years old with disturbances in the SPE, indicating that early diagnosis and treatment of MG were effectively facilitated. Studies have shown that changes in the gamma band of the SPE pattern are observed in polyclonal gammopathy (PG), MG, and hypogam-

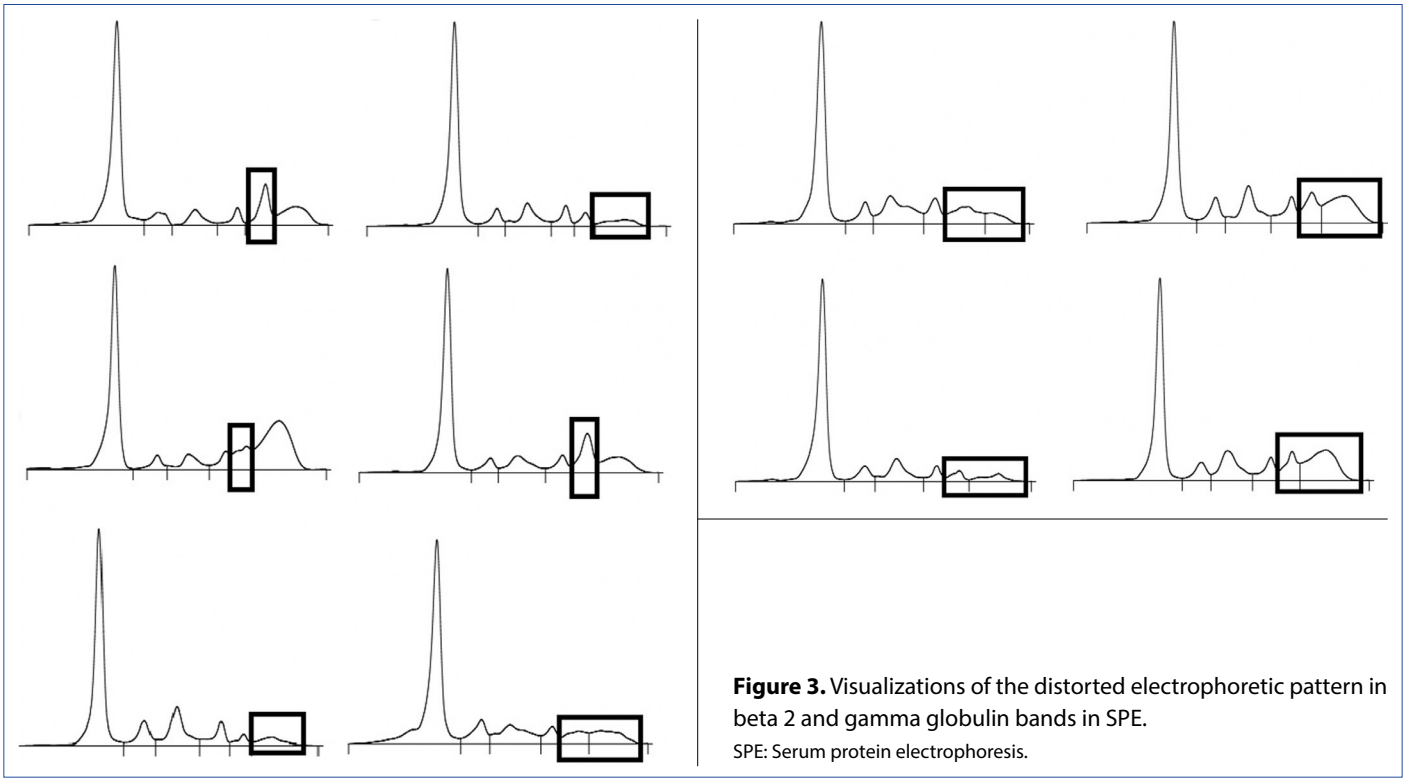

Figure 3. Visualizations of the distorted electrophoretic pattern in beta 2 and gamma globulin bands in SPE.
SPE: Serum protein electrophoresis.

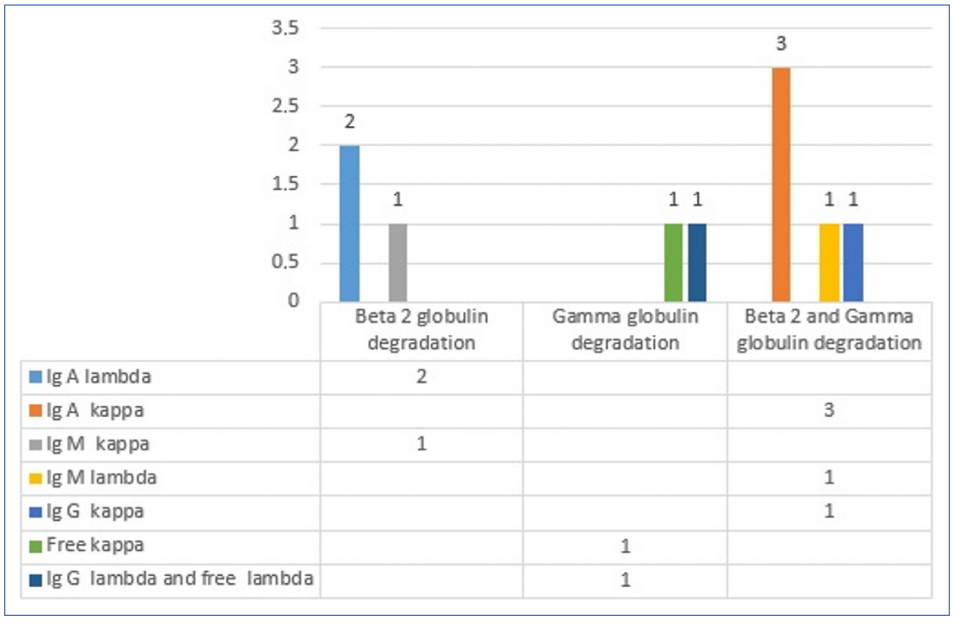

Figure 4. Distrubution of MCB's observed with IFE in patients with deterioration in SPE.
MCB: Monoclonalband; SPE: Serum protein electrophoresis.

maglobulinemia. Of PG patients, 61% have liver disease, 28% have connective tissue diseases, and 8% have chronic infections. Hypogammaglobulinemia has also been observed due to hereditary reasons or during the course of malignant or benign diseases. In the literature, MGUS was observed in 51% of MG patients, MM in 18%, AL amyloidosis in 11%, lymphoproliferative diseases in 4%, smoldering multiple myeloma (SMM) in 6%, WM in 3%, and solitary or extramedullary plasmacytomas in 1% of cases [16, 17]. In our study, among the patients with disturbances in the SPE (n=52), 36.5 % were from hematology, 36.5 % from rheumatology, 17.3% from internal medicine, and 7.69% from nephrology. Among those with disturbances in the SPE, 70% of the IFE tests that detected MCBs were from hematology, 20% were from internal medicine, and 10% were from rheumatology. The higher frequency of MCB detection in hematology was due to the greater number of patients diagnosed and fol-

lowed for MM, MG, and other paraproteinemias. In the internal medicine department, IFE was requested for patients suspected of MG, and the frequency of MCB detection was higher. In rheumatology, although SPE was frequently requested as part of the screening process, MCB detection was lower due to the lower frequency of MG and other paraproteinemias.

Diseases with paraprotein-associated kidney involvement require a collaborative approach between hematology and nephrology. Cast nephropathy is the most common renal lesion seen in MM and is one of the most important causes of kidney failure. MG of renal significance (MGRS) is used in nephrology practice to describe cases where monoclonal paraprotein causes renal damage without meeting the criteria for hematologic malignancy. MGRS is defined as a clonal proliferative disorder that does not meet the criteria for hematologic malignancy but produces nephrotoxic monoclonal immunoglobulins. MGRS encompasses all B-cell lymphoproliferative and plasma cell proliferative disorders that do not meet the criteria for MM, WM, chronic lymphocytic leukemia (CLL), or Malignant Lymphoma. Kidney lesions associated with monoclonal Ig in low-grade CLL and low-grade B-cell non-Hodgkin lymphoma are also included in MGRS. Additionally, if kidney involvement is present in MM, primary AL amyloidosis, or MGUS, it is also considered MGRS [18]. In MGRS, kidney damage can be glomerular, interstitial, mesangial, or tubular. Without treatment to suppress the clonal production, MGRS can progress to end-stage kidney failure. In our study, among the two patients with disturbances in the SPE from nephrology, no MCBs were detected in their IFE results. For patients suspected of MG and/or MGRS, kidney biopsies should include immunofluorescent and immunohistochemical evaluations alongside light microscopy, and electron microscopy should also be considered for a more comprehensive assessment. In our study, of the patients with disturbances in the SPE (n=52), 34.62% had joint pain, 23.08% had general symptoms such as fatigue and vitamin deficiencies, 19.22% had MG and/or MM, 11.54% had hematologic symptoms, and 11.54% had kidney diseases. Among the patients with disturbances in the SPE who had MCBs detected by IFE (n=10), 60% were diagnosed with MG and/or MM, 20% had joint pain, and 20% had hematologic symptoms. Joint pain, fatigue, and vitamin deficiencies are common clinical symptoms observed in many hematologic malignancies, including MG and MM [3, 8]. These clinical symptoms are frequently seen in nonspecific clinical departments such as rheumatology, nephrology, neurology, and orthopedics. Since SPE is requested as part of the screening process in these settings, the detection rate of MCBs by IFE is lower. Since the diagnostic and monitoring criterion for MG and/or MM is the detection of MCBs in serum, IFE is more frequently used to detect MCBs in patients with disturbances in their SPE. In the literature, it has been shown that even when serum total protein, beta, or gamma globulin levels are within normal limits, a small MCB may be hidden in the beta or gamma regions [19, 20]. In MG screenings, when MCBs are hidden, measurements of immunoglobulins (IgG, IgA, IgM) and total light chains (kappa and lambda) by SPE are often insufficient. Therefore, IFE, which has higher sensitivity than SPE, and quantitative measurements of free light chains in serum are recommended for diagnosing MG [20]. In patients with MCBs detected by IFE, IgG kappa was found in 35.3%, IgG lambda in 10.8%, and IgA kappa in 6.1% of cases [20–22]. In our study, among the patients with disturbances in the SPE, MCBs detected by IFE were found in 30% of cases as IgA kappa, 20% as IgA lambda, 10% as IgM kappa, 10% as IgM lambda, 10% as IgG kappa, 10% as free kappa, and 10% as two or more immunoglobulins with light chains (IgG lambda and free lambda). Capillary electrophoresis (CE) plays a critical role in the early detection and monitoring of monoclonal gammopathies due to its high resolution and automation capabilities. Its ability to efficiently separate protein fractions allows for the identification of subtle abnormalities that may indicate the presence of monoclonal proteins. Compared to traditional gel-based methods, CE offers faster turnaround times, reduced sample volume requirements, and improved reproducibility, making it highly valuable in routine clinical laboratories. The detection of electrophoretic disturbances, particularly in the beta and gamma regions, by CE enables the timely recommendation of confirmatory tests such as immunofixation electrophoresis. Therefore, incorporating capillary electrophoresis into diagnostic workflows significantly enhances the sensitivity of screening for monoclonal gammopathies and supports early clinical decision-making. The most important limitation of our study is that IFE, which is a costly test, cannot be performed on every patient for whom SPE is requested. In addition, since ethics committee approval was obtained for adult patients from internal medicine and its subspecialties, data from adult patients from other branches could not be included in the study. Samples that are preanalytically inappropriate in the laboratory (hemolysis, lipemia) are also not included in the study.

Conclusion

In our retrospective study, the frequency of MCBs was found to be high in patients with disturbances in their SPE who were subsequently tested with IFE. This highlights the importance of SPE in the early diagnosis and follow-up of MG, which can present in various clinical scenarios. The detection of MCBs in MG can be effectively screened with SPE, which is faster and more cost-effective compared to IFE. We recommend performing IFE as a reflex test in patients with abnormalities detected in their SPE electrophoretic pattern, as it significantly aids clinicians in making diagnostic and treatment decisions.

One of the main limitations of this study is the study population was restricted to adult patients from internal medicine and its subspecialties, limiting the generalizability of the findings to other clinical departments. Additionally, pediatric patients and samples with pre-analytical issues such as hemolysis, lipemia, or icterus were excluded, potentially reducing the sample diversity. The retrospective design of the study may also introduce selection bias, as only cases with available laboratory records were included. Finally, the lack of long-term clinical follow-up data prevents evaluating the progression of detected monoclonal bands to clinically significant disease.

Ethics Committee Approval: The study was approved by the Basaksehir Cam and Sakura Hospital Clinical Reseach Ethics Committee (no: 2023-36, date: 25/01/2023).

Informed Consent: Informed consent was obtained from all participants.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Research Article

Immature granulocyte, immature granulocyte-lymphocyte ratio, and other hematological inflammatory parameters in Alzheimer disease

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Abstract

Objectives: Persistent overactivation of inflammatory responses has been associated with various neurodegenerative disorders, including Alzheimer disease. This study aimed to investigate whether parameters derived from complete blood count, such as white blood cell populations, platelet counts, and hemogram-derived parameters like platelet-lymphocyte ratio, immature granulocyte-lymphocyte ratio, systemic immune-inflammation index, and platelet-neutrophil ratio, which can be easily detected without additional cost, could have diagnostic value in the pathogenesis of Alzheimer disease. Additionally, the co-occurrence rates of Alzheimer disease with other diseases (such as Parkinson disease, anxiety, diabetes mellitus, cancer, osteoporosis and kidney disease) were analyzed.

Methods: Complete blood count data of 231 patients diagnosed with Alzheimer disease after pre-screening were retrospectively reviewed. Complete blood count parameters were generated using hemogram device data. Platelet-lymphocyte ratio, immature granulocyte-lymphocyte ratio, systemic immune-inflammation index, and platelet-neutrophil ratio were calculated using neutrophil, lymphocyte, and platelet counts. 593 patients diagnosed with Alzheimer disease during pre-screening were retrospectively screened again and Alzheimer disease comorbidities were analyzed.

Results: The immature granulocyte, immature granulocyte %, immature granulocyte-lymphocyte ratio values were found to be statistically significantly higher in Alzheimer's patients compared to the control group. However, the receiver operating characteristic analysis did not provide sufficient discrimination. The diseases accompanying Alzheimer disease were determined and the numbers found were expressed as percentages. The most common diseases were anxiety disorder, vitamin D deficiency and hypertension, respectively. The comorbidity with Parkinson disease was found to be 13.8%.

Conclusion: Since immature granulocyte, immature granulocyte %, immature granulocyte-lymphocyte ratio values, which do not require extra cost and can be easily detected with complete blood count, were determined to have discriminatory value in some diseases, we hope that our study will guide future research.

Keywords: Alzheimer disease, anxiety, immature granulocyte, immature granulocyte-lymphocyte ratio, parkinson disease

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Alzheimer disease (AD) is the most common form of dementia, accounting for 60–70% of dementia patients [1]. AD is defined by the buildup of abnormal protein aggregates, such as amyloid plaques made of beta-amyloid peptides and neurofibrillary tangles resulting from hyperphosphorylated tau protein [2]. The prolonged overactivation of pro-inflammatory responses has been linked to various neurodegenerative disorders, including AD [3]. Neuroinflammation plays a role in various neurological and behavioral disorders, including AD, Parkinson disease, and depression. The previously held view that the brain is an "immune-privileged" organ has shifted, based on more recent findings, towards an understanding that recognizes the significant connection between the immune response in the central nervous system and the rest of the body [4]. It is

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now widely recognized that systemic inflammation can initiate or worsen the brain's inflammatory state, thereby contributing to chronic neuroinflammation and neurodegeneration [5]. Increased immature granulocyte (IG) counts in peripheral blood are a sign of improved bone marrow activity [6] Although IGs are typically absent in peripheral blood, their levels rise in conditions like bacterial sepsis, inflammation, trauma, cancer, steroid therapy, and myeloproliferative disorders [7]. Some studies have shown that IGs are more effective than markers like C-reactive protein and neutrophil-to-lymphocyte ratio (NLR) in determining the severity of infection [8]. Intensive research is being conducted to diagnose and prevent the disease before symptomatic period [9]. Early detection for the prevention and treatment of AD is highly clinically significant. Blood samples, in contrast to cerebrospinal fluid, are easier to collect and can be taken more frequently. As a result, identifying potential early diagnostic markers in blood is particularly crucial [10]. The platelet-lymphocyte ratio (PLR), platelet-neutrophil ratio (PNR), systemic immune-inflammation index (SII), and Immature granulocyte-lymphocyte ratio (IGLR) are values commonly used in clinical practice based on blood cell counts and are also recognized as novel inflammatory markers [11, 12]. The platelet-lymphocyte ratio (PLR) can be used to assess platelet activation induced by inflammatory-coagulation reactions, severe coagulation disorders, and systemic inflammatory responses. The systemic immune-inflammation index (SII) is a novel biomarker for malignancy and inflammatory diseases [13].

This study aimed to investigate the role of white blood cell (WBC) populations (IG%, IG count), lymphocyte percentage and count, platelet parameters (platelet count, mean platelet volume: MPV, platelet large ratio: P-LCR, platecrit: PCT) and derived hematological parameters such as PLR, PNR, IGLR and SII as potential biomarkers in the differential diagnosis of AD. Additionally, the co-occurrence rates of AD with other diseases (such as Parkinson disease, anxiety, diabetes mellitus, cancer, osteoporosis and kidney disease) were analyzed.

Materials and Methods

The study was approved by the Bursa City Hospital Clinical Research Ethics Committee (Date 01/02/2023, No: 2023-3/4) The study was designed in accordance with the Helsinki Declaration.

This current study was designed as a retrospective study. During the preliminary screening, patient data records of 593 patients diagnosed with AD between 01.01.2023 and 28.02.2022 were retrospectively reviewed. Patients diagnosed with "early-onset Alzheimer disease" were not included in the study. In addition, subjects with acute/chronic infectious diseases, systemic inflammatory disorders, diseases affecting hematological parameters, thyroid dysfunctions, cerebrovascular diseases, hematological malignancies, kidney failure, malignancies, and those with positive C-reactive protein were excluded. Complete blood count (CBC) data of 231 patients obtained after screening were examined. A total of 51 (45.1%) men and 62 (54.9%) women were included in the study. Characteristics such as age, sex, and disease diagnosis codes (ICD-10: International Statistical Classification of Diseases and Related Health Problems) were obtained from patient records. The number of healthy controls was determined as 113 using the G*Power statistical program version 3.1.9.4 (University of Düsseldorf, Germany). CBC parameters were measured using the Sysmex XN-10 Automated Hematology Analyzer (Kobe 651-0073 Japan) in the laboratory with EDTA-containing hemogram tubes. Parameters measured included platelet (PLT) count, platelet distribution width (PDW), mean platelet volume (MPV), plateletcrit (PCT) value, and white blood cell (WBC), neutrophil (NEUT), lymphocyte (LYMPH), monocyte (MONO), basophil (BASO), eosinophil (EO), and immature granulocyte (IG) counts from the CBC analysis. PLR (platelet-lymphocyte ratio), IGLR (immature granulocyte-lymphocyte ratio), SII (systemic immune-inflammation index), and PNR (platelet-neutrophil ratio) were calculated using neutrophil, lymphocyte, and platelet counts. The calculations were made as follows: PLR=Platelet count / Lymphocyte count, PNR=Platelet count / Neutrophil count, IGLR=Immature Granulocyte count/ Lymphocyte count, SII=Systemic Immune-Inflammation Index=(Platelet×Neutrophil) / Lymphocyte. The results were compared with those of the healthy control group.

During the pre-screening, 593 patients diagnosed with AD were retrospectively scanned again, and this time, the diseases accompanying AD were analyzed. Disease diagnosis codes (ICD-10: International Statistical Classification of Diseases and Related Health Problems) were obtained from the patient file data records. Thus, the diseases accompanying AD and their numbers were determined. In addition, this number was expressed as a percentage by comparing it to the total number of patients (total 593).

Statistical analysis

Continuous variables were expressed as mean±standard deviation, and categorical data as numbers and percentages. Normality analyses of continuous variables were performed using the Kolmogorov-Smirnov Goodness-of-Fit Test. For intergroup analyses of normally distributed data, the Student's T-test was used, and for non-normally distributed data, the Mann-Whitney U Test was applied. Categorical data comparisons were made using the chi-square test. Cutoff values for IG, IG%, and IGLR in Alzheimer patients were examined using Receiver Operating Characteristics (ROC) curve analysis. If significant threshold values were present, the sensitivity, specificity, positive, and negative predictive values of these thresholds were calculated. A Type 1 error level below 5% was considered to indicate that the test's diagnostic value was statistically significant. Analyses were conducted using IBM SPSS version 27.0 (IBM Corporation, Armonk, NY, USA), and a p-value of <0.05 was accepted as the threshold for statistical significance.

Results

There was no statistically significant difference in terms of age and gender between the patient and control groups (p=0.131 and p=0.118, respectively) (Table 1).

Table 1. Comparison of AD and control groups in terms of age and gender

	Control group (n=113)		Alzheimer group (n=231)		p
	n	%	n	%	
Age (years) (mean±SD)	70.33±8.71		71.39±4.31		0.131*
Gender					
Female	62	54.9	147	63.6	0.118**
Male	51	45.1	84	36.4	

*: Student's t test, **: Chi-square test

In AD, IG, IG%, and IGLR values were found to be statistically significantly higher compared to the control group (p=0.002, p=0.001, and p=0.016, respectively). No statistically significant differences were found between the groups in terms of WBC, NEUT, LYMPH, MONO, EO, BASO, MONO%, EO%, PLT, PDW, MPV, P-LCR, PCT, NEUT%, LYMPH%, BASO%, PLR, PNR, and SII values (p>0.05) (Table 2). IG, IG%, and IGLR values were found to be statistically significantly higher compared to the control group (Table 2).

However, the ROC analysis did not provide sufficient discrimination (Table 3) (Fig. 1).

The number of comorbidities of AD with other diseases (such as Parkinson disease, anxiety, diabetes mellitus, cancer,

Table 2. Comparison of some blood parameters between the groups

Parameters	Control group (n=113)	Alzheimer group (n=231)	p
WBC [median (min-max)]	6.5 (4.6–10.7)	6.9 (4.1–12.4)	0.054*
NEUT [median (min-max)]	3.8 (2.4–6.4)	4.1 (1.8–8.3)	0.112*
LYMPH (Mean±SD)	2.04±0.47	2.09±0.46	0.370**
MONO [median (min-max)]	0.53 (0.26–0.81)	0.54 (0.28–1.03)	0.376*
EO [median (min-max)]	0.14 (0.02–0.55)	0.14 (0.0–0.81)	0.816*
BASO [median (min-max)]	0.04 (0.01–0.14)	0.04 (0.0–0.11)	0.745*
IG (Mean±SD)	0.018±0.008	0.025±0.029	0.002**
MONO% [median (min-max)]	7.9 (4.7–12.8)	8.0 (4.5–13.9)	0.814*
EO% [median (min-max)]	2 (0.3–8.0)	2.1 (0.0–10.1)	0.989*
PLT [median (min-max)]	244 (152 - 381)	237 (127 - 474)	0.983*
PDW [median (min-max)]	12.1 (8.4–22.9)	11.9 (8.3–21.5)	0.736*
MPV [median (min-max)]	10.4 (8.7–13.9)	10.4 (8.4–13.8)	0.742*
P-LCR [median (min-max)]	28.4 (14.9–55.6)	29 (12.1–54.4)	0.607*
PCT [median (min-max)]	0.25 (0.16–0.39)	0.25 (0.15–0.47)	0.941*
NEUT% [median (min-max)]	57.7 (42.3–71.6)	59.9 (43.2–73.8)	0.501*
LYMPH% [median (min-max)]	30.9 (19.5–44.7)	29.1 (14.1–44.5)	0.449*
BASO% [median (min-max)]	0.6 (0.1–2)	0.6 (0.0–1.4)	0.202*
IG% (Mean±SD)	0.26±0.11	0.33±0.31	0.001**
PLR [median (min-max)]	122 (64.0–218.2)	113.1 (47.6–305.6)	0.458*
PNR [median (min-max)]	62.4 (30.2–106.1)	59.7 (26.1–136.3)	0.278*
IGLR [median (min-max)]	0.0089 (0.00–0.023)	0.0099 (0.00–0.23)	0.016**
SII [median (min-max)]	455.6 (168.7–1369.4)	493.2 (137.3–1511.8)	0.470*

*: Mann Whitney U test, **: Student's t test. WBC: White blood cell, NEUT: Neutrophil, LYMPH: Lymphocyte, MONO: Monocyte, EO: Eosinophil, BASO: Basophil, IG: Immature granulocyte, PLT: Platelet, PDW: Platelet distribution width, MPV: Mean platelet volume, P-LCR: Platelet large cell ratio, PCT: Platecrit, PLR: Platelet-lymphocyte ratio, PNR: Platelet-neutrophil ratio, IGLR: Immature granulocyte-lymphocyte ratio, SII: Systemic immune-inflammation index, SD: Standard deviation.

Table 3. ROC analysis results and some cut-off values for IG, IG%, and IGLR in AD

	Diagnostic test					ROC curve		
	Cut-off	Sensitivity	Specificity	PPV	NPV	AUC	CI 95%	p
IG	≥0.020	70.4	36.3	69.2	37.6	**0.591**	0.530–0.652	**0.006***
IG %	≥0.25	64.3	46.0	70.8	38.8	**0.598**	0.537–0.659	**0.003***
IGLR	≥0.009	59.6	49.1	70.6	37.2	**0.580**	0.518–0.641	**0.016***

*: ROC analysis. ROC: Receiver operating characteristics; IG: Immature granulocyte; IGLR: Immature granulocyte/lymphocyte ratio; AD: Alzheimer disease; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the curve, CI: Confidence interval.

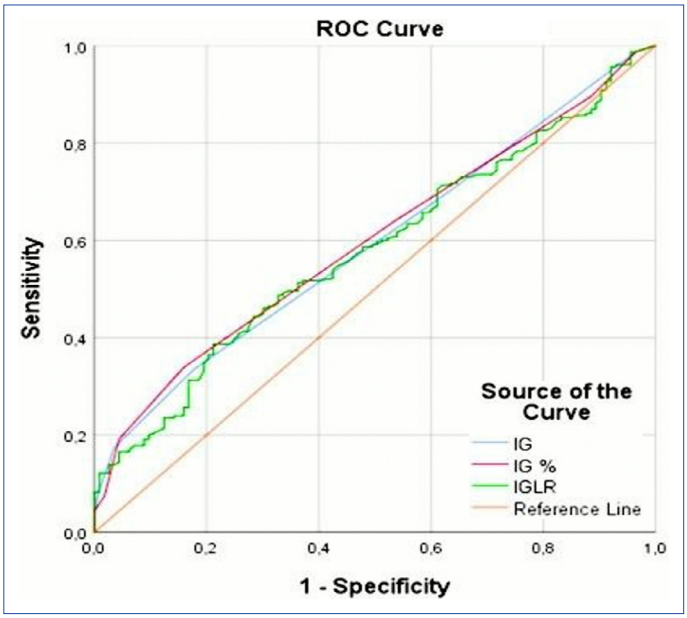

Figure 1. ROC Curve Graph For IG, IG%, and IGLR Values in AD.

ROC: Receiver operating characteristics; IG: Immature granulocyte; IGLR: Immature granulocyte/lymphocyte ratio; AD: Alzheimer disease.

osteoporosis and kidney disease) was determined. The numbers found were presented as a percentage by comparing to a total of 593 patients (Table 4).

Table 4. Numbers and rates of comorbidities in 593 patients diagnosed with AD

Diseases accompanying AD	Number of patients	% of the number of patients
Anxiety disorder	348	58.7
Vitamin D deficiency	321	54.1
Hypertension	299	50.4
Diabetes mellitus	180	30.4
Vitamin B-12 deficiency	161	27.2
Iron deficiency	86	14.5
Parkinson disease	82	13.8
Hyperlipidemia	72	12.1
Depressive episodes	70	11.8
Kidney failure	61	10.3
Thyroid disorders	55	9.3
Epilepsy	38	6.4
Delirium	35	5.9
Folic acid deficiency	33	5.6
Psychosis	30	5.1
Osteoporosis	28	4.7
Malignancy	24	4.0
Bipolar disorder	7	1.2
Migraines	5	0.8
Multiple sclerosis	2	0.3

AD: Alzheimer disease.

Discussion

AD, characterized by a progressive decline in memory and cognitive abilities, is the most common neurodegenerative disorder and significantly impairs the daily activities of elderly individuals [14]. Inflammation is believed to play a key role in the development of AD [15]. Given the role of inflammation in AD pathology, we proposed that routine blood parameters could hold diagnostic significance in AD. This study aimed to investigate the levels of total white blood cell (WBC) populations (such as IG, lymphocytes, etc.), platelet counts, and hemogram-derived parameters such as PLR, PNR, IGLR, and SII in the differential diagnosis of dementia. Human inflammatory markers (PLR, SII etc.) have been noted to potentially be associated with various health risks, including cardiovascular and cerebrovascular diseases in the elderly [16]. Many studies have reported a decrease in lymphocyte count in AD [16, 17]. However, when we compared the lymphocyte count and percentage values of the group with AD in this study to the control group, no significant difference was found between them (p=0.370, p=0.449, respectively) (Table 2). Fu et al. [18], demonstrated that P-LCR was significantly elevated in AD patients compared with controls. They found an increasing trend in the rate of change in P-LCR with disease progression. They showed that P-LCR may be a risk factor for AD after adjusting for age, sex, APOE4, and body mass index. In this study, no statistically significant difference was found between the groups in terms of P-LCR value (p>0.05) (Table 2). Systemic inflammation can be evaluated using various biochemical or hematological indicators, which are typically measured in routine blood tests or calculated from those results [19]. The systemic immune-inflammation index (SII) is new inflammatory biomarker calculated by (platelet count×neutrophil count) / lymphocyte count [20, 21]. In a study by Van der Willik et al. [22], higher levels of GLR, PLR, and SII ratios in the general population were found to be associated with an increased risk of dementia. In this study, when we compared the PLR and SII values of patients with AD to the control group, no statistically significant difference was found between them (p=0.458 and p=0.470, respectively) (Table 2). In a study by Wang et al. [23], MPV and PDW were found to be significantly lower in patients with AD compared to controls. In this study, no statistically significant difference was found between AD and the control group in terms of DW, MPV, and PNR values (p>0.05) (Table 2). Previous studies have used immature granulocyte (IG) count, a marker of heightened bone marrow activity, and IG percentage (IG%), the ratio of IGs to total WBC, in conditions such as acute necrotizing pancreatitis, pyelonephritis, sepsis, thyroid malignancies, and renal cell carcinoma [24]. However, there appears to be limited research in the literature on the relationship between IG, which stands out as an early marker of inflammation, and AD. Some other studies related to immature granulocytes are as follows: Gülten et al. [25], found immature granulocytes valuable in predicting short-term mortality in acute myocardial infarction. Another study [26], conducted on migraine patients found that IG count and NLR levels were significantly higher compared to the healthy

control group. In a different study [27], IG was found effective in predicting severe bacterial infection in the pediatric population. In a study by Sengul et al. [28], IG count and IG% were found not to be useful in determining the severity of H. pylori and inflammation. Another study [29], on subacute thyroiditis showed that IG significantly decreased after treatment, and IG and NLR could be used to evaluate treatment response. In another study by Ünal Y. [30], IG count has been shown to be important in the diagnosis of AA and the differential diagnosis of CAA with a cutoff value of >0.104 (10^3/uL), with 93% sensitivity and 93.8% specificity. When evaluating this study group, at the cut-off point of IG≥0.020 (10^3/uL), sensitivity was found to be 70.4% and specificity was 36.3% (AUC: 0.591, p=0.006) (Table 3). In the research conducted by Ünal et al. [31], an increased IG% was identified as an effective and reliable marker for the early detection of acute cholecystitis severity. They demonstrated that IG% had significantly greater predictive accuracy for stage 3 acute cholecystitis compared to other stages, with an AUC of 0.95, sensitivity of 92.5%, specificity of 84.2%, PPV of 32.5%, and NPV of 99.3%. In this study, when comparing AD with the control group, the difference in IG% was significant (p=0.012) (Table 2). However, despite this observed significance, when evaluated by ROC analysis, it was found that the ROC analysis, as shown in Table 3 and Figure 1, did not have sufficient effectiveness. (Area under the curve (AUC): 0.598, sensitivity: 64.3%, specificity: 46.0%, positive predictive value (PPV): 70.8%, and negative predictive value (NPV):38,8%). Kubat et al. [32], investigated the immature granulocyte-lymphocyte ratio (IGLR) in patients with acute appendicitis and concluded that it was useful to distinguish between complicated and uncomplicated acute appendicitis groups. For the immature granulocyte-lymphocyte ratio: AUC: 0.782, sensitivity: 71.8%, specificity: 74.3%, and NPV:95%. When we look at this study group, the difference between AD and the control group was significant (p=0.015) (Table 2). However, as shown in Table 3, for IGLR, the values were determined as (AUC: 0.580, sensitivity: 60.0%, specificity: 53.6%, PPV: 89.6%, NPV: 16.8). Although a significant difference was found between the groups, when evaluated by ROC analysis, it was determined that this distinction did not have sufficient effectiveness.

Recent research has uncovered the relationships between neurodegenerative disorders such as AD and Parkinson disease with other chronic diseases. Many common comorbid medical conditions, such as cancer, diabetes, cardiovascular disease, stroke, stress, seizures, osteoporosis, and kidney disease, have been associated with AD and Parkinson disease [33]. Although there are phenotypic and neuropathological differences among dementia, Parkinson disease, multiple sclerosis, and motor neuron diseases, research thus far has indicated that these four conditions are influenced by the interplay of genetic and environmental factors, which similarly result in central nervous system and immune dysfunctions [34]. The highly correlated prevalences of these diseases suggest that they share common etiological factors [35]. Considering that many common comorbid medical conditions have been associated with AD, the rates of co-occurrence of other diseases in AD patients were also determined in this study. Among the 593 patients diagnosed with AD, we found that 58.7% (348 patients) had anxiety disorder, 54.1% had vitamin D deficiency (321 patients), 50.4% had hypertension (299 patients), 30.4% had diabetes mellitus (180 patients), 27.2% had vitamin B-12 deficiency (161 patients), 14.5% had iron deficiency (86 patients), 13.8% had Parkinson disease (82 patients), 12.1% had hyperlipidemia (72 patients), 11.8% had depressive episodes (70 patients), 10.3% had kidney failure (61 patients), 9.3% had thyroid disorders (55 patients), 6.4% had epilepsy (38 patients), 5.9% had delirium (35 patients), 5.6% had folic acid deficiency (33 patients), 5.1% had psychosis (30 patients), 4.7% had osteoporosis (28 patients), 4% had malignancy (24 patients), 1.2% had bipolar disorder (7 patients), 0.8% had migraine (5 patients), and 0.3% had multiple sclerosis (2 patients) (Tablo 4). The most common diseases were anxiety disorder, vitamin D deficiency and hypertension, respectively. The comorbidity with Parkinson's disease was found to be 13.8%. It was thought that determining the accompanying diseases would be useful in terms of providing a horizon for new research.

In this study, IG, IG%, and IGLR values were found to be significantly higher in the AD group compared to the control group. However, ROC analysis did not provide sufficient discrimination. Since IG, IG%, and IGLR values are easily detected by complete blood count, do not require additional costs, and provide rapid results, their significant elevation in AD, along with their diagnostic differentiation in other diseases, highlights the importance of further studies investigating the usability of these parameters in AD. We believe this study is a pioneering one and hope it serves as a guide for future research into the usability of these parameters.

Conclusion

Since IG, IG%, and IGLR values, which do not require extra cost and can be easily detected with complete blood count, were determined to have discriminatory value in some diseases, we hope that this study will guide future research.

Ethics Committee Approval: The study was approved by the Bursa City Hospital Clinical Research Ethics Committee (no: 2023-3/4, date: 01/02/2023).

Informed Consent: Informed consent was obtained from all participants.

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Research Article

Assessment of relationship between triglyceride/HDL-C ratio and incident type 2 diabetes mellitus risk

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Abstract

Objectives: The most prevalent endocrine condition in the world today is diabetes mellitus (DM). In addition to the recognized markers for assessing glycemic control and insulin resistance (IR), easily available, accurate, and repeatable markers are required. In order to assess the use of the triglyceride (TG), HDL cholesterol ratio (THR) as a marker for insulin resistance and glycemic management, our study was conducted.

Methods: We looked back at the TG, fasting serum glucose (FSG), and Fasting Insulin levels of 953 samples that were concurrently evaluated in our Faculty of Medicine Hospital Laboratory from March 2023 to August 2023. In terms of their homeostasis model assessment-estimated insulin resistance (HOMA-IR) values, the patients were split into two groups: those with good glycemic control and those with poor glycemic control. The THR's capacity to distinguish between good and poor glycemic control was assessed using ROC analysis. The accepted level of statistical significance was $p<0.05$. Additionally, a multivariate logistic regression analysis was conducted.

Results: The mean age was 40.83±16.78 years. All the patients had significant differences ($p<0.001$) in gender, FSG, HOMA-IR, FI, TG, and THR based on glycemic control, except age ($p=0.613$). In pairwise correlation, THR had moderate negative correlation ($r=-0.555$, $p<0.001$) with HDL, while strong positive correlation with TG ($r=0.959$, $p<0.001$). THR had the high selectivity and positive predictive value (PPV) with a cutoff value of ≥2.64 (AUC:0,72, Se:65%, Sp:70% ($p<0.001$:95% CI:0,66-0,78)). Men are 2.247 times more likely than women to have poor glycemic control ($p=0.022$). Poor glycemic control risk rised by 1.045 times with age, and by 1.056 times with glucose ($p=0.007$).

Conclusion: Based on the current results, we think that the THR may be a useful marker of glycemic control and IR.

Keywords: Diabetes mellitus, glycemic control, HDL-C, insulin resistance, triglyceride

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One of the biggest socioeconomic and health issues is diabetes mellitus. Diabetes mellitus type 2 (T2DM) is becoming more prevalent globally. According to numerous regional and national studies, the overall prevalence of T2DM in Türkiye ranges from 12.7% to 14.7% [1–4]. Additionally, the frequency of early fatalities brought on by macro- and microvascular complications in diabetes is increasing [5]. The pathophysiology of diabetes and metabolic syndrome is significantly influenced by insulin resistance (IR), which is the reduced insulin sensitivity of peripheral tissues. It may manifest one to two decades prior to the official diagnosis of T2DM [6]. IR's value as a predictor of future diabetes or insulin-sensitizing drugs' ability to prevent T2DM lends credence to this notion [7]. Diabetic dyslipidemia is another cardiovascular disease (CVD) risk factor in individuals with T2DM. Increased triglycerides (TG), decreased HDL-C (high-density lipoprotein cholesterol), and

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postprandial lipemia are its constituents. The atherogenic index of plasma, which measures the ratio of blood triglycerides to high-density lipoprotein cholesterol (THR), is a significant risk factor for cardiovascular disease and metabolic syndrome [8, 9]. There is a correlation between endothelial dysfunction and a higher THR. Additionally, the THR has been suggested as an indicator of IR [10]. This is because lipid metabolism is altered by the metabolic processes that induce IR, and these alterations are mirrored in serum triglyceride and HDL cholesterol levels [11]. THR has also been demonstrated by Quispe et al. [12] to be a measure of glycemic control, particularly in obese individuals with T2DM. A further indicator linked to IR is the triglyceride to glucose (TyG) index. It assists in identifying asymptomatic T2DM patients who are at high risk of CVD [13]. THR and IR in diabetic individuals have been the subject of the majority of research to date [14].

In order to evaluate insulin sensitivity, the medical profession has therefore looked for substitute, indirect biomarkers. Strong predictive ability, high specificity, and sensitivity make Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) and Fasting Insulin (FI) popular surrogate markers for IR assessment. However, although being more practical than the hyperinsulinemic–euglycemic clamp technique, they are still difficult to use in day-to-day situations [11, 15]. Since insulin is not measured in every hospital, and due to transportation problems of blood samples from small family medicine offices to the hospital laboratories or HOMA-IR measurement handicaps, the quest for easier biomarkers to incorporate into a routine test is ongoing. Because measurement of triglyceride, HDL, and glucose levels is common and reasonably priced, THR can be calculated more frequently in clinical practice [16]. Over the past 20 years, efforts have been made to define the precise predictive power, constraints, and idiosyncrasies of THR, because of the unquestionable ease of use and accessibility of these two biomarkers.

Studies that sought to evaluate the relationship between glycemic control and the THR are scarce in our country, nevertheless. Thus, this study's objective was to assess the relationship between our population's THR and incident T2DM risk.

Materials and Methods

After taking approval from the ethical committee of Tokat Gaziosmanpaşa University Faculty of Medicine Clinical Research Ethics Committee (Date 26/10/2023, No: 23-KAEK-252), this cross-sectional retrospective study was conducted in Tokat Gaziosmanpaşa University. The study was designed in accordance with the Helsinki Declaration. All 953 participants aged between 18–75 years old, had blood tests for fasting serum glucose (FSG), HOMA-IR, FI, and TG levels between March and August 2023 were included in the study. THR was calculated according to following formula "serum triglyceride(mg/dL)/serum HDL(mg/dL)" [17] and HOMA-IR was derivedu (FSG): (mg/dL) / 405 [18]. We excluded participants under 18 years, having chronic thyroid disease, liver diseases, chronic kidney disease, hematological disorders or malignancies, systemic inflammatory or infectious diseases, a history of metabolic or bariatric surgery and use of anti-inflammatory or steroid therapy.

All the information of the patients was collected retrospectively from our hospital data system (ENLIL HBYS Co. Türkiye). Cobas c501 (Roche Diagnostics, GmbH, Manheim, Germany) instrument was used to estimate FSG, TG, levels. Serum FI estimation was performed on Cobas e411 (Roche Diagnostics, GmbH, Mannheim, Germany).

Statistical analysis

Participants were grouped on the basis of HOMA-IR values and evaluated according to their cutoff value as 2.5 [19]. After evaluating the qualitative variables, we looked at the distributions of the quantitative variables.

The relationship between the significant variables and THR was evaluated for HOMA-IR group. We defined the cutoff values and The Receiver Operating Characteristic (ROC) curves of THR for HOMA-IR group. Then, we performed logistic regression analysis of the selected variables based on our study group.

To learn more about the overall traits of the research group, descriptive analyses were carried out. Continuous variable data are displayed as mean±standard deviation, whereas categorical variable data are displayed as n (%). Independent sample t test was used to compare the normally distributed age, FSG, HDL variables between two groups and Mann-Whitney U Test was used to compare the non-normally distributed HOMA-IR, FI, TG, THR variables between two groups. To find performance metrics for predicting THR variable, ROC analysis was employed. In examining the relationships among the variables THR and age, FSG, HOMA-IR, HDL, FI, and TG, Spearman correlation coefficient was used. A multivariate logistic regression model was implemented to determine relation among selected variables and HOMA-IR. If a P value was less than 0.05, it was deemed statistically significant. For the computations, pre-made statistical software (SPSS 22.0 Chicago, IL, USA) was utilized.

Results

Participants in the study included 953 patients, whose mean age was 40.83±16.78. Research groups had significant differences ($p<0.001$) in FSG, HOMA-IR, FI, TG, and THR parameters, based on HOMA-IR cut off value of 2.5 (Table 1). In research groups, THR had a moderate negative correlation with HDL ($r=-0,555$, $p<0.001$), while strong positive correlation with TG ($r=0,959$, $p<0.001$) in pairwise correlation (Table 2). For HOMA-IR, THR had the highest selectivity and positive predictive value (PPV) with a cut off value of ≥2.64 (AUC:0,72, Se:65%, Sp:70% ($p<0.001$: 95% CI:0,66–0,78)) and ROC analysis results for THR are shown in Table 3. THR had the value of Area Under Curve (AUC) of 0.72 for HOMA-IR group in Receiver Operating Characteristic (ROC) analysis (Fig. 1). According to multivariate logistic regression analysis, men are 2.247 times more likely than women to have poor glycemic control and this difference is statistically significant ($p=0.022$). The risk of having poor glycemic control rises by 1.045 times with each unit of age, and this difference is statisti-

Table 1. Distribution of quantitative variables

Variables	Total (n=953) Mean±SD	HOMA-IR group <2.5 (n=379) Mean±SD	HOMA-IR group ≥2.5 (n=574) Mean±SD	p
Age (Year)	40.83±16.78	41.42±16.6	40.5±16.89	0.130
FSG (mg/dL)	115.28±59.91	99.88±34.48	123.77±68.67	<0.001
HDL-C (mg/dL)	50.97±12.51	56.62±13.93	47.94±10.53	<0.001
HOMA-IR	3.2 [2.08–5.22]	1.82 [1.34–2.24]	4.64 [3.47–7.13]	<0.001*
FI (mIU/mL)	12.63 [8.14–19.36]	7.78 [5.32–9.36]	17.7 [13.79–25.12]	<0.001*
TG (mg/dL)	127 [93.3–179]	109 [77–141.95]	145.1 [107–204]	<0.001*
THR	2.65 [1.54–4.07]	1.74 [1.2–2.89]	3.26 [1.96–5.12]	<0.001*

Data are shown as mean±standard deviation or median [Quartile 1-Quartile 3]. Independent Samples t test was used. *: Mann Whitney U test was used. HOMA-IR: Homeostasis model assessment-estimated insulin resistance; SD: Standard deviation; FSG: Fasting serum glucose; HDL-C: High-density lipoprotein cholesterol; FI: Fasting insulin; TG: Triglycerides; THR: Triglyceride/HDL-C ratio

Table 2. Pairwise correlation between variables

Variables	Total THR	HOMA-IR <2.5 THR	HOMA-IR ≥2.5 THR
Age (year)			
r	0.069	0.268*	0.111
p	0.258	0.005	0.165
FSG (mg/dL)			
r	0.276*	0.399*	0.219*
p	<0.001	<0.001	0.006
HOMA-IR			
r	0.372*	0.239*	0.292*
p	<0.001	0.012	<0.001
HDL (mg/dL)			
r	-0.555*	-0.613*	-0.549*
p	<0.001	<0.001	<0.001
FI (mIU/mL)			
r	0.254*	0.087	0.146
p	<0.001	0.364	0.066
TG (mg/dL)			
r	0.959*	0.924*	0.962*
p	<0.001	<0.001	<0.001

Spearman correlation coefficient was used. *: Statistically significant positive correlations. HOMA-IR: Homeostasis model assessment-estimated insulin resistance; THR: Triglyceride/HDL-C ratio; FSG: Fasting serum glucose; HDL: High-density lipoprotein; FI: Fasting insulin; TG: Triglycerides.

cally significant (p=0.007). The likelihood of having poor glycemic control rises by 1.056 times for every unit of FSG, and this increase is statistically significant (p=0.001) (Table 4).

Discussion

Patients in our study who had uncontrolled T2DM had elevated THR levels as well. Additionally, there was a negative association between THR and HDL levels, while there was a significant high positive association with TG, and a low positive association with FSG and HOMA-IR. These findings imply that THR can be considered independently as a major predictor to determine the increased risk of acquiring incident T2DM.

IR and reduced βeta-cell activity are characteristics of incident T2DM [20]. The presence of IR causes hyperglycemia and hyperlipidemia in a variety of tissues, including muscle, liver, adipose, and pancreatic β-cells [21]. Triglycerides reduce glucokinase activity and glucose-stimulated insulin release in islets during hypertriglyceridemia [22]. Hyperglycemia results in ongoing oxidative stress on islet cells, even if the cells themselves have a lower antioxidant capacity [23]. Therefore, lipotoxicity and glucose toxicity may have an effect on β-cell failure [21].

Considering its critical role in T2DM and metabolic syndrome, IR assessment is important. In epidemiological studies and clinical practice, HOMA is a widely used and proven technique to measure IR from FSG and insulin [24]. In 28 studies, HOMA-IR was the most commonly used technique to measure IR [18]. In our study, HOMA-IR was significantly increased in poor glycemic control group and it was positively and significantly associated with THR, which was consistent with the existing research results.

Baneu et al. [25] stated in their review that ROC curve analysis for the assessment of IR was used in 17 studies with an AUC greater than 0.7, indicating a reasonable predictive power. In line with this result, we found an AUC of 0.72 in our study, indicating a moderate predictive power of THR.

FI is a measurement that assesses insulin levels in the blood following an overnight fast. Despite its simplicity, it does not provide a complete picture of insulin sensitivity, which limits its usefulness [26, 27]. FI was significantly increased in poor glycemic control group and it had a positive significant association with THR in our study.

The hyperinsulinemic-euglycemic clamp technique is the gold standard for determining insulin sensitivity and resistance. This approach is labor-intensive, expensive, and re-

Tablo 3. ROC analysis results for THR

Variable	Cut-off	AUC (95% CI)	Se	Sp	PPV	NPV	p
THR	≥2.64	0.72 (0.66–0.78)	0.65	0.70	0.76	0.58	<0.001

ROC: Receiver operating characteristic; THR: Triglyceride/HDL-C ratio; AUC: Area under curve, CI: Confidence interval; Se: Sensitiviy; Sp: Specifity; PPV: Positive predictive value; NPV: Negative predictive value.

Table 4. Logistic regression analysis of selected variables

Model	Univarite				Multivariate			
	p	Odds ratio	95% CI for odds ratio		p	Odds ratio	95% CI for odds ratio	
			Lower	Upper			Lower	Upper
Gender (F/M)	0.026	1.382	1.040	1.835	**0.022**	2.247	1.051	4.504
Age (year)	0.130	1.007	0.986	1.002	**0.001**	1.056	1.033	1.081
FSG (mg/dL)	<0.001	1.016	1.010	1.021	0.007	1.045	1.012	1.079
HDL_cholesterol	<0.001	1.066	0.918	0.958	**0.032**	1.053	1.004	1.103
TG (mg/dL)	<0.001	1.009	1.006	1.012	0.884	1.002	0.979	1.025
THR	<0.001	1.534	1.295	1.818	0.991	0.996	0.487	2.034

Reference category: Women for Gender. CI: Confidence interval; FSG: Fasting serum glucose; HDL: High-density lipoprotein; TG: Triglycerides; THR: Triglyceride/HDL-C ratio.

quires extensive knowledge [28, 29]. We could not evaluate this technique in our cross-sectional study.

According to diabetic medical care guidelines, asymptomatic adult patients with high blood levels of TG and low HDL cholesterol are at risk for developing pre-diabetes and diabetes [30]. Finding relevant biomarkers like THR for T2DM can aid in the follow-up and development of new treatment plans to increase patient survival [31].

In the study by Jabeen et al. [17], THR levels were found to be increased in patients with uncontrolled T2DM in line with our results. In the study by Gedikli et al. [32]. increased FSG levels were positively associated with THR in Chinese T2DM patients, which was consistent with our findings. THR was significantly increased in poor glycemic control group.

Cutoff levels for THR are an important consideration in clinical practice. In 50% of research, authors provided particular cutoff values, whereas the rest treated the THR as a continuous variable. When exact ratios were provided, they were either generic for the entire group or based on race or gender, with many cutoffs proposed. According to three research studies conducted between 2005 and 2008 [33–35], the highest score was 3.5 for both genders. The median cutoff value for women was 2.53, while men's was 2.8. Li et al. [36] addressed the ethnicity question in 2008 and found no significant difference in Odd Ratio (OR) in 3 separate subpopulations of their study, non-Hispanic whites, non-Hispanic blacks, and Mexican Americans, respectively; using ethnicity-specific cutoff points, the THR was 3.0 for Caucasians and Mexican Americans and 2.0 for African Americans. In our study, the THR cutoff value was determined as 2.64 with a moderate predictive value. In addition, men were 2.2 times more likely than women to have poor glycemic control. Poor glycemic control risk rised by 1.045 times with age and by 1.1 times with FSG levels.

Strengths of the study

It includes a large community-based sample size across a wide age range, high participation rates, standardized high-quality clinical and laboratory procedures, and adjustment for numerous potential confounding factors.

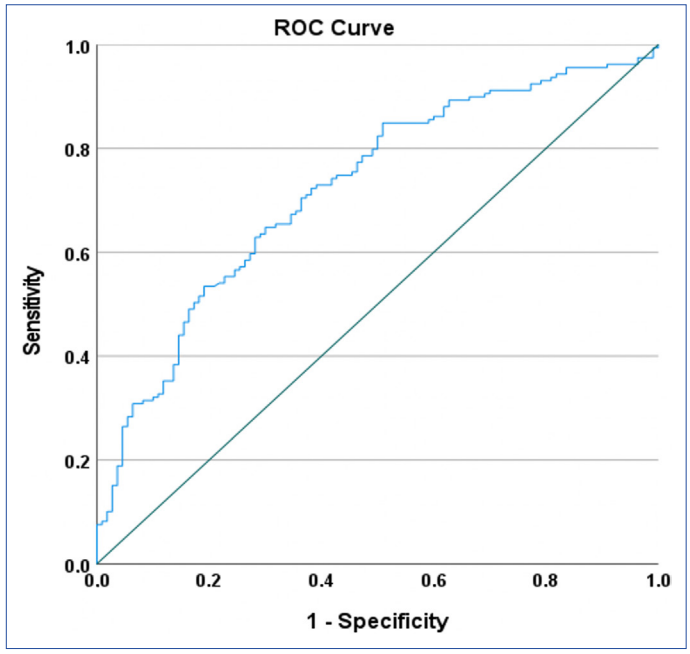

Figure 1. ROC curve of THR.
ROC: Receiver operating characteristic; THR: Triglyceride/HDL-C ratio.

Limitations of our study

The main limitations are that the study was a retrospective cross-sectional study and its relationship with T2DM complications was not assessed, the progression of diabetes was not followed and any causal relationship between our findings was not inferred. Although the results appear significant, they should be confirmed by the euglycemic clamp method.

Conclusion

Our results raise the prospect of using THR for diabetes risk assessment in actual clinical settings or extensive epidemiologic research because it is simple to compute from standard laboratory data.

Ethics Committee Approval: The study was approved by the Tokat Gaziosmanpaşa University Faculty of Medicine Clinical Research Ethics Committee (no: 23-KAEK-252, date: 26/10/2023).

Informed Consent: Informed consent was obtained from all participants.

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Research Article

Study the genetic variations of GRIAs genes to investigate association to methamphetamine addiction

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Abstract

Objectives: Drug addictions are chronic complicated disorders characterized by the repeated and irresistible intake of specific drugs that produce transient bliss. Methamphetamine, an amphetamine derivative, is an addictive psychostimulant substance used all over the world, especially in South East Asia. Chronic Methamphetamine consumption not only causes dependence but also frequently promotes psychotic symptoms, auditory hallucinations, and paranoid delusions, which are similar to the positive symptoms of schizophrenia. This study looked at the genetic variation of the *GRIA1* and *GRIA3* genes to see if they were associated with methamphetamine addiction in Iraqi males.

Methods: A total of 150 male participants were enrolled in this case-control study (100 methamphetamine-dependent and 50 control), ages between 20 to 45 years. *GRIA1* (rs2195450) and *GRIA3* (rs3761555) polymorphism detection was achieved by using high-resolution melting (HRM) real-time PCR analysis.

Results: The findings of the current study showed a highly significant difference in genotype distribution and allele frequency of rs2195450 SNP between the methamphetamine-dependent and the control groups, according to GA genotype and A allele, and in rs3761555 based on CC genotype and C allele, displaying a positive association with addiction.

Conclusion: According to the result of present study GA and CC genotypes have a positive correlation with disease these could be considered as a risk factor that makes people more susceptible to methamphetamine addiction.

Keywords: Drug addiction, genetic variation, *GRIA1*, *GRIA3*, methamphetamine

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Methamphetamine is the second most often abused substance worldwide. Methamphetamine addiction can lead to weight loss, memory loss, tremors, convulsions, psychosis, paranoia, hallucinations, Parkinson's-like symptoms, and cardiovascular collapse. The intensity of effects of withdrawal differs from person to person [1, 2]. The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid glutamate receptor (AMPA or GluR) is an ionotropic glutamate receptor subtype with four subunits: GluR1–4. The functional unit of the AMPA receptor is made up of GluR1R2, GluR2R3, or GluR2-lacking combinations. These subunits are expressed in various brain areas, including the hippocampus, nucleus accumbens, dorsal striatum, and prefrontal cortex [3, 4]. The AMPA receptor has an essential role in activities of brain plasticity of synapses, including learning, memory, and drug addiction [5, 6]. Thus, genes encoding AMPA glutamate receptors (GRIAs) are intriguing candidate genes for vulnerability to methamphetamine use and related psychosis [7]. Glutamate Ionotropic Receptor AMPA Type Subunit 1 (*GRIA1*) is situated on chromosome 5q33; earlier research suggested that *GRIA1* may be implicated in the therapeutic effect of major depressive disorder (MDD). Furthermore, it has been reported to be responsible for psychotic characteristics in schizophrenia and bipolar disorder [3, 8]. *GRIA3* is located on Xq25 and is frequently linked to the risk of schizophrenia, bipolar disorder, and alcoholism [9]. Thus, genetic variations in GRIA subunit genes have been linked to both mental disease and

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drug dependence. However, there is no indication that they are associated with either METH dependency or METH-dependent psychosis [9, 10]. Therefore, the goal of this study was to evaluate the relationship of common and previously investigated polymorphisms in *GRIA1* and *GRIA3* with METH dependency in the Iraqi male population. Addiction is connected to both hereditary and environmental causes. Examining the significance of genetic differences in the etiology of addiction might enhance the effectiveness of medications and assist in avoiding diseases. Both genome-wide techniques and candidate gene investigations seek for the fundamental significance of the genetic factor that causes drug addiction by identifying genes that are critical to neural adaption [11, 12].

Materials and Methods

This study was approved by the Ethics Committee of the Ministry of justice in Iraq/ Iraqi correction service (No: 24303/3/1/13, Date: 15/09/2021). This study involving human subjects is in accordance with the Helsinki Declaration of 1975 as revised in 2000.

Study groups and sample collection

This case-control study included 150 participants (100 methamphetamine dependent, 50 control) males ethically aged 20–45. Methamphetamine dependent subjects came from the Iraqi Correction Service. The primary inclusion criterion was diagnosis by a psychiatrist as meeting the criteria for substance dependence according to the Diagnostic and Statistical Manual of Mental Disorders (DSM) [13]. The control subjects were free from any reported personal or family history of drug misuse or psychiatric disorders. All subjects were given a complete illustration of the study. The signed written consent was taken from all participants in the study and the personal information for each participant was presented in questionnaire form under the supervision of the consultant and after obtaining approval for obtaining samples from all participants. The venous blood samples were collected in an EDTA tube from both study groups and genomic DNA was extracted by using ReliaPrep™ Blood gDNA Miniprep System and kept at -20°C for genotyping [14]. The purity and concentration of DNA solu¬tion extracted from blood samples were determined using Nanodrop at 260 nm, and the DNA purity was calculated as a ratio of A260/A280.

Genotyping analysis

The primers of *GRIA1* and *GRIA3* genes were designed for the current study. These primers were supplied by Alpha DNA company in a lyophilized form. Lyophilized primer was dissolved in free nuclease water to give a final concentration of 10 Pmol/ µl as a stock solution. *GRIA1* (rs2195450) and *GRIA3* (rs3761555) polymorphisms were chosen to investigate, these SNPs detection were achieved by using high-resolution melting (HRM) real-time PCR analysis. The reaction of HRM analysis for genotype using quantitative real-time PCR was performed in 20 µL total volume which comprised: 2xTransStart® Tip Green qPCR Super Mix, DNA template, 10 Pmol of Primers for rs2195450 genotyping (forward: 5`GAGGCAGTGCTTTCTCTGCT 3`) and reverse: (5`TTCAGTGGCTGCAATATACCC3`). While (forward: 5` CCTTCACTCCTGTCCATGAAA 3`) and reverse:(5` TCATTCTGATAATGCATAATTTCCTC 3`) for rs3761555 genotyping, then complete volume with nuclease free water. The HRM-PCR program of rs2195450 and rs3761555 polymorphisms displayed in Table 1.

Data analysis

The Statistical Package of Social Science (SPSS) version 26 was used to evaluate the effect of Diverse factors on study parameters. Chi-square was employed to test count variance. Alleles and genotype of gene SNPs. were presented as number and percentage frequencies. Hardy-Weinberg equilibrium analysis of genotype frequency was performed using Fisher test. A mean and standard deviation of continuous variables were calculated (SD). The student's t-test was applied to compare group differences. Statistical differences were defining significant as * $p \leq 0.05$ or ** $p \leq 0.01$ [15, 16].

Results

Demographic results

In this study, the results of some demographic features of two investigated groups are described in Table 2. They were measured by analyzing a questionnaire that was completed during a direct interview with all participants. According to the age mean±SD of the study groups was 28.62 ±8.25 for the methamphetamine dependent group and 27.01±5.14 for the control group, the result found that there was no significant association between both groups studied p=0.08. As mentioned in the Marital status, results showed no significant differences between the control and methamphetamine dependent groups (p=0.1). Then, depending on the occupation the findings of the present study showed significant differences between two studied groups (p=0.001). According to anxiety and depression, self-confidence, improve memory, self-hart and smoking the methamphetamine dependent was comprised of (53%, 56%, 43%, 60%, 86%, 8%) respectively.

Table 1. The HRM-PCR program of rs2195450 and rs3761555 polymorphisms

Step	Temperature (°C)	Time (sec.)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	
Annealing			
(rs2195450)	60	15	40
(rs3761555)	58		
Extension	72	20	
HRM	55–95	0.2sec for 1 degree	

HRM: High resolution melting; PCR: Polymerase chain reaction.

Table 2. The study groups' anthropometric characteristics

Variables	Methamphetamine dependent n=100		Control n=50		p
	n	%	n	%	
Age	28.62±8.25		27.01±5.14	0.08	NS
Marital status					
Single	54	54.00	28	56.00	0.1 NS
Married	46	46.00	22	44.00	
Occupation					
No	77	77.00	18	36.00	0.001*
Employ	23	23.00	32	64.00	
Anxiety and depression	53	53	–	–	
Self confidence	56	56	–	–	
Improve memory	43	43	–	–	
Self-hart	60	60	–	–	
Smoking	86	86	–	–	
Drink	8	8	–	–	

*: Significant. NS: Non-significant.

GRIA1 and *GRIA3* genotyping using HRM real-time PCR

DNA samples from all research groups were genotyped for rs2195450 and rs3761555 SNPs using HRM real-time PCR reaction. The picture depicts the thermocycler output from the HRM analysis process for the amplification of DNA, as shown in Figure 1.

The rs2195450 was the SNP of the *GRIA1* gene, located on chromosome 5 (5:153491449 (GRCH38)) with allele variation G>A. The genotype result for SNP rs2195450 displayed in Figure 2, where A refer to the HRM result, while B represents the wild, heterozygous and mutant genotypes.

In contrast, the rs3761555 was the SNP of the *GRIA3* gene, located on chromosome X at locus123182584, located in the promotor region with allele variation T>A,C. The genotype result for SNP rs3761555 clarified in Figure 3, where A refer to the HRM result, while B represents the wild, heterozygous and mutant genotypes.

The results displayed in Table 3, there were a highly significant differences in genotype distribution and allele frequency of rs2195450 SNP between the methamphetamine dependent and the control groups, according to GA genotype and A allele, These have a positive correlation with disease (odds ratio

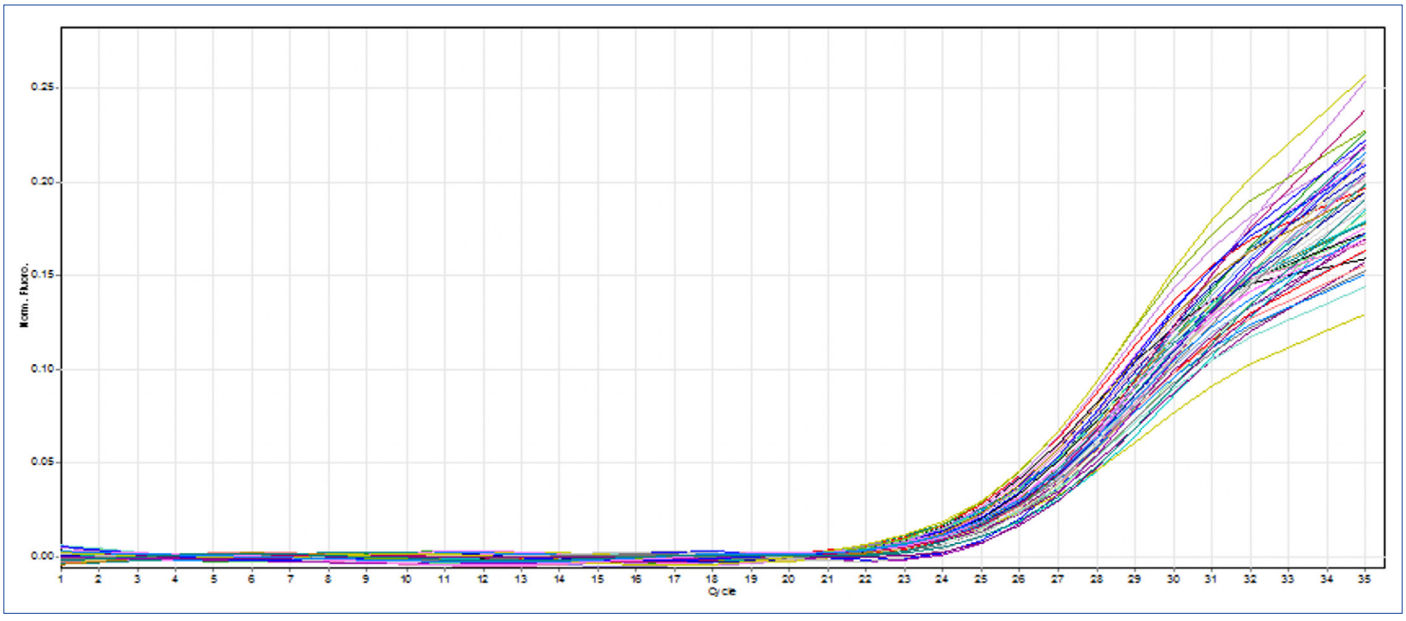

Figure 1. Amplification of DNA, the picture was taken directly from HRM analysis software.

HRM: High-resolution melting.

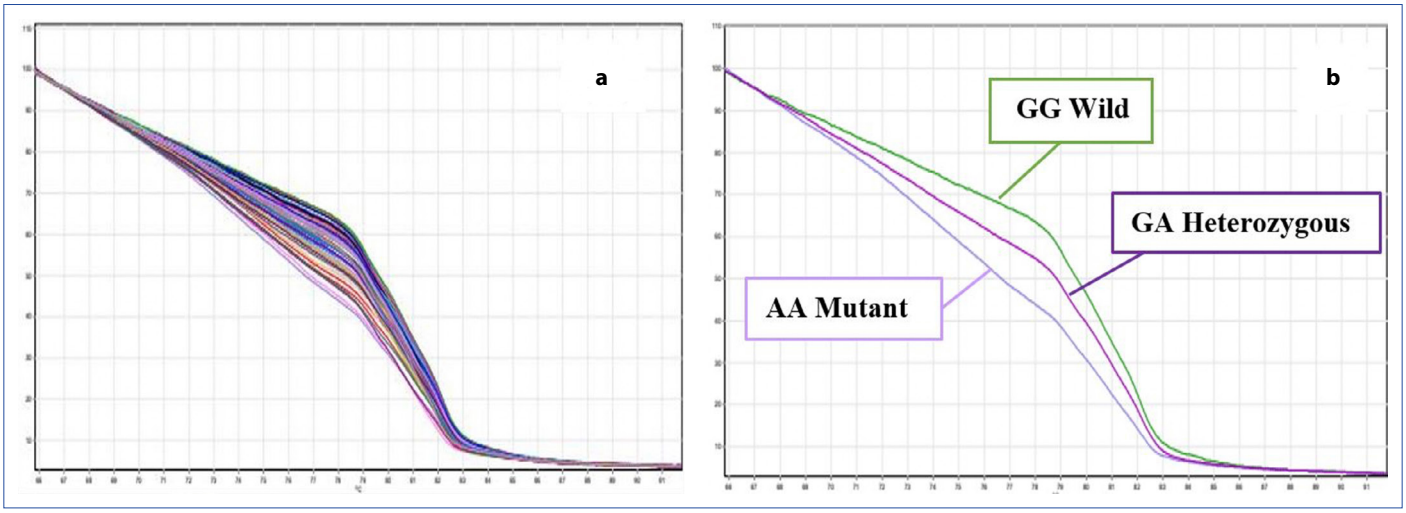

Figure 2. The genotype result for SNP rs2195450, (a) represents the HRM result, (b) represents the wild, heterozygous and mutant genotypes.
GG: Wild genotypes; GA: Heterozygous genotypes; AA: Mutant genotypes; HRM: High-resolution melting.

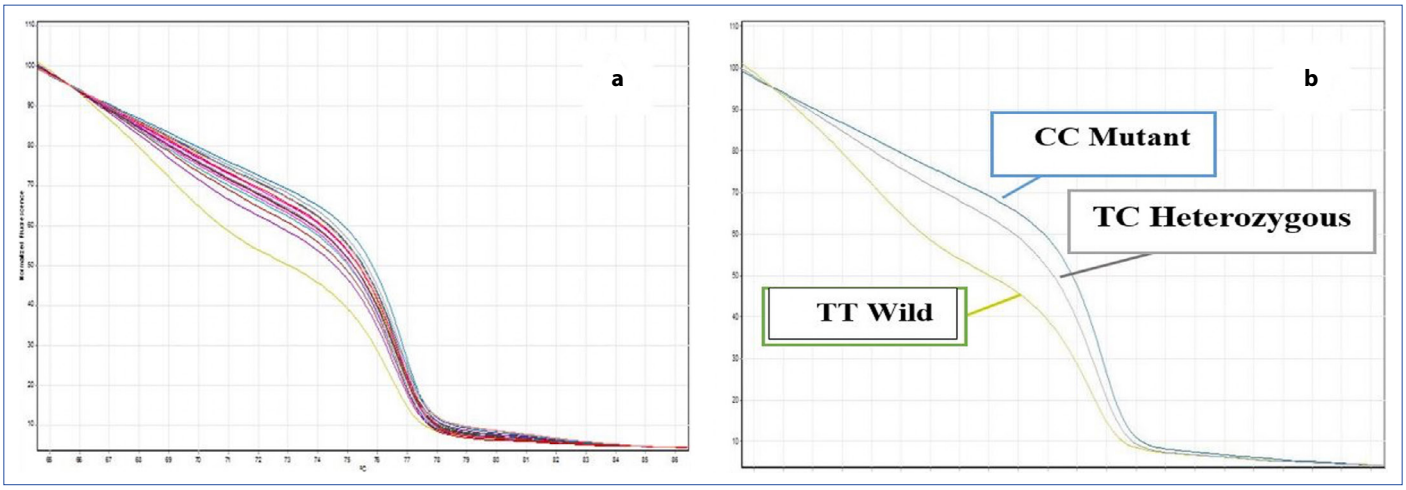

Figure 3. The genotype result for SNP rs3761555, (a) represents the HRM result, (b) represents the wild, heterozygous and mutant genotypes.
TT: Wild genotypes; TC: Heterozygous genotypes; CC: Mutant genotypes.

6 and 4.36 respectively) these could be considered as a risk factor that makes people more susceptible to drug addiction. On the other hand, there were a highly significant differences in rs3761555 genotype distribution and allele frequency between two studied groups, based on CC genotype and C allele, The odd ratio of these (6.09 and 6.75 respectively), displaying a positive association with addiction, which might be seen as a risk factor that makes people more vulnerable to addiction.

The observed genotype frequencies of rs2195450 SNP exhibited no significant differences from those predicted, it was 1.65 in Methamphetamine dependent group and 1.71 in controls, respectively, according to the Hardy-Weinberg Equilibrium. Because the total observed recorded for this locus was highly ratio at the genotype AA, it was 93 may be considered the common genotype in the Iraqi population, the other genotype, GA and, GG were 51, 6 correspondingly. In contrast, the genotype frequencies of rs3761555 SNP observed in methamphetamine dependent group were much greater than those expected by the Hardy-Weinberg Equilibrium hypothesis it was 37.69 while it was less than expected by H.W.E in the control group it was 26.52. TT genotype was shown to be the most common genotype in the Iraqi population with a total observed 99 followed by TC genotype with a total observed 20 CC genotype was present with a total observed 31, as shown in Table 4.

Discussion

This study analyzed the genetic variation of *GRIA1* and *GRIA3* genes and investigated the genetic susceptibility to methamphetamine addiction among sample of Iraqi males. No significant effects were found for AA and GG genotype of *GRIA1* SNP also, TT and TC genotype of *GRIA3* SNP that leading to indicate no impact of these SNPs on methamphetamine addiction. On the other hand, GA and CC have a favorable link with addiction, these could be regarded risk factors for methamphetamine addiction. The results are consistent with the extensive evidence for a disruption of glutamatergic neu-

Table 3. Genotype distribution of *GRIA1* and *GRIA3* polymorphisms in methamphetamine dependent and control groups

Subjects	Genotype frequency						Allele frequency			
***GRIA1* rs2195450**	**AA**		**GA**		**GG**		**A**		**G**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Methamphetamine dependent (n=100)	5	5	45	45	50	50	55	27.5	145	72.5
Control (n=50)	1	2	6	12	43	86	8	8	92	92
p	0.5 NS		0.0001**		–		0.0001**		–	
Odd ratio (95% CI)	2.58 (0.34–62.54)		6.00 (2.4–16.58)		1.00		4.36 (2.05–10.17)		1.00	
GRIA3 rs3761555	TT		TC		CC		T		C	
Methamphetamine dependent (n=100)	54	54	18	18	28	28	126	63	74	37
Control (n=50)	45	90	2	4	3	6	92	92	8	8
p	–		0.01**		0.001**		–		0.0001**	
Odd ratio (95% CI)	1.00		5.27 (1.32–34.61)		6.09 (1.90–26.25)		1.00		6.75 (3.20–15.60)	

**: Significant. GRIA: Glutamate Ionotropic receptor AMPA gene; GG: Wild genotypes; GA: Heterozygous genotypes; AA: Mutant genotypes; NS: non-significant; CI: Confidence interval.

Table 4. Expected frequencies of *GRIA1* and *GRIA3* polymorphisms in methamphetamine dependent and control groups using Hardy-Weinberg Equilibrium

***GRIA1* rs2195450**	**AA**	**GA**	**GG**	**χ^2**	**p**
Methamphetamine dependent genotype					
Observed no.	50	45	5	1.65	0.4 NS
Expected no.	52.56	39.87	7.56		
Control genotype					
Observed no.	43	6	1	1.71	0.4 NS
Expected no.	42.32	7.36	0.32		
Total observed	93	51	6		
***GRIA3* rs3761555**	**TT**	**TC**	**CC**	**χ^2**	**p**
Methamphetamine dependent genotype					
Observed no.	54	18	28	37.69	0.0001**
Expected no.	39.69	46.62	13.69		
Control genotype					
Observed no.	45	2	3	26.52	0.0001**
Expected no.	42.32	7.36	0.32		
Total observed	99	20	31		

**: Significant. NS: non-significant; GRIA: Glutamate Ionotropic receptor AMPA gene; GG, TT: Wild genotypes; GA, TC: Heterozygous genotypes; AA, CC: Mutant genotypes.

rotransmission in the brain in METH-induced psychosis [17]. As previously stated, this glutamatergic dysregulation is similar in mechanism and pathophysiology to that postulated for schizophrenia. The positive symptoms are similar to those of METH dependency [18, 19]. Furthermore, *GRIA3* DNA hypermethylation, as well as a significant decrease in *GRIA3* relative gene expression evaluated in blood samples, has been associated to the increased likelihood of schizophrenia [20]. The BDNF promotes the production of dopamine D3 receptors, which is very crucial in addiction [21, 22]. Taken together, these results reveal potential interaction effects of BDNF and AMPA receptor in boosting dopaminergic neurotransmission, critically altering the mesolimbic dopaminergic pathway in response to exposure to drugs of abuse. On the other hand, the results of this study did not support previous evidence implicating a role for *GRIA1* in METH addiction susceptibility [7], who described was an absence of substantial relationships between *GRIA1* gene polymorphisms and METH dependency in male Thai subjects. Another study by [23] have indicated the role of a *GRIA1* polymorphism in psychotic symptoms, while [8] displayed that GRIA1 has been related with schizophrenia in a large Korean sample. The previous studies by [24, 25], who

discovered a statistically significant result is that the *GRIA1* rs2195450 variant and the rs3761555 SNP of *GRIA3* gene is a potential genetic risk factor for female migraine in the Chinese Han population from the southern Fujian province of China.

Conclusion

According to the result of the present study GA genotype of rs2195450 SNP in *GRIA1* gene and the CC genotype of rs3761555 SNP in *GRIA3* gene have a positive correlation with disease these could be considered as a risk factor that makes people more susceptible to methamphetamine addiction.

Ethics Committee Approval: The study was approved by the Ministry of justice in Iraq/Iraqi correction service Ethics Committee (no: 24303/3/1/13, date: 15/09/2021).

Informed Consent: Informed consent was obtained from all participants.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Research Article

Assessing the pathogenicity of missense single nucleotide polymorphisms in the human *FUCA1* gene using multiple bioinformatics tools

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Abstract

Objectives: Fucosidosis is a rare lysosomal storage disorder caused by mutations in the *FUCA1* gene leading to a deficiency in α-L-fucosidase. This study aimed to investigate the pathogenic missense mutations in the *FUCA1* gene and their effects on protein stability using various bioinformatics tools.

Methods: Initially, 438 missense mutations were retrieved from the NCBI database, of which 43 mutations were identified by SIFT. The impact of these mutations on protein stability was assessed using I-MUTANT2.0 and MUPRO. Additionally, protein flexibility was analyzed using MEDUSA.

Results: Among the 43 mutations, SIFT predicted 20 mutations as "deleterious". PANTHER database predicted 21 mutations as "probably damaging" and 4 mutations as "possibly damaging", PolyPhen-2 tool identified 14 mutations as "probably damaging", and 6 mutations as "possibly damaging", PHD-SNP tool predicted 21 mutations as "disease-related", PROVEAN tool predicted 27 mutations as "deleterious", PMUT tool predicted 18 mutations as "disease-related", and SNP&GO tool predicted 24 mutations as "disease-related". Nine mutations (R173Q, W145R, W188C, R36C, R162Q, R308H, R162W, G425R, A368T) were commonly predicted by all the seven tools. The impact of these mutations on protein stability was assessed using I-MUTANT2.0 and MUPRO tools. The I-MUTANT2.0 tool indicated that all nine mutations result in a decrease in protein stability. Similarly, MUPRO tool showed that eight of the nine mutations decrease protein stability, and one mutation, G425R, was found to increase protein stability. Additionally, protein flexibility was analyzed using MEDUSA tool, which revealed that the positions of all 9 SNPs were rigid, except R36C and G425R which were flexible.

Conclusion: We hope that these findings could contribute to understanding the molecular basis of diseases associated with *FUCA1* gene mutations. However, Experimental validation is recommended to confirm these results and guide future therapeutic strategies.

Keywords: *FUCA1*, fucosidosis, missense, pathogenicity prediction, single nucleotide polymorphisms

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Fucosidosis is a rare lysosomal storage disorder [1], caused by mutations in the *FUCA1* gene leading to a deficiency in α-L-fucosidase [2]. It is inherited in an autosomal recessive pattern [1].

The disease is classified into two primary types based on age and clinical severity. Type I typically manifests before the age of one year and progresses rapidly, with affected individuals usually dying between 5 and 10 years of age. In contrast, type II generally begins later than two years old and often allows patients to survive until adulthood [3]. Clinical features include growth retardation, dysostosis multiplex, recurrent upper respiratory infections, coarse facial features, and angiokeratoma corporis diffusum [2]. Other symptoms, frequently observed in fucosidosis patients include hepatosplenomegaly, epilepsy, and inguinal hernia [3].

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The *FUCA1* gene is located on chromosome 1p34.11-1p36.11 and consists of eight exons and seven introns [4]. It encodes a homotetramer protein of 461 amino acids known as α-L-fucosidase [4]. Alpha-fucosidases are hydrolytic enzymes, that act on α-L-fucosyl linkages. They belong to the glycoside hydrolase families GH29 and GH95 [5].

The enzyme is made up of subunits with varying molecular weights (50 to 60 kDa), resulting from differences in N-glycosylation and proteolytic processing [6]. A deficiency or absence of this enzyme in fucosidosis disrupts the degradation of fucosylated glycoproteins and glycolipids within lysosomes, causing the accumulation of fucosylated substrates in various tissues [7]. Both homozygous mutations and heterozygous mutations in the *FUCA1* locus can result in reduced or complete loss of α-L-fucosidase activity [8].

Bioinformatics tools have been employed to predict the effects of novel alterations on the structure and function of protein [9]. This is the first in silico study of the human *FUCA1* gene, utilizing various bioinformatics tools to analyze the structural and functional effects of missense single nucleotide polymorphisms (SNPs) in the *FUCA1* gene, which may contribute to predicting pathogenic SNPs that may serve as diagnostic markers for fucosidosis.

Materials and Methods

Data set

SNPs associated with the *FUCA1* gene were retrieved from the single nucleotide polymorphism database (dbSNP). These SNPs are identified by their unique reference sequence IDs (rsID).

Prediction of deleterious missense SNPs

To predict the functional and pathogenic effects of missense SNPs in the coding region of the *FUCA1* gene, seven bioinformatics tools were employed (Fig. 1). All tools were utilized with their default settings unless specified otherwise.

SIFT: Sorting Intolerant from Tolerant (SIFT) is a bioinformatics tool that uses homology-based sequence analysis to assess the impact of missense SNPs. It calculates normalized probabilities for all possible substitutions from the alignment to differentiate between deleterious and tolerated missense SNPs. SNPs with scores ≤0.05 are classified as deleterious, while those >0.05 are considered tolerated [10].

PolyPhen-2: Polymorphism phenotyping v2 (Polyphene-2) is a web-based tool integrates multiple sequence alignment and 3D structural analysis to predict the effects of amino acid substitutions on protein structure and function. The

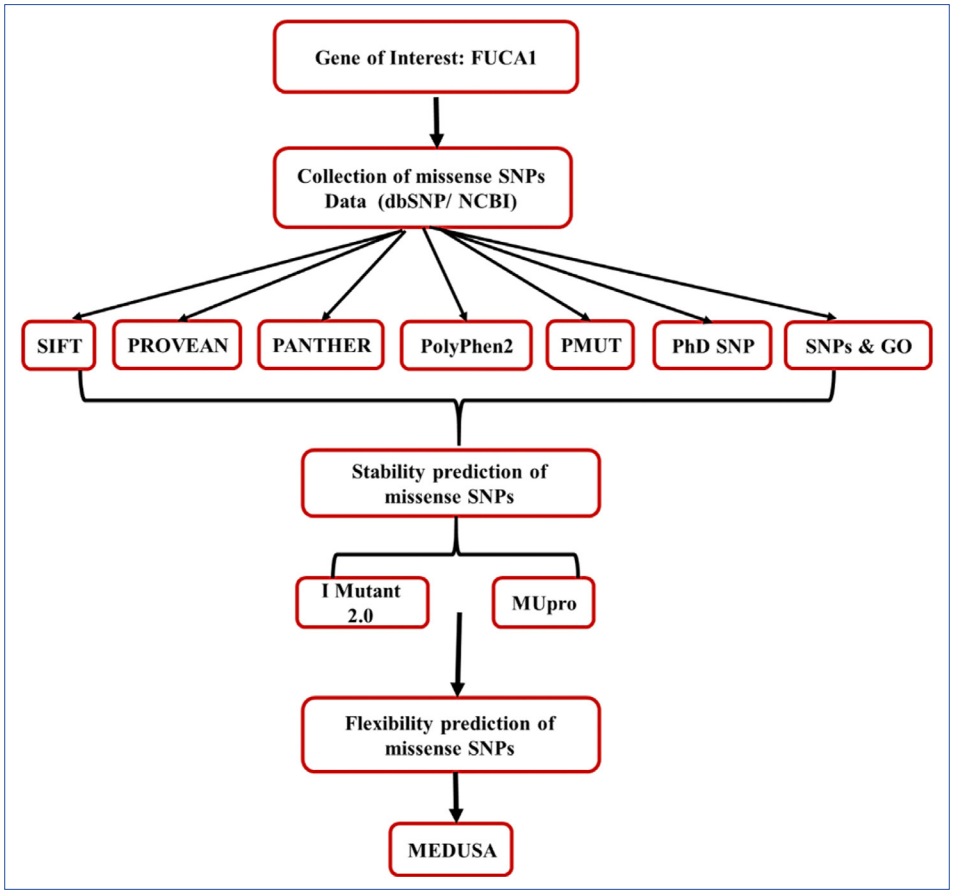

Figure 1. Workflow of in silico tools for computational analysis of *FUCA1* gene.

SNP: Single nucleotide polymorphisms; NCBI: National center for biotechnology information; SIFT: Sorting intolerant from tolerant; PROVEAN: Protein variation effect analyzer; PANTHER: Protein analysis through evolutionary relationship; PolyPhen-2: Polymorphism phenotyping v2; PMUT: Pathogenic mutation prediction.

tool calculates position-specific independent count (PSIC) scores for each of the two variants and determines the PSIC score difference between them [11]. SNPs are classified as benign (0.00–0.45), possibly damaging (0.45–0.95), or probably damaging (0.95–1) [10].

PhD-SNP: The PhD-SNP is a web server designed to assess whether a mutation occurs is benign or associated with inherited diseases, using support vector machines (SVMs). It provides an accuracy of 78% and a score range of 0–9 [12]. FASTA sequence of the corresponding protein and residue changes were submitted as inputs in the PhD-SNP server.

PANTHER: Protein Analysis Through Evolutionary Relationship (PANTHER) is a bioinformatics tool that predicts the likelihood of a mutation being harmful by estimating the evolutionary conservation of the amino acid position using a Hidden Markov Model and multiple sequence alignments [11]. The Substitution Position-Specific Evolutionary Preservation (PSEP) score indicates how long a site has been preserved during evolution:

- **Probably damaging:** >450 million years
- **Possibly damaging:** 200–450 million years
- **Probably benign:** <200 million years

PMUT: Utilizes neural networks' intelligence to predict whether SNPs are neutral or disease-causing, with an accuracy of 80% [13]. SNPs are classified as neutral (0–0.5) or pathological (0.5–1) [14].

PROVEAN: Is a web-based tool that determines the functional impact of amino acid substitutions based on sequence homology. Mutations with a score ≤-2.5 are predicted as "deleterious," while those with scores above -2.5 are considered "neutral" [13].

SNPs&GO: Single nucleotide polymorphism database and gene ontology (SNPs&GO) is a web server that combines sequence features, evolutionary data, and Gene Ontology (GO) annotations to predict disease relevance of mutations using an SVM model [14]. The result is based on the combination of Panther, PHD-SNP, and SNPs&GO results. SNPs with scores ≥0.5 are considered disease-causing, while those <0.5 are neutral.

Prediction of protein stability

Two different computational tools were used to predict the effects of single amino acid substitution on the stability of the human *FUCA1* protein.

I-mutant 2.0 is an automated web server based on SVM, that predicts changes in protein stability due to single-point mutations. It predicts the protein stability free energy change (Delta-DeltaG, or DDG) in kcal/mol. A negative DDG value indicates a decrease in stability, while a positive DDG value suggests an increase in stability [15]. DDG values for neutral mutations range from -0.5 ≤DDG ≤0.5, with values <-0.5 classified as a "large decrease" in stability and values >0.5 as a "large increase".

MUpro (mupro.proteomic.ics.uci.edu) is a web server that uses both neural network (NN) and support vector machine (SVM) models and generates confidence scores ranging between -1 and 1 [16]. A negative value indicates that the mutation is predicted to decrease protein stability, while a positive value suggests increased stability. The magnitude of the score reflects the confidence level of the prediction—the closer the score is to -1 or 1, the higher the confidence in the predicted effect.

Prediction of protein flexibility

MEDUSA is a web server used to predict the flexibility of proteins. It categorizes each residue as either flexible or rigid, considering evolutionary origin and physicochemical properties [10].

Results

Prediction of nsSNPs pathogenicity

A comprehensive study was conducted to identify pathogenic mutations in the *FUCA1* gene using seven bioinformatics tools. Initially, 438 missense mutations were collected from the dbSNP NCBI database. Of these, 43 mutations were identified by the SIFT server (Table 1), while the remaining mutations were classified as "not found." Among the 43 identified mutations, SIFT predicted 20 mutations as "deleterious" with a tolerance index score ≤0.05, and 23 mutations as 'tolerated'. These 43 mutations were subsequently analyzed using six additional bioinformatics tools. Panther predicted 21 mutations as "probably damaging" and 4 mutations as "possibly damaging" and 18 mutations were "probably benign". PolyPhen-2 identified 14 mutations as "probably damaging", 6 mutations as "possibly damaging" and 23 mutations were "benign". PHD-SNP classified 21 mutations as "disease-related" and 22 mutations as "neutral" (Table 2). PROVEAN indicated that 27 mutations were "deleterious" and 16 mutations were "neutral". PMUT categorized 18 mutations as "disease-related," 15 as "neutral" and 9 as "not found." SNP&GO predicted 24 mutations as "disease-related" and 19 as "neutral" (Table 3).

To refine the analysis, we performed an intersection of the pathogenic mutations identified across the different tools. The intersection revealed that nine missense SNPs (R173Q, W145R, W188C, R36C, R162Q, R308H, R162W, G425R, A368T) were common and were observed as deleterious, disease-associated, probably damaging, and possibly damaging (Table 4).

Prediction of protein stability

The impact of the nine mutations on protein stability was evaluated using I-MUTANT2.0 and MUPRO, based on the free energy change value (Delta Delta G, DDG) and the confidence score.

The I-MUTANT2.0 tool indicated that all nine mutations result in a decrease in protein stability. Similarly, MUPRO showed that eight of the nine mutations decrease protein stability, and one mutation, G425R, was found to increase protein stability according to MUPRO results based on stability and confidence score (Table 5).

Table 1. Tolerated and deleterious missense SNPs predicted by SIFT

No	SNP ID	CHR	REF allele	ALT allele	Amino acid change	Region	SIFT score	SIFT prediction
1	rs665	1	C	T	V260I	CDS	0.058	TOLERATED
2	rs13551	1	T	C	Q286R	CDS	0.074	TOLERATED
3	rs2070956	1	G	C	P10R	CDS	0.3	TOLERATED
4	rs61996282	1	C	G	A3P	CDS	0.084	TOLERATED
5	rs80358195	1	C	G	E380Q	CDS	0.134	TOLERATED
6	rs138378067	1	G	A	P87L	CDS	0.054	TOLERATED
7	rs143691289	1	C	G	L134F	CDS	0.159	TOLERATED
8	rs145537354	1	C	T	R178H	CDS	0.9	TOLERATED
9	rs145603001	1	C	T	R376Q	CDS	0.033	DELETERIOUS
10	rs148194937	1	C	T	R173Q	CDS	0.016	DELETERIOUS
11	rs149168482	1	G	A	S393L	CDS	0.2	TOLERATED
12	rs149540896	1	G	A	R178C	CDS	0.023	DELETERIOUS
13	rs150062050	1	A	G	W145R	CDS	0	DELETERIOUS
14	rs150422575	1	C	T	G425E	CDS	0.017	DELETERIOUS
15	rs189315801	1	C	A	W188C	CDS	0	DELETERIOUS
16	rs201209052	1	G	A	R36C	CDS	0.008	DELETERIOUS
17	rs201499886	1	C	T	E318K	CDS	0.869	TOLERATED
18	rs372045495	1	C	T	R162Q	CDS	0.021	DELETERIOUS
19	rs367641377	1	G	C	S46C	CDS	0.003	DELETERIOUS
20	rs372537257	1	C	T	R308H	CDS	0.011	DELETERIOUS
21	rs375270869	1	G	A	R162W	CDS	0	DELETERIOUS
22	rs377360830	1	C	T	A454T	CDS	0.314	TOLERATED
23	rs11549095	1	C	T	R113H	CDS	0.038	DELETERIOUS
24	rs75146878	1	C	T	D290N	CDS	0.442	TOLERATED
25	rs143545485	1	T	A	T38S	CDS	0.646	TOLERATED
26	rs149277420	1	G	A	S323L	CDS	0.336	TOLERATED
27	rs144919088	1	T	C	S287G	CDS	0.405	TOLERATED
28	rs199940255	1	G	A	R94C	CDS	0.012	DELETERIOUS
29	rs200493075	1	C	T	C235Y	CDS	0.354	TOLERATED
30	rs200678715	1	T	G	D309A	CDS	0.079	TOLERATED
31	rs202079642	1	T	C	I227V	CDS	0.255	TOLERATED
32	rs367783196	1	T	G	D344A	CDS	0.047	DELETERIOUS
33	rs202239236	1	G	A	R91C	CDS	0.033	DELETERIOUS
34	rs368369988	1	G	A	P34S	CDS	0.843	TOLERATED
35	rs368693347	1	T	C	S102G	CDS	0.082	TOLERATED
36	rs369435617	1	T	C	N144D	CDS	0.02	DELETERIOUS
37	rs371134787	1	G	A	P39L	CDS	0.001	DELETERIOUS
38	rs373007532	1	G	A	P10S	CDS	0.482	TOLERATED
39	rs373805274	1	T	C	N96S	CDS	0.079	TOLERATED
40	rs373999438	1	C	T	G425R	CDS	0.001	DELETERIOUS
41	rs374427540	1	C	T	A368T	CDS	0.003	DELETERIOUS
42	rs377191399	1	T	C	I442V	CDS	0.725	TOLERATED
43	rs2228424	1	G	A	P146L	CDS	0.009	DELETERIOUS

SNP: Single nucleotide polymorphism; SIFT: Sorting intolerant from tolerant; CHR: Chromosome; REF: Reference allele; ALT: Alternative allele; CDS: Coding DNA sequence.

Prediction of protein flexibility

Based on the flexibility prediction of *FUCA1* protein by MEDUSA (7=rigid, 2=flexible), the positions of all 9 SNPs were rigid, except R36C and G425R, which were flexible. The amino acid sequence positions W145R, W188C and A368T had a confidence score>0.75, while the positions R173Q, R36C and G425R had the confidence score of 0.6–0.75 and positions R162Q, R308H and R162W had a confidence score<0.6 (Fig. 2).

Table 2. Prediction of the pathogenicity of the missense SNPs using PANTHER, PolyPhen-2, and PHD-SNP servers

No	SNP ID	PANTHER		PolyPhen-2		PHD-SNP	
		Score (Pdel)	Prediction	Score	Prediction	Score	Prediction
1	rs665	0.86	PROBABLY DAMAGING	0.538	POSSIBLY DAMAGING	6	NEUTRAL
2	rs13551	0.19	PROBABLY BENIGN	0.001	BENIGN	6	NEUTRA
3	rs2070956	0.27	PROBABLY BENIGN	0.272	BENIGN	7	NEUTRAL
4	rs61996282	0.13	PROBABLY BENIGN	0.000	BENIGN	9	NEUTRAL
5	rs80358195	0.57	PROBABLY DAMAGING	0.007	BENIGN	3	DISEASE
6	rs138378067	0.85	PROBABLY DAMAGING	0.029	BENIGN	2	DISEASE
7	rs143691289	0.86	PROBABLY DAMAGING	0.112	BENIGN	2	NEUTRAL
8	rs145537354	0.27	PROBABLY BENIGN	0.003	BENIGN	2	NEUTRAL
9	rs145603001	0.86	PROBABLY DAMAGING	0.351	BENIGN	6	DISEASE
10	rs148194937	0.86	PROBABLY DAMAGING	0.956	POSSIBLY DAMAGING	8	DISEASE
11	rs149168482	0.5	POSSIBLY DAMAGING	0.004	BENIGN	8	DISEASE
12	rs149540896	0.27	PROBABLY BENIGN	0.998	PROBABLY DAMAGING	8	DISEASE
13	rs150062050	0.95	PROBABLY DAMAGING	0.999	PROBABLY DAMAGING	5	DISEASE
14	rs150422575	0.86	PROBABLY DAMAGING	0.992	PROBABLY DAMAGING	7	DISEASE
15	rs189315801	0.86	PROBABLY DAMAGING	1.000	PROBABLY DAMAGING	7	DISEASE
16	rs201209052	0.74	PROBABLY DAMAGING	0.954	POSSIBLY DAMAGING	2	DISEASE
17	rs201499886	0.5	POSSIBLY DAMAGING	0.307	BENIGN	8	NEUTRAL
18	rs372045495	0.86	PROBABLY DAMAGING	1.000	PROBABLY DAMAGING	7	DISEASE
19	rs367641377	0.27	PROBABLY BENIGN	0.988	PROBABLY DAMAGING	6	DISEASE
20	rs372537257	0.86	PROBABLY DAMAGING	0.976	PROBABLY DAMAGING	2	DISEASE
21	rs375270869	0.86	PROBABLY DAMAGING	1.000	PROBABLY DAMAGING	8	DISEASE
22	rs377360830	0.19	PROBABLY BENIGN	0.000	BENIGN	5	NEUTRAL
23	rs11549095	0.86	PROBABLY DAMAGING	0.008	BENIGN		NEUTRAL
24	rs75146878	0.27	PROBABLY BENIGN	0.001	BENIGN	8	NEUTRAL
25	rs143545485	0.57	PROBABLY DAMAGING	0.97	BENIGN	2	NEUTRAL
26	rs149277420	0.27	PROBABLY BENIGN	0.014	BENIGN	7	NEUTRAL
27	rs144919088	0.19	PROBABLY BENIGN	0.001	BENIGN	8	NEUTRAL
28	rs199940255	0.19	PROBABLY BENIGN	0.981	PROBABLY DAMAGING	5	DISEASE
29	rs200493075	0.27	PROBABLY BENIGN	0.000	BENIGN	3	DISEASE
30	rs200678715	0.19	PROBABLY BENIGN	0.006	BENIGN	4	DISEASE
31	rs202079642	0.57	PROBABLY DAMAGING	0.476	POSSIBLY DAMAGING	5	NEUTRAL
32	rs367783196	0.5	POSSIBLY DAMAGING	0.124	BENIGN	7	DISEASE
33	rs202239236	0.27	PROBABLY BENIGN	0.989	PROBABLY DAMAGING	3	DISEASE
34	rs368369988	0.27	PROBABLY BENIGN	0.000	BENIGN	5	NEUTRAL
35	rs368693347	0.27	PROBABLY BENIGN	0.005	BENIGN	7	NEUTRAL
36	rs369435617	0.86	PROBABLY DAMAGING	0.992	PROBABLY DAMAGING	1	NEUTRAL
37	rs371134787	0.86	PROBABLY DAMAGING	0.997	PROBABLY DAMAGING	0	NEUTRAL
38	rs373007532	0.27	PROBABLY BENIGN	0.013	BENIGN	9	NEUTRAL
39	rs373805274	0.86	PROBABLY DAMAGING	0.576	POSSIBLY DAMAGING	1	NEUTRAL
40	rs373999438	0.86	PROBABLY DAMAGING	1.000	PROBABLY DAMAGING	6	DISEASE
41	rs374427540	0.86	PROBABLY DAMAGING	0.628	POSSIBLY DAMAGING	8	DISEASE
42	rs377191399	0.27	PROBABLY BENIGN	0.000	BENIGN	8	NEUTRAL
43	rs2228424	0.5	POSSIBLY DAMAGING	0.996	PROBABLY DAMAGING	2	NEUTRAL

SNP: Single nucleotide polymorphisms; PANTHER: Protein analysis through evolutionary relationship; PolyPhen: Polymorphism phenotyping; PHD-SNP: Predictor of human deleterious single nucleotide polymorphisms.

Discussion

The human genome shares approximately 99.9% identical DNA sequences, with the remaining 0.1% accounting for individual variations [17]. SNPs are the most common type of DNA alterations, involving changes at a single nucleotide within the genomic sequence [12]. These genomic variations can affect protein structure and function, potentially

Table 3. Prediction of the pathogenicity of the missense SNPs using PROVEAN, PMUT, SNP&GO servers

No	SNP ID	PROVEAN		PMUT		SNP&GO		
		Score	Prediction	Score	Prediction	RI	Probability	Prediction
1	rs665	-0.79	NEUTRAL	-	-	6	0.191	NEUTRAL
2	rs13551	-	NEUTRAL	-	-	6	0.206	NEUTRAL
3	rs2070956	-0.15	NEUTRAL	-	-	3	0.331	NEUTRAL
4	rs61996282	-0.34	NEUTRAL	0.43 (85%)	NEUTRAL	8	0.116	NEUTRAL
5	rs80358195	-2.23	NEUTRAL	0.64 (84%)	DISEASE	1	0.428	NEUTRAL
6	rs138378067	-6.08	DELETERIOUS	-	-	1	0.539	DISEASE
7	rs143691289	-3.27	DELETERIOUS	0.30 (89%)	NEUTRAL	2	0.386	NEUTRAL
8	rs145537354	2.10	NEUTRAL	-	-	2	0.416	NEUTRAL
9	rs145603001	-2.71	DELETERIOUS	0.85 (82%)	DISEASE	0	0.492	NEUTRAL
10	rs148194937	-3.94	DELETERIOUS	0.39 (86%)	NEUTRAL	7	0.867	DISEASE
11	rs149168482	-2.99	DELETERIOUS	0.88 (92%)	DISEASE	6	0.777	DISEASE
12	rs149540896	-4.13	DELETERIOUS	0.90 (93%)	DISEASE	6	0.781	DISEASE
13	rs150062050	-13.98	DELETERIOUS	-	-	7	0.861	DISEASE
14	rs150422575	-7.46	DELETERIOUS	0.89 (92%)	DISEASE	5	0.760	DISEASE
15	rs189315801	-12.21	DELETERIOUS	0.55 (81%)	DISEASE	8	0.921	DISEASE
16	rs201209052	-4.01	DELETERIOUS	0.39 (86%)	NEUTRAL	2	0.602	DISEASE
17	rs201499886	-0.83	NEUTRAL	0.70 (86%)	DISEASE	8	0.122	NEUTRAL
18	rs372045495	-3.99	DELETERIOUS	0.86 (91%)	DISEASE	7	0.826	DISEASE
19	rs367641377	-3.62	DELETERIOUS	0.51 (79%)	DISEASE	2	0.578	DISEASE
20	rs372537257	-4.32	DELETERIOUS	0.90 (93%)	DISEASE	2	0.604	DISEASE
21	rs375270869	-7.99	DELETERIOUS	0.02 (98%)	NEUTRAL	8	0.895	DISEASE
22	rs377360830	-0.72	NEUTRAL	0.10 (96%)	NEUTRAL	6	0.177	NEUTRAL
23	rs11549095	-0.83	NEUTRAL	0.14 (94%)	NEUTRAL	6	0.212	NEUTRAL
24	rs75146878	-0.09	NEUTRAL	-	-	7	0.153	NEUTRAL
25	rs143545485	-1.14	NEUTRAL	-	-	7	0.127	NEUTRAL
26	rs149277420	-3.27	DELETERIOUS	0.18 (93%)	NEUTRAL	7	0.152	NEUTRAL
27	rs144919088	-2.10	NEUTRAL	0.11 (95%)	NEUTRAL	8	0.089	NEUTRAL
28	rs199940255	-4.18	DELETERIOUS	0.53 (80%)	DISEASE	3	0.645	DISEASE
29	rs200493075	-2.59	DELETERIOUS	0.13 (94%)	NEUTRAL	2	0.600	DISEASE
30	rs200678715	-0.51	NEUTRAL	0.42 (85%)	NEUTRAL	2	0.618	DISEASE
31	rs202079642	-6.43	DELETERIOUS	0.31 (89%)	NEUTRAL	3	0.361	NEUTRAL
32	rs367783196	-3.02	DELETERIOUS	0.64 (84%)	DISEASE	7	0.840	DISEASE
33	rs202239236	0.41	NEUTRAL	0.81 (89%)	DISEASE	1	0.567	DISEASE
34	rs368369988	-3.01	DELETERIOUS	0.07 (97%)	NEUTRAL	7	0.163	NEUTRAL
35	rs368693347	-3.93	DELETERIOUS	0.11 (95%)	NEUTRAL	6	0.210	NEUTRAL
36	rs369435617	-9.14	DELETERIOUS	0.82 (90%)	DISEASE	2	0.417	NEUTRAL
37	rs371134787	0.31	NEUTRAL	0.80 (89%)	DISEASE	1	0.454	NEUTRAL
38	rs373007532	-4.39	DELETERIOUS	0.05 (97%)	NEUTRAL	7	0.159	NEUTRAL
39	rs373805274	-7.95	DELETERIOUS	0.72 (87%)	DISEASE	3	0.362	NEUTRAL
40	rs373999438	-3.73	DELETERIOUS	0.76 (88%)	DISEASE	6	0.782	DISEASE
41	rs374427540	0.13	NEUTRAL	0.60 (83%)	DISEASE	5	0.728	DISEASE
42	rs377191399	-6.72	DELETERIOUS	0.29 (90%)	NEUTRAL	9	0.048	NEUTRAL
43	rs2228424	-3.93	DELETERIOUS	-	-	0	0.505	DISEASE

SNP: Single nucleotide polymorphisms; PROVEAN: Protein variation effect analyzer; PMUT: Pathogenic mutation prediction; SNP&GO: Single nucleotide polymorphism&gene ontology.

altering the normal characteristics of an organism. A single nucleotide substitution can result in either a missense or nonsense mutation. In a missense mutation, one amino acid is replaced by another, whereas nonsense mutations replace a coding codon with a stop codon, leading to protein truncation [18].

Table 4. Common deleterious SNPs in *FUCA1* gene as predicted by seven in silico tools

No	SNP ID	Amino acid	SIFT	PMUT	PANTHER	PROVEAN	PHD_SNP	SNP_GO	POLYPHEN-2
1	rs148194937	R173Q	DELETERIOUS	DISEASE	Probably damaging	DELETERIOUS	DISEASE	DISEASE	Possibly damaging
2	rs150062050	W145R	DELETERIOUS	DISEASE	Probably damaging	DELETERIOUS	DISEASE	DISEASE	Possibly damaging
3	rs189315801	W188C	DELETERIOUS	DISEASE	Probably damaging	DELETERIOUS	DISEASE	DISEASE	Possibly damaging
4	rs201209052	R36C	DELETERIOUS	DISEASE	Probably damaging	DELETERIOUS	DISEASE	DISEASE	Possibly damaging
5	rs372045495	R162Q	DELETERIOUS	DISEASE	Probably damaging	DELETERIOUS	DISEASE	DISEASE	Possibly damaging
6	rs372537257	R308H	DELETERIOUS	DISEASE	Probably damaging	DELETERIOUS	DISEASE	DISEASE	Possibly damaging
7	rs375270869	R162W	DELETERIOUS	DISEASE	Probably damaging	DELETERIOUS	DISEASE	DISEASE	Possibly damaging
8	rs373999438	G425R	DELETERIOUS	DISEASE	Probably damaging	DELETERIOUS	DISEASE	DISEASE	Possibly damaging
9	rs374427540	A368T	DELETERIOUS	DISEASE	Probably damaging	DELETERIOUS	DISEASE	DISEASE	Possibly damaging

SNP: Single nucleotide polymorphisms; SIFT: Sorting intolerant from tolerant; PMUT: Pathogenic mutation prediction; PANTHER: Protein analysis through evolutionary relationship; PROVEAN: Protein variation effect analyzer; PHD_SNP: Predictor of human deleterious single nucleotide polymorphisms; SNP&GO: Single nucleotide polymorphism&gene ontology.

Table 5. The effect of missense SNPs on protein stability

No	SNP ID	I-Mutant 2.0		MUpro		MUpro (SVM)		MUpro (neural network)	
		Stability	DDG	Stability	DDG	Stability	Confidence score	Stability	Confidence score
1	rs148194937	Decrease	-0.42	Decrease	-0.92804456	Decrease	-0.53267036	Decrease	**-0.81992480**
2	rs150062050	Decrease	-1.11	Decrease	-0.79418806	Decrease	-0.71590402	Decrease	**-0.72355086**
3	rs189315801	Decrease	-1.94	Decrease	-1.2967016	Decrease	-1	Decrease	**-0.96074062**
4	rs201209052	Decrease	-0.79	Decrease	-0.93061864	Decrease	-1	Decrease	**-0.90201448**
5	rs372045495	Decrease	-1.27	Decrease	-1.0057917	Decrease	-0.4656619	Decrease	**-0.86043601**
6	rs372537257	Decrease	-2.02	Decrease	-1.403344	Decrease	-0.7006115	Decrease	**-0.85234457**
7	rs375270869	Decrease	-0.72	Decrease	-0.82555437	Decrease	-0.12250558	Decrease	**-0.77772743**
8	rs373999438	Decrease	-1.82	Decrease	-0.41414478	Decrease	0.22525227	Decrease	**0.6359362**
9	rs374427540	Decrease	-0.71	Decrease	-0.54392681	Decrease	-0.25394459	Decrease	**-0.78975651**

SNP: Single nucleotide polymorphisms; SVM: Support vector machine; DDG: Delta delta G.

Identifying SNPs that impact morphology is essential for understanding the genetic basis of diseases and phenotypic diversity. Furthermore, this knowledge aids in selecting markers for use in population-based association studies [12].

In the current study, the pathogenic missense mutations in the *FUCA1* gene and their effects on protein stability and flexibility were assessed using various bioinformatics tools.

The findings illustrate significant variability in the predictions among different tools, emphasizing the importance of cross-referencing outputs to improve the reliability of pathogenic mutation identification. We performed an intersection of the pathogenic mutations identified across the different tools. This approach was employed to ensure greater accuracy by focusing on mutations consistently predicted as pathogenic by multiple tools. Nine mutations were commonly identified as pathogenic across all tools. This consistency in results between different independent tools, which evaluate various aspects of protein structure, function, and evolutionary conservation, adds strength to the reliability of these predictions. Such predictions suggest that these mutations may disrupt the normal folding and stability of the *FUCA1* protein, potentially leading to the pathogenic phenotypes observed in diseases like fucosidosis. Furthermore, these mutations may interfere with the protein's function by altering critical functional sites, which could include regions essential for substrate binding or catalysis, ultimately impairing protein activity.

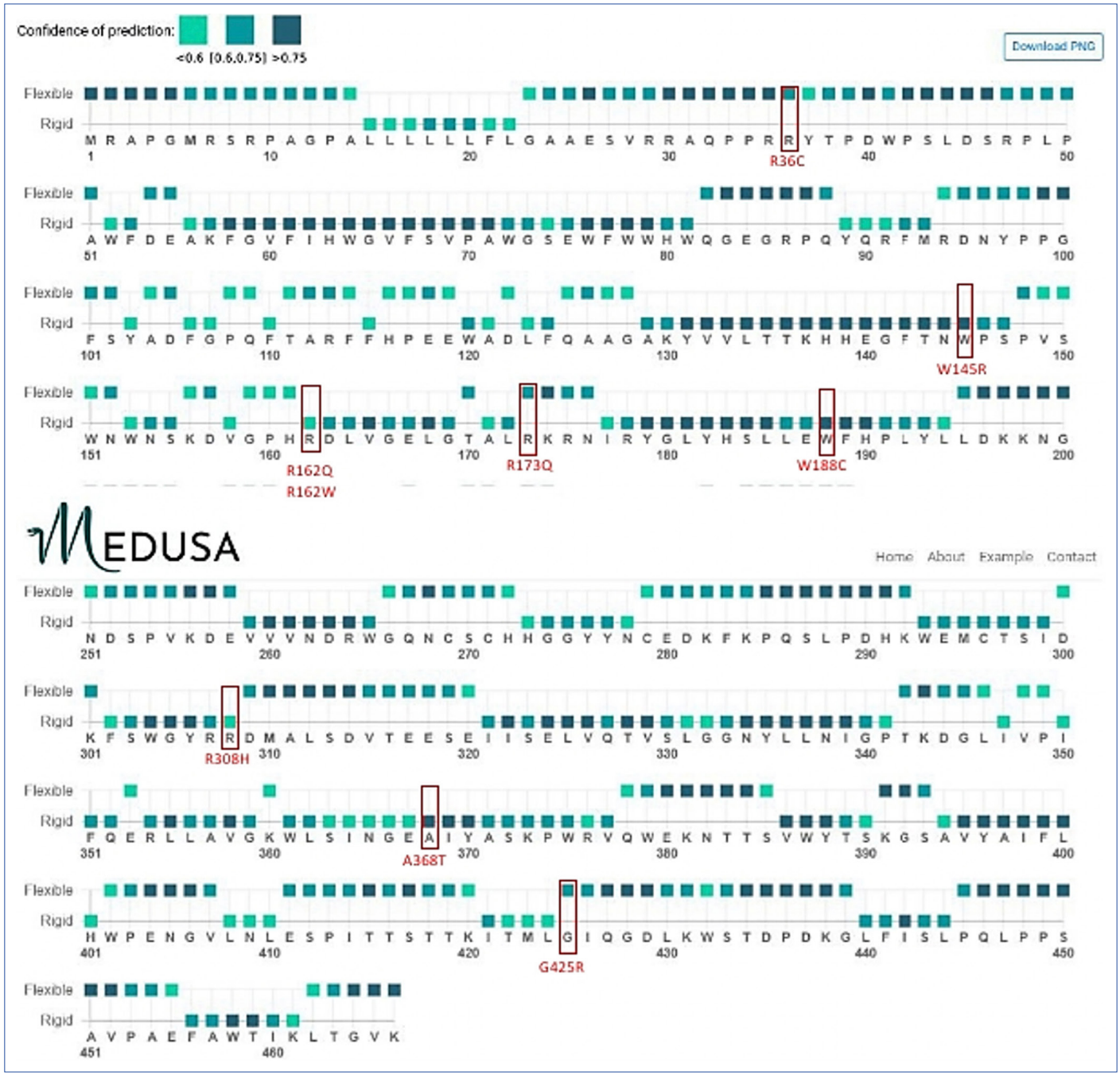

Figure 2. Flexibility prediction of missense SNPs on α-L-fucosidase protein.

SNP: Single nucleotide polymorphisms.

Rs148194937 (C/T mutation) leads to the conversion of arginine (R) to glutamine (Q) at position 173 (R173Q). The molecular weight of glutamine is smaller than that of arginine, and unlike arginine, which is positively charged, glutamine is neutral at physiological pH. This mutation can result in the loss of protein function due to changes in charge and potential alterations in hydrogen bonding.

Rs150062050 (A/G mutation) results in the conversion of tryptophan (W) to arginine (R) at position 145 (W145R). The molecular weight of arginine is smaller than that of tryptophan, and arginine is positively charged. The elongated side chain of arginine can form multiple hydrogen bonds, leading to incorrect folding and aggregation of the protein, thus impairing its function.

Rs189315801 (C/A mutation) causes the conversion of tryptophan (W) to cysteine (C) at position 188 (W188C). Tryptophan has a higher molecular weight than cysteine, and the thiol group (-SH) of cysteine is a nucleophile, making it reactive and prone to forming disulfide bonds. This mutation can disrupt the protein's stability and lead to a loss of function.

Rs201209052 (G/A mutation) leads to the substitution of arginine (R) with cysteine (C) at position 36 (R36C). Cysteine has a lower molecular weight than arginine and contains a thiol group. This mutation is known to cause disease due to its impact on protein folding and stability.

Rs372045495 (C/T mutation) causes the conversion of arginine (R) to glutamine (Q) at position 162 (R162Q). Glutamine has a smaller molecular weight than arginine and is neutral at physiological pH. This mutation has been established as deleterious, likely due to the loss of positive charge.

Rs372537257 (C/T mutation) results in the substitution of arginine (R) with histidine (H) at position 308 (R308H). Histidine has a smaller molecular weight than arginine but retains a positive charge under physiological conditions. This mutation is considered deleterious due to its impact on the protein's functional and structural properties.

Rs375270869 (G/A mutation) causes the conversion of arginine (R) to tryptophan (W) at position 162 (R162W). Tryptophan has a higher molecular weight than arginine and is neutral. This substitution disrupts the charge balance and is predicted to be damaging to the protein due to altered hydrophobicity and structural interactions.

Rs373999438 (C/T mutation) leads to the replacement of glycine (G) with arginine (R) at position 425 (G425R). Arginine has a higher molecular weight and is polar, whereas glycine is non-polar and lacks a substantial side chain. This mutation introduces a charged residue, which disrupts the local structural conformation, making it damaging to the protein.

Rs374427540 (C/T mutation) results in the substitution of alanine (A) with threonine (T) at position 368 (A368T). Threonine has a higher molecular weight than alanine and is polar due to its hydroxyl group, allowing it to form hydrogen bonds. Alanine is non-polar and cannot form such interactions. This mutation is deleterious because it alters the hydrophobicity and hydrogen bonding potential of the protein [19].

After identifying the nine mutations common across the seven tools, their impact on protein stability was evaluated using I-MUTANT2.0 and MUPRO. The I-MUTANT2.0 tool indicated that all nine mutations result in a decrease in protein stability. This aligns with the general understanding that missense mutations in conserved regions of proteins often destabilize their native structures, rendering them nonfunctional. MUPRO showed that eight of the nine mutations decrease protein stability, and one mutation, G425R, was found to increase protein stability, suggesting that this mutation might lead to a more compact protein structure. However, this does not necessarily indicate a functional gain, as increased stability could potentially hinder the protein's conformational flexibility or its ability to interact with substrates or other proteins, further complicating the interpretation of its biological effect. The effect of G425R warrants further experimental investigation, as in silico predictions alone may not fully capture the nuances of its impact on protein function.

However, the discrepancy observed in the G425R mutation may be attributed to the computational methods used by MUPRO, which include SVM and neural network approaches that incorporate additional structural or physicochemical features into their predictions. These methods may consider certain compensatory effects or unique interactions within the protein structure that stabilize the protein despite the mutation. Neural networks are designed to recognize complex patterns and may account for subtle, non-linear interactions in the protein structure that are missed by SVM or other simpler algorithms. For instance, the G425R mutation might induce specific structural changes, such as enhanced intramolecular interactions or improved packing within the protein, that offset the destabilizing effects typically caused by mutations. These stabilizing effects could be captured by the neural network's ability to analyze a broader range of protein features, leading to the prediction of increased stability for this mutation. However, this variation underscores the complexity of protein stability predictions and highlights the importance of using multiple tools to gain a more comprehensive understanding of mutation impacts. Further experimental studies, such as molecular dynamics simulations or thermal stability assays, are recommended to validate these computational predictions and elucidate the precise effect of the G425R mutation on protein stability.

The MEDUSA analysis was conducted to evaluate the flexibility of the protein, and the results revealed that the positions of all 9 SNPs were rigid, except R36C and G425R, which were flexible.

Rigid regions in a protein are often critical for maintaining structural integrity and ensuring proper folding and stability. Mutations in these regions are likely to disrupt the protein's core structure, leading to a loss of functional efficiency or complete misfolding. Such structural disturbances may explain the pathogenic nature of these mutations, as *FUCA1* plays a key enzymatic role, and any alteration in its stable regions could hinder its catalytic activity.

On the other hand, flexible regions are typically involved in dynamic processes, such as substrate binding, enzyme activation, or interactions with other biomolecules. Mutations in these regions might alter the dynamic behavior of the protein, leading to improper substrate accommodation or destabilization of transient conformations necessary for enzymatic function.

Conclusion

This study analyzed the effects of amino acid variations on α-L-fucosidase protein structure, function, and disease association. Among 438 mutations initially retrieved, nine were identified as commonly pathogenic across seven computational tools. Most mutations decrease protein stability, with one exception (G425R), which showed an increase in stability under specific conditions. Additionally, flexibility analysis using MEDUSA demonstrated that seven mutations were located in rigid regions of the protein, while two were found in flexible regions. These findings highlight the importance of computational approaches to understand the molecular basis of *FUCA1*-related diseases. Experimental validation is recommended to confirm the precise effects of these mutations and their contributions to disease phenotypes and guide future therapeutic strategies.

Informed Consent: The study is purely bioinformatics-based and does not involve any human subjects or patient data.

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Research Article

Machine learning-assisted prediction of positive urine cultures using urinalysis and hemogram data: A retrospective cohort study

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Abstract

Objectives: Urinary tract infections (UTIs) are common and often lead to unnecessary urine culture testing, increasing costs and delaying treatment. This study aims to develop a machine learning (ML) model using urinalysis and hemogram data to predict urine culture positivity and reduce unnecessary testing.

Methods: We retrospectively analyzed data from 12,433 patients who underwent urinalysis, urine culture, complete blood count, and CRP testing. After preprocessing and exclusion criteria, data were split into training, test, and validation sets. H_2O AutoML was employed to develop and evaluate various ML algorithms.

Results: The gradient boosting model demonstrated an AUC-ROC of 0.822 with high sensitivity (73.8%) and negative predictive value (90.4%), making it reliable in ruling out negative cases. Urinary leukocytes, nitrite, and bacterial count were identified as top predictors.

Conclusion: ML-based models can improve diagnostic accuracy and reduce unnecessary urine cultures. These models have the potential to be integrated into clinical workflows to enhance cost-effectiveness and minimize empirical antibiotic use.

Keywords: Artificial intelligence, diagnostic model, machine learning, predictive analytics, urinalysis, urinary tract infection, urine culture

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Urinary tract infections (UTIs) are among the most prevalent bacterial infections encountered in clinical practice, particularly affecting women. They impose a significant burden on both individual health and healthcare systems worldwide. If left untreated or inadequately managed, UTIs can lead to serious complications, including kidney damage and sepsis, while also contributing to the rise in antimicrobial resistance (AMR) rates [1, 2].

The diagnosis of UTIs involves a combination of clinical evaluation, laboratory tests, and advanced diagnostic techniques. Patients commonly present with symptoms such as dysuria, increased urinary frequency, urgency, and, in some cases, hematuria. Initial assessment typically includes microscopic urinalysis and dipstick testing, which together constitute standard urinalysis (UA). Although widely used, microscopic urinalysis is time-consuming and prone to human error. When combined

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with dipstick tests—specifically leucocyte esterase and nitrite assessments—diagnostic accuracy improves significantly [3, 4].

Despite the utility of these methods, urine culture remains the gold standard for UTI diagnosis. However, it requires a minimum turnaround time of 24 hours, and culture positivity is observed in only 50–80% of suspected UTI cases. This variability results from multiple factors, including symptom severity and duration, patient characteristics, sample quality, etiological diversity, and methodological differences in testing [5–7]. Additionally, urine cultures are often unavailable in primary care settings and are typically limited to hospital microbiology laboratories. Consequently, empirical antibiotic therapy is frequently initiated based on clinical presentation and urinalysis results, with treatment adjustments made following culture outcomes [8].

The emergence of artificial intelligence (AI) holds promise for enhancing diagnostic accuracy and efficiency, potentially reducing delays in UTI diagnosis. While traditional diagnostic methods remain essential, the integration of AI could revolutionize the field by delivering more rapid and precise results. Importantly, AI-based diagnostics should complement—not replace—standard laboratory approaches to ensure comprehensive patient care [9]. These models leverage parameters such as patient age, bacterial presence, and specific analytical markers to accurately classify negative samples, thereby optimizing laboratory workflows and potentially reducing diagnostic costs by up to €40,000 annually [10].

This study aims to apply machine learning algorithms to urinalysis data in order to predict the necessity of urine culture testing. By doing so, we seek to minimize unnecessary culture requests and improve the accuracy of treatment decisions, ultimately contributing to more efficient and cost-effective management of UTIs.

Materials and Methods

Study population / subjects

This study was conducted at Tepecik Education and Research Hospital. Patients who presented to this center and its affiliated hospital (AH) between January 1, 2023, and December 31, 2023, and underwent first-time urinalysis (UA), urine culture, complete blood count (CBC), and C-reactive protein (CRP) testing were included. The baseline characteristics of the study population are presented in Table 1. Patients with incomplete test results, missing sub-parameters, or urine cultures identifying non-bacterial agents were excluded from the study.

Urinalysis samples were analyzed using the Zybio Corporation U2610 (Chongqing, China), CBC samples with the Sysmex Corporation XN-1000 (Kobe, Japan), and CRP testing with the Beckman Coulter AU-5800 (California, USA).

For urine culture, midstream samples were collected in sterile containers concurrently with urinalysis and processed following standard microbiological procedures. Samples with no growth signal after 24 hours were incubated for an additional 48 hours. If no growth was observed, the result was reported as "no growth." All reagents and calibrators used were certified and obtained from their respective manufacturers. Quality control materials were sourced from Bio-Rad (California, USA).

Study design

Ethical approval was obtained from the Tepecik Training and Research Hospital Ethics Committee prior to study initiation (No: 2024/07-13, Date: 19/08/2024). This study was performed in accordance with the ethical standards set by the Declaration of Helsinki. Patient identifiers were anonymized, and a dataset including age, sex, CRP, CBC, urinalysis, and urine culture results from 13,475 patients (12,085 from the main building and 1,390 from the affiliated hospital) was compiled using Microsoft Excel 2021 (USA).

After applying exclusion criteria, the final dataset included 12,433 patients (11,189 from the main hospital and 1,244 from the affiliated hospital).

In dipstick testing, semi-quantitative parameters were encoded as follows: 'negative'=0, 'trace'=0.5, and values 1, 2, or 3 for increasing levels of positivity. Urine color and appearance variables were also recategorized by merging similar classes to enhance data standardization.

The cleaned dataset was randomly divided into training and testing sets using an 80:20 ratio with stratified sampling to preserve class distribution. An additional external test set was used to evaluate model generalizability. The subject flow is outlined in the Standards for Reporting Diagnostic Accuracy (STARD) diagram (Fig. 1).

Data preprocessing and training of machine learning algorithms

Patient results were first exported to Microsoft Excel for initial preprocessing. Cases with missing values were excluded. Urine cultures with bacterial growth exceeding 10,000 colony-forming units/mL (CFU/mL) were classified as positive. Mixed flora, colonization, or growth below this threshold were labeled negative. Outcomes were binary coded: Negative (0), Positive (1).

Dipstick test results—such as glucose, protein, and nitrite—were converted into binary values. The final dataset was analyzed in Python 3.10 using the H_2O AutoML framework (version 3.46) [11]. AutoML was chosen due to its ability to automate complex processes such as feature engineering, model selection, and hyperparameter tuning—especially valuable when the user lacks deep data science expertise. Despite its growing relevance, AutoML has rarely been applied in clinical laboratory contexts [12].

Fifteen machine learning algorithms were evaluated (Appendix 1). The model with the highest AUC (area under the curve) was selected. The final model was trained on the following variables:

- Demographic variables: Age, sex,
- Hematologic variables: WBC (white blood cells), neutrophil, lymphocyte, monocyte, eosinophil, basophil, hemoglobin,

Table 1. Characteristics of the study population (no analysis was performed for semi-quantitative tests)

Characteristics	Units	Central Hospital (n=11189) Mean±SD	Affiliated Hospital (n=1244) Mean±SD	Reference interval
Age (years)		38.30±27.55	38.18±28.11	
Male (min-max)		40.11±28.86 (0–99)	38.74±29.26 (0–95)	
Female (min-max)		37.27±26.72 (1–104)	27.85±27.42 (1–95)	
Sex, n (%)				
Male		4065 (36.3)	462 (37.1)	
Female		7124 (63.7)	782 (62.9)	
WBC	$10^3/\mu L$	8.56±5.35	8.45±3.28	3.91–8.77
Neutrophil	$10^3/\mu L$	5.07±3.58	5.08±2.95	1.78–5.38
Lymphocyte	$10^3/\mu L$	2.56±3.68	2.46±1.34	0.85–3.0
Monocyte	$10^3/\mu L$	0.68±0.44	0.67±0.31	0.2–0.8
Eosinophil	$10^3/\mu L$	0.21±0.24	0.20±0.20	0.1–0.4
Basophil	$10^3/\mu L$	0.04±0.06	0.04±0.04	0.02–0.1
Hemoglobin	g/dL	12.23±1.89	12.19±1.86	11.9–15.4
Urine density	---	1018±0.007	1016±0.007	1010–1030
pH (urine)	---	5.96±0.77	5.99±0.77	5–9
Bacteria (urine)	/HPF	39.89±152.94	39.15±150.01	0–5
Leucocyte (urine)	/HPF	48.79±230.56	51.41±257.35	0–4
Yeast (urine)	/HPF	0.47±7.53	0.47±4.61	0
C-reactive protein	mg/L	10.42±40.57	8.40±33.88	0–5
Urine culture results	Train (+/-)	6700/2003 (77/23%)		
	Test (+/-)	1913/573 (77/23%)	958/286 (77/23%)	

SD: Standard deviation; min: Minimum, max: Maximum; WBC: White blood cells; HPF: High power field.

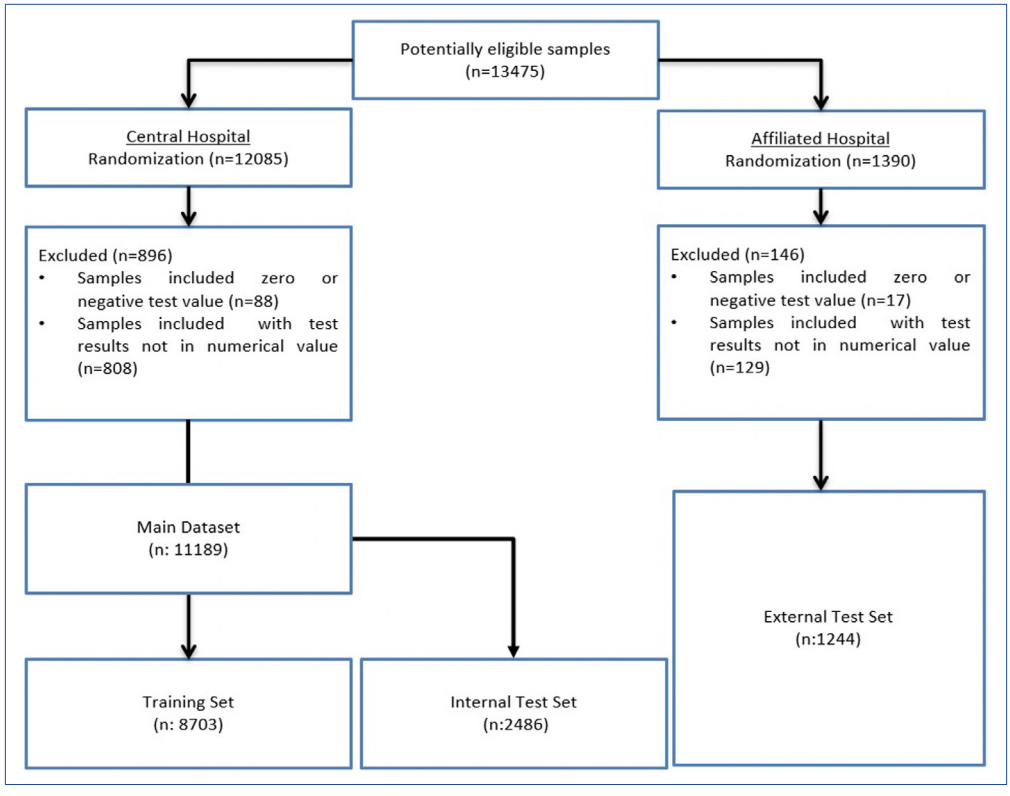

Figure 1. The standards for reporting diagnostic accuracy diagram.

- Urine dipstick variables: Appearance, urobilinogen, bilirubin, nitrite, ketone, leucocyte esterase, glucose, protein, pH, blood,
- Other urinalysis variables: Urine color, urine density, cylinder, mucus,
- Flow cytometry variable: Bacteria count, leucocyte count, yeast count.

Following model training, performance evaluation was conducted using the test dataset.

Performance evaluation

Scikit-learn, Pandas, NumPy, Shap, StatsModels, $H_2$0.automl and Matplotlib/Seaborn—among Python's most robust libraries for machine learning and statistical analysis—were employed in this project. The modeling process underwent comprehensive evaluation, including hyperparameter tuning and model selection through internal cross-validation. Model performance was assessed using multiple evaluation metrics. The following criteria were used for classification:

1. Classification performance metrics
    - Area Under the Receiver Operating Characteristic Curve (AUC-ROC),
    - Area Under the Precision-Recall Curve (AUC-PR),
    - Confusion matrix analysis,
    - Sensitivity, Specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV), Positive Likelihood Ratio (PLR), Negative Likelihood Ratio (NLR) F1 score, odds ratio.
2. Model interpretability metrics
    - Feature importance analysis,
    - SHAP (Shapley Additive Explanations) graphs.
3. Validation results of the predictive models were analyzed to ensure a comprehensive assessment. This structured and multifaceted evaluation approach provides a robust framework for predicting treatment modality outcomes based on laboratory-derived data. The algorithm was further validated using data from the affiliated hospital (external test set), which, while functioning as a distinct clinical site, operates under the same institutional umbrella as the central hospital. This approach aligns with the IFCC recommendations for assessing model generalizability across internal institutional subpopulations [13].

Results

Dataset description and data pre-processing

The dataset used in this study included a total of 11.189 records, consisting of 8.703 entries in the training set, 2.486 in the internal test set, and an additional 1.244 records in the external set. All datasets contained urinalysis and hemogram parameters alongside demographic data, allowing for comprehensive baseline characterization.

Baseline demographic characteristics of the study population are presented in Table 1. The mean age was 38.30±27.63 years in the training set, 38.33±27.28 years in the internal test set, and similar in the external test set at 38.18±28.11 years ($p>0.05$). When stratified by sex, male participants were slightly older than female participants within each subset ($p>0.05$).

Regarding sex distribution, males comprised 36.2% of the training set, 36.7% of the internal test set, and 37.1% of the external test set, while females made up 63.8%, 63.3%, and 62.9%, respectively. These differences were not statistically significant ($p=0.783$), indicating a relatively balanced gender distribution across the subsets.

Descriptive statistics for hemogram and urinary biomarkers are presented in Table 1. Most variables showed no statistically significant differences between the Central and Affiliated Hospital datasets—including blood WBC, neutrophil, monocyte, eosinophil, basophil, hemoglobin, CRP, urine density, pH, bacteria count, urinary leucocytes, and yeast (all $p>0.05$). Although a statistically marginal difference in lymphocyte counts was observed ($p=0.046$), the magnitude of difference was too small to be clinically meaningful. Overall, this observed homogeneity across subsets supports the robustness and comparability of subsequent analyses and model validation.

The performance of H_2O AutoML was comparatively evaluated based on predictive capabilities, classification metrics, and interpretability. Classification metrics such as F1 score, sensitivity, specificity, and AUC-ROC were used to assess the models' ability to discriminate between classes.

Comparison of classification performance metrics

The H_2O AutoML framework was employed to systematically explore a wide range of algorithms and hyperparameter configurations. Among the candidate models generated, a Gradient Boosting Machine (GBM) emerged as the most performant, striking an optimal trade-off between discrimination and calibration metrics—specifically AUC-ROC and log loss. The selected model (ID: GBM_1_AutoML_12_20250410_211225) achieved an AUC-ROC of 0. 0.818 and a log loss of 0.399, indicating both high classification accuracy and well-calibrated probabilistic outputs.

The performance metrics of the internal test set was summarized in Tables 2 and Figure 2a–c. The model achieved balanced classification performance with a sensitivity and specificity of 73.8%, and a high negative predictive value (NPV) of 90.4%, indicating strong reliability in ruling out negative cases. The positive predictive value (PPV) was 45.8%, and the resulting odds ratio of 7.95 further supported its overall discriminative capacity. Accuracy reached 73.8%, with an F1 score of 0.565, reflecting a moderate balance between precision and recall.

As visualized in Figure 2a, the model demonstrated an AUC-ROC of 0.822 and an AUC-PR of 0.649, confirming strong discriminative ability, particularly under class imbalance. The confusion matrix (Fig. 2c) further supports this consistent performance, underscoring the model's applicability in clinical diagnostic settings.

Table 2. Performance metrics of internal test set and external test set

Set	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Positive likelihood ratio	Negative likelihood ratio	Odds ratio	Accuracy	F1 score
Internal test set	0.738	0.738	0.458	0.904	2.819	0.355	7.948	0.738	0.565
External test set	0.738	0.735	0.453	0.904	2.783	0.357	7.798	0.736	0.562

External test set results of the models

In accordance with the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommendations, model validation was performed using data from the affiliated hospital to assess generalizability across institutional subpopulations. The results, summarized in Table 2 and Figure 2b, c, were consistent with the test sets, supporting the model's robustness and external applicability. Sensitivity and specificity were 73.8% and 73.5%, respectively, with a PPV of 45.3% and NPV of 90.4%, reflecting balanced

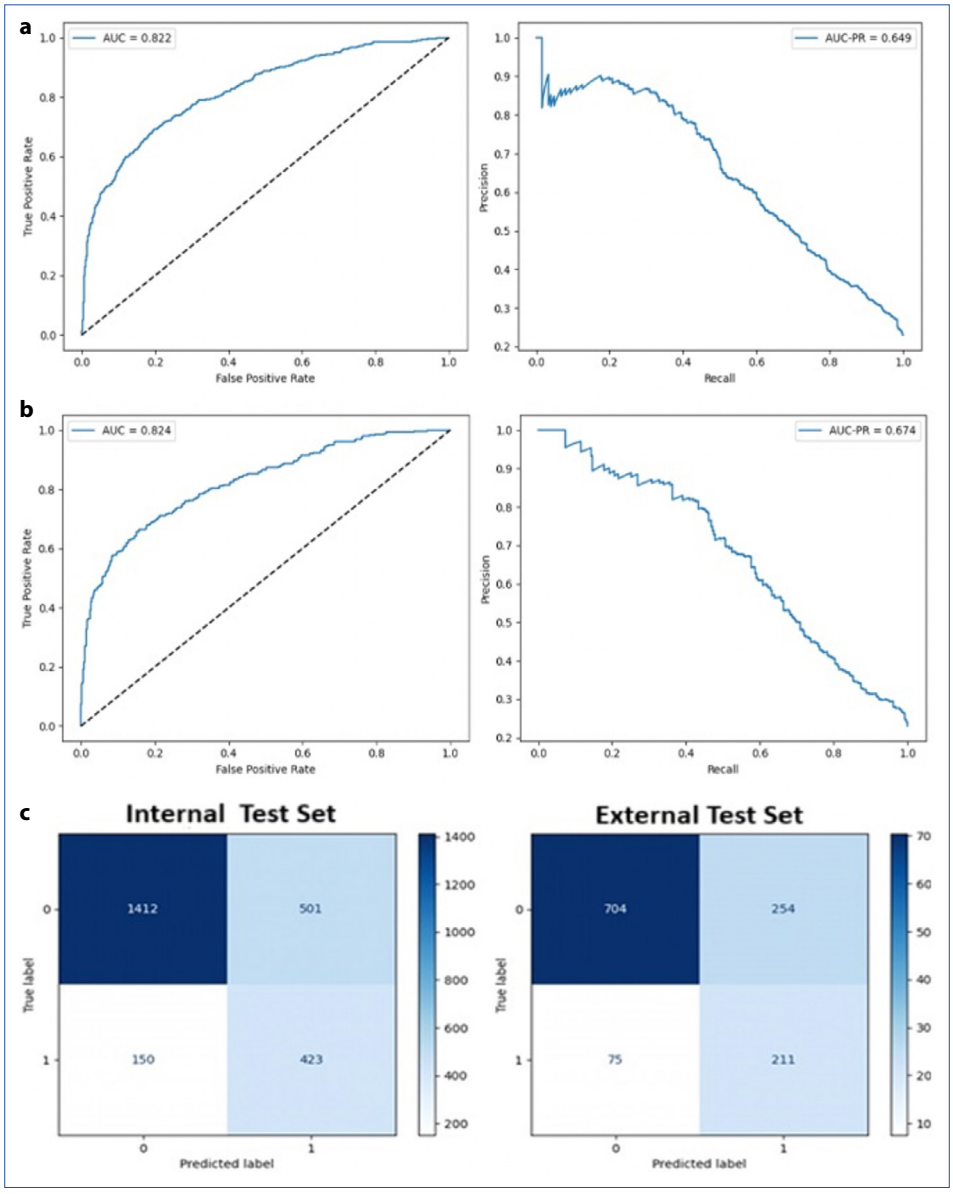

Figure 2. Performance outputs for our model. (a) AUC-ROC and AUC-PR graph for internal test set. (b) AUC-ROC and AUC-PR graph for external test set. (c) Confusion matrixes for internal and external test sets.

AUC: Area under the curve; ROC: Receiver operating characteristic; PR: Precision-recall.

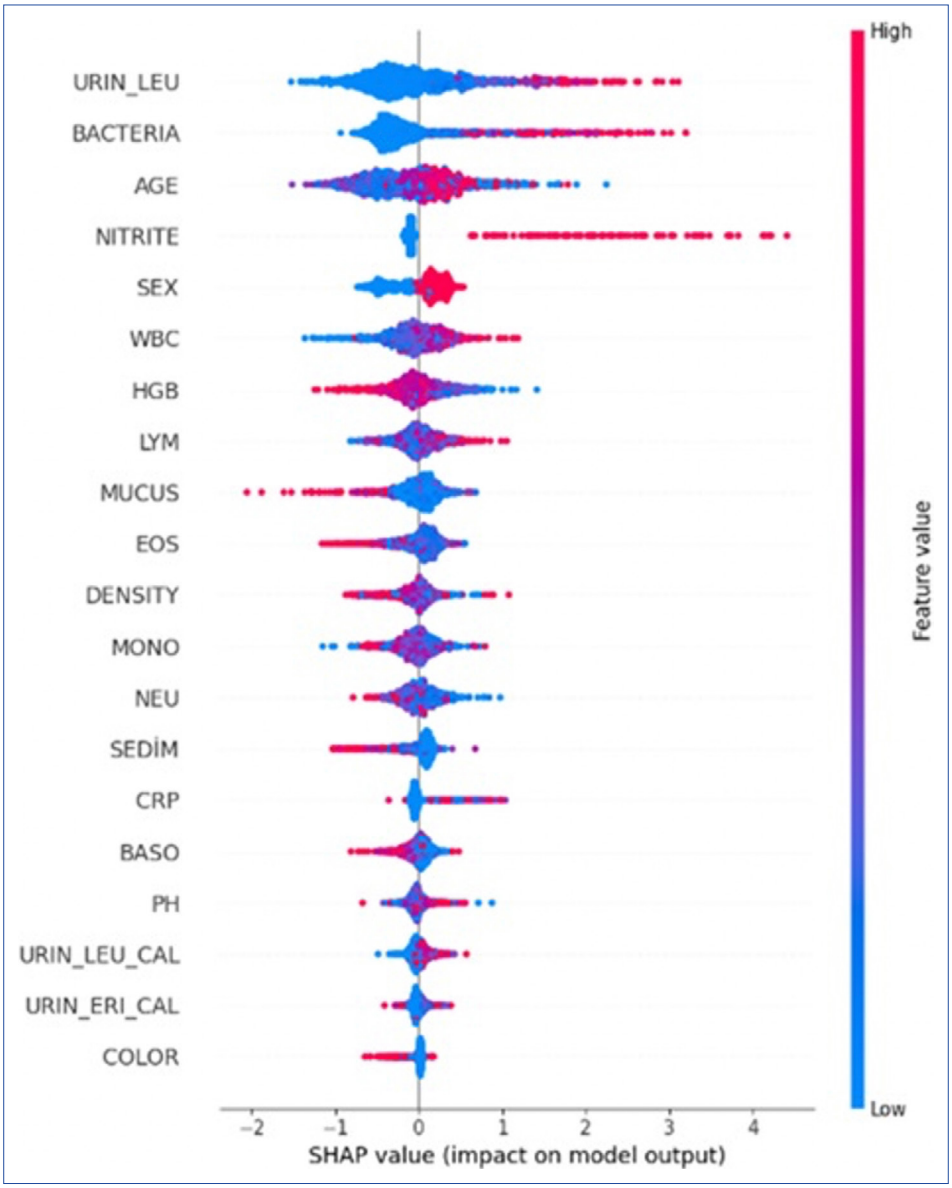

Figure 3. SHAP plot of our model.
SHAP: Shapley additive explanations.

classification and reliable identification of negative cases. The odds ratio (7.80), accuracy (73.6%), and F1 score (0.562) reinforced overall diagnostic utility.

As shown in Figure 2b, the model achieved an AUC-ROC of 0.824 and an AUC-PR of 0.674, slightly outperforming the test set and confirming its strong discriminative power. The confusion matrix (Fig. 2c) reflected similar prediction patterns, underscoring the model's consistency, generalizability, and clinical relevance within real-world healthcare settings.

Interpretability and threshold-based diagnostic performance of key variables

SHAP value analysis in Figure 3 highlights the most influential features driving the model's predictions. Urinary leucocyte (URIN_LEU) levels and bacterial count ranked highest, with elevated values strongly associated with increased likelihood of a positive classification—consistent with their well-established clinical importance in urinary tract infections. Age and nitrite also demonstrated substantial impact, particularly at higher levels. Moderate contributions were observed for sex, WBC, hemoglobin, and lymphocyte count, indicating context-specific influence on prediction.

Features such as urine pH, CRP, basophil, and sediment parameters had lower SHAP importance, though they may hold relevance in certain clinical subgroups. The distinct SHAP distributions reinforce the model's interpretability and alignment with biological plausibility.

Complementing SHAP analysis, Table 3 compares ROC-based and model-based diagnostic metrics for key infection-related variables commonly referenced in clinical

Table 3. Diagnostic metrics of key biomarkers

Variable	ROC cut-off	Sensitivity (ROC)	Specificity (ROC)	PPV (ROC)	NPV (ROC)	Sensitivity (model)	Specificity (model)	PPV (model)	NPV (model)
Leucocyte count (URIN_LEU)	12	0.561	0.906	0.781	0.776	0.563	0.819	0.482	0.863
Bacteria count	12	0.469	0.882	0.703	0.736	0.552	0.841	0.510	0.863
Leucocyte esterase (URIN_LEU_CAL)	1	0.688	0.804	0.677	0.812	0.668	0.706	0.404	0.877
Nitrite	1	0.198	0.997	0.979	0.676	0.266	0.981	0.809	0.817
Age	57	0.535	0.784	0.57	0.739	0.528	0.722	0.362	0.837

ROC: Receiver operating characteristic; PPV: Positive predictive value; NPV: Negative predictive value.

practice. Urinary leucocytes (URIN_LEU) demonstrated high ROC-based specificity (0.906) and PPV (0.781), alongside a strong model-based NPV (0.863), confirming its value in ruling out infection.

Leucocyte esterase (URIN_LEU_CAL), a point-of-care proxy for white blood cells, achieved robust standalone diagnostic performance (ROC sensitivity 0.688, specificity 0.804) and retained value in the model (NPV 0.877). Similarly, urinary bacteria showed high specificity (0.882) and consistent predictive power across both methods.

Although nitrite yielded an exceptionally high PPV (0.979) with ROC thresholds, its low sensitivity (0.198) and modest model contribution suggest it should be interpreted alongside complementary features. Age demonstrated moderate discriminative performance but provided consistent support across methods.

Together, these findings emphasize that routinely used clinical biomarkers—especially leucocyte esterase and urinary leucocytes—retain both individual and integrative predictive value within threshold-based and machine learning-driven diagnostic frameworks.

Discussion

This study evaluated the usability of machine learning models based on urinalysis in predicting the necessity of urine culture and classifying potential positive cases. The findings align with the literature supporting the clinical use potential of AI-based approaches in diagnosing urinary tract infections (UTIs).

Although symptomatology was not available in the dataset, the classification approach using $\geq 10^4$ CFU/mL as the threshold for positive urine cultures proved effective in the context of microbiological diagnostics. Notably, the model demonstrated strong performance in differentiating cases based on laboratory parameters alone. The high predictive contribution of urinary bacterial count suggests that the model could successfully identify patterns indicative of asymptomatic bacteriuria, catheter-associated infections, and uncomplicated cystitis. This outcome implies that even in the absence of clinical symptoms, machine learning algorithms can leverage routine urinalysis to support diagnostic differentiation across varied patient subgroups.

AI-based urinalysis risk scores have been shown to accelerate and improve diagnostic accuracy by predicting the need for culture testing, thereby reducing unnecessary diagnostics [14]. Integrating highly specific predictive models into clinical practice may help rationalize empirical antibiotic use and support more targeted treatment strategies [15]. By assessing key parameters such as leucocyte esterase, nitrite, and bacterial count, these systems offer significant cost savings for healthcare systems [16]. Future integration into clinical decision support systems is expected to enhance diagnostic workflows, improve patient safety, and contribute to infection control and the fight against antibiotic resistance. Recently, machine learning algorithms—particularly logistic regression, support vector machines, random forests, and deep learning—have shown promise in early UTI diagnosis by improving accuracy and reducing false positives, thus limiting unnecessary antibiotic use [3, 6, 7, 10]. The class imbalance observed in the dataset—characterized by a predominance of negative urine cultures—is a common feature in clinical laboratory data. To address this, stratified sampling was employed to ensure balanced class representation across all data splits. Furthermore, the use of H_2O AutoML provided automated internal handling of imbalance-related challenges and hyperparameter optimization, supporting reliable model calibration without the need for external resampling techniques. This workflow reflects real-world diagnostic settings and contributes to the model's practical applicability. These attributes collectively make H_2O AutoML an ideal framework for addressing the complexities of clinical laboratory data.

However, traditional studies have also reported significant findings on the diagnostic accuracy of urinalysis and microbiological tests. Price et al. [5] demonstrated that 30% of urine culture-positive patients were initially misdiagnosed as negative using dipstick tests. Similarly, Williams et all.'s [6] meta-analysis found that rapid urine tests had a sensitivity of 53–65% and a specificity of 85–90%.

In our study, CRP testing did not emerge as one of the most valuable features. It is well known that CRP levels do not typi-

cally rise in lower urinary tract infections due to the absence of a systemic inflammatory response [17]. Since patients in this study were not grouped based on symptoms or diagnosis, this phenomenon could not be explicitly explained.

Comparison of model performance with traditional methods

The best-performing model, GBM_1_AutoML_12_20250410_211225, demonstrated strong predictive performance on the test sets, with a sensitivity of 73.8%, specificity of 73.8%, positive predictive value (PPV) of 45.8%, negative predictive value (NPV) of 90.4%, positive likelihood ratio (PLR) of 2.82, negative likelihood ratio (NLR) of 0.36, an F1 score of 56.5%, and accuracy of 73.8%. The sensitivity of our model was lower compared to Heytens et al.'s [18] PCR-based analysis (70%).

In traditional studies, urinalysis-based diagnostic tests are often compared to urine cultures. Hooton et al. [19] reported that standard urinalysis in female patients had a sensitivity of 50–60% and specificity of 80–90%. Huysal et al. [20] found that routine laboratory tests had a sensitivity of 47% and specificity of 91.1%. Gupta et al. [21] indicated that dipstick tests were sufficient in terms of specificity for UTI diagnosis but had lower sensitivity (45–55%). These findings suggest that our model exhibits similar sensitivity to traditional methods but slightly lower performs them in specificity.

The reasons for these differences include the structural characteristics of the dataset used, variations in the patient population, and different preprocessing steps. Additionally, while traditional methods primarily employ univariate analyses, our model is based on multivariate analyses, which may result in higher sensitivity but lower specificity.

Comparison with other machine learning models

When compared with other machine learning models, Li et al.'s [22] machine learning-based models exhibited AUC-ROC values ranging from 0.68 to 0.97, sensitivity between 63–90%, and specificity between 69–86% [22]. The study by Burton et al. [23] in different patient groups reported an AUC-ROC value of 0.90, sensitivity ranging from 70–90.7%, specificity between 52–89%, accuracy of 63–85%, PPV of 40–71%, and NPV of 90–97%.

Seheult et al. [24] conducted studies using decision tree algorithms in different age groups, reporting an AUC-ROC range of 0.79–0.48, with an average sensitivity of 82.4%, specificity between 52–89%, accuracy of 65.8%, PPV of 46.3%, and NPV of 91.3%.

Flores et al. [25] developed a model combining neural networks and random forest algorithms, achieving an AUC-ROC of 0.81–85, sensitivity of 78–87%, specificity of 83%, accuracy of 80–85%, PPV of 86–83%, NPV of 74–87%, PLR of 4.6–5.07, and NLR of 0.26–0.16. Yen et al. [26] found the AUC-ROC value to be 0.83, sensitivity 88%, specificity 59%, accuracy 69.1%, F1 Score 65.2% in their study to identify high-risk patients with urinary tract infections that may cause critical outcomes in the emergency department. These studies had similar performance metric rates to our study.

While the model demonstrated high negative predictive value (NPV≈90%), which supports its utility as a rule-out tool, the relatively modest positive predictive value (PPV≈45%) indicates that a substantial number of predicted positive cases may not correspond to true infections. This imbalance raises important clinical considerations, particularly in the context of antibiotic stewardship and avoiding unnecessary interventions. However, we would like to emphasize that, from a patient safety perspective, missing a true infection (undertreatment) is clinically more critical than administering antibiotics unnecessarily. The potential financial and antimicrobial burden associated with overtreatment may be considered an acceptable trade-off when weighed against the risk of clinical deterioration due to untreated urinary tract infections.

Given the model's relatively modest positive predictive value (PPV≈45%) and moderate F1 score, its primary clinical utility may currently lie in ruling out infections rather than confirming them. The high negative predictive value (NPV≈90%) supports its role as a screening tool to exclude unnecessary culture testing in low-risk cases. Therefore, the model may be more effective as a "rule-out" aid in diagnostic workflows, helping reduce the burden of unwarranted laboratory procedures and antibiotic prescriptions until further improvements increase its confirmatory strength.

This trade-off between high NPV and lower PPV is common in diagnostic screening tools and reflects the real-world prevalence and distribution of urinary tract infections. Future improvements—such as threshold tuning, inclusion of symptom-based variables, or integration of additional inflammatory or microbiological markers—may enhance PPV without significantly compromising sensitivity, thereby expanding the model's practical applicability in clinical decision-making.

The model's interpretability, as assessed through SHAP analysis, revealed strong alignment with clinical intuition. Key variables such as urinary leucocytes, leucocyte esterase, and bacteria—which are already central to UTI diagnosis—emerged as the top contributors to prediction outcomes. Importantly, these features not only performed well in data-driven ML ranking but also retained their diagnostic strength under traditional ROC-based threshold analysis. This convergence highlights the potential of interpreting ML tools to bridge conventional clinical reasoning with algorithmic decision-making. The marginal performance of nitrite—despite high PPV but low sensitivity—further illustrates the value of multivariate modeling, where limitations of individual biomarkers can be mitigated by their collective interactions. These findings underscore the feasibility of using ML not just for black-box prediction, but as a transparent, clinically synergistic tool to enhance diagnostic efficiency in routine care.

Although urinalysis parameters are traditionally the primary focus in urinary tract infection diagnostics, this study also incorporated hemogram data to evaluate its additive predictive

value. Variables such as white blood cell count, lymphocyte percentage, and hemoglobin contributed moderately to the model's predictions according to SHAP analysis. While these parameters did not emerge as top-ranking features, their inclusion slightly improved the model's performance and may reflect systemic inflammatory responses in certain patient subgroups.

Notably, hemogram data are rarely emphasized in prior machine learning models for UTI detection. Our findings suggest that, although not dominant predictors, hematologic variables offer supplementary information that can enhance model robustness, particularly when urinalysis results are borderline or ambiguous. This reinforces the potential role of composite laboratory data in improving infection risk stratification through interpretable AI.

In studies conducted in recent years, AUC-ROC range, PPV/NPV, PLR/NLR balance were generally observed similar to our study. In contrast to this, although there are studies similar to the sensitivity/specificity balance in our study, there are studies that declare the opposite of these values. The main reasons for these differences include the variable selection of different machine learning models, hyperparameter optimization strategies, and the scope of the dataset used to train the model. Some studies included more clinical variables, while our study used only specific biomarkers. Furthermore, while some studies turn to deep learning methods, our model is based on traditional machine learning algorithms.

Limitations

This study has several limitations. First, as a retrospective analysis, the model's performance may vary across demographic subgroups and disease severity levels. Notably, females exhibited higher urine culture positivity rates than males across all subsets, as shown in Appendix 2, suggesting the need for future sex-stratified performance evaluations. Secondly, only urinalysis and basic hematologic data were used; clinical history, symptoms, and additional biomarkers were not included. Incorporating such features may improve model accuracy and applicability. Lastly, the integration of machine learning models into real-time clinical decision support systems and evaluation of their clinical impact remain essential future steps.

Conclusion

Our study shows that machine learning-based models can be effective in the early diagnosis of urinary tract infections. Although the sensitivity of our model is lower compared to some studies, its specificity is quite high. This suggests that the model can prevent unnecessary antibiotic use by reducing false positives. Future studies should test the model in different patient groups, add symptomatic data and validate it in real-time clinical applications. The recommendations of the IFCC working group on the application of artificial intelligence in laboratory medicine also suggest cautious application of these technologies in a clinical context. In this context, additional studies are needed to improve the usability of machine learning models in the hospital setting.

Ethics Committee Approval: The study was approved by the Tepecik Training and Research Hospital Non-interventional Ethics Committee (no: 2024/07-13, date: 19/08/2024).

Informed Consent: Informed consent was obtained from all participants.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Authorship Contributions: Concept – F.D., D.I.T.; Design – F.D., D.I.T.; Supervision – F.D., I.A., Y.A.; Materials – F.D., I.A., Y.A.; Data collection and/or processing – F.D., I.A., Y.A.; Data analysis and/or interpretation – F.D., D.I.T.; Literature search – F.D., D.I.T.; Writing – F.D., D.I.T.; Critical review – F.D., D.I.T.

Peer-review: Externally peer-reviewed.

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Appendix 1

	Model_id	AUC	Logloss
1	GBM_1_AutoML_12_20250410_211225	0.818256	0.398633
2	GBM_5_AutoML_12_20250410_211225	0.816776	0.400571
3	GBM_2_AutoML_12_20250410_211225	0.816195	0.402202
4	XGBoost_grid_1_AutoML_12_20250410_211225_model_1	0.815258	0.400285
5	GBM_grid_1_AutoML_12_20250410_211225_model_1	0.811711	0.402868
6	GBM_3_AutoML_12_20250410_211225	0.809752	0.407616
7	DRF_1_AutoML_12_20250410_211225	0.809508	0.442237
8	XGBoost_3_AutoML_12_20250410_211225	0.808496	0.471458
9	XGBoost_grid_1_AutoML_12_20250410_211225_model_2	0.807497	0.409993
10	GBM_4_AutoML_12_20250410_211225	0.807266	0.415546
11	XGBoost_grid_1_AutoML_12_20250410_211225_model_3	0.805151	0.414008
12	GBM_grid_1_AutoML_12_20250410_211225_model_2	0.804209	0.419474
13	XRT_1_AutoML_12_20250410_211225	0.800781	0.445000
14	XGBoost_1_AutoML_12_20250410_211225	0.794679	0.475173
15	DeepLearning_grid_1_AutoML_12_20250410_211225_...	0.794134	0.434181

H_2O AutoML models and performance metrics. AUC: Area under the curve.

Appendix 2

Dataset-sex	Negative (n)	Positive (n)	Negative (%)	Positive (%)
Train set-male	2571.0	582.0	81.54	18.46
Train set-female	4129.0	1421.0	74.40	25.60
Internal test set-male	736.0	176.0	80.70	19.30
Internal test set-female	1177.0	397.0	74.78	25.22
External test set-male	374.0	88.0	80.95	19.05
Externa test set-female	584.0	198.0	74.68	25.32

Urine culture distribution by sex and dataset.

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Research Article

The serum NF-κB and adiponectin levels in patients with acute pancreatitis

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Abstract

Objectives: This study aimed to evaluate whether serum nuclear factor-kappa B (NF-κB) and adiponectin levels can provide insight into disease progression and serve as potential biomarkers for predicting disease severity in patients with acute pancreatitis (AP).

Methods: A total of 49 patients diagnosed with AP and admitted to the Emergency Department of Gaziosmanpaşa Training and Research Hospital were enrolled. An age-matched control group of 49 healthy individuals without AP was also included. Serum levels of NF-κB and adiponectin were measured and compared between groups.

Results: Patients with AP exhibited significantly elevated serum NF-κB levels and reduced adiponectin levels compared to the control group (both $p<0.001$). A strong negative correlation was observed between adiponectin and NF-κB levels in the AP group ($r=-0.865$, $p<0.05$). Receiver operating characteristic (ROC) analysis determined the optimal cut-off value for adiponectin as 3.4, with a sensitivity and specificity of 1.000. The optimal cut-off for NF-κB was 1.8, with a sensitivity of 1.000 and specificity of 0.96.

Conclusion: The findings suggest that serum NF-κB and adiponectin levels may be valuable biomarkers for assessing disease severity in AP. Their combined use or integration with existing scoring systems could enhance prognostic accuracy. Further experimental and clinical studies are necessary to evaluate the therapeutic potential of adiponectin and to validate these biomarkers for routine clinical use.

Keywords: Acute pancreatitis, adiponectin, disease severity, inflammation, nuclear factor kappa B

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Acute pancreatitis (AP) is a serious condition marked by sudden inflammation of the pancreas, which can result in significant complications and may necessitate intensive care. The disease is defined by an inflammatory response that causes swelling and bleeding in pancreatic tissue. In severe instances, it may lead to the necrosis of pancreatic tissue due to premature enzyme activation within the organ [1]. It is often associated with gallstones and excessive alcohol consumption. AP starts with the initiation of the process of autodigestion, which leads to the pancreas digesting itself, resulting in damage to tissues through activation of enzymes. Clinically, AP can present in both mild and severe forms; the severe form carries a risk of developing systemic inflammatory response syndrome (SIRS) and multiple organ failure [2].

Gallstones and chronic alcohol abuse account for approximately two-thirds of cases in the etiology of acute pancreatitis. Other common causes include the development of AP following endoscopic retrograde cholangiopancreatography

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(ERCP) and hypertriglyceridemia [3]. Recent studies have demonstrated that metabolic abnormalities such as diabetes, hypertriglyceridemia, morbid obesity, and vitamin D deficiency are closely associated with the severity and prognosis of AP [4]. Since insulin resistance (IR) is a chronic, low-grade inflammatory state, it is hypothesized that IR plays a pathogenic role in other inflammatory diseases such as AP. This hypothesis is supported by evidence that pre-existing diabetes increases the risk of developing AP and progression to severe AP by increasing local and systemic complications in AP [5].

Nuclear Factor-B (NF-κB) is a modulator of cytokine receptors and adhesion biological activity. Recent research has shown that NF-κB is known as a transcription factor that controls cell apoptosis, adaptive immunity, cell proliferation and aging, as well as regulating gene expression of autoimmune molecules [6]. The kinase cascade mediates activation of NF-κB, a transcription factor commonly associated with activation of inflammation genes, and other signal transducers and activators of transcription that have been shown to lead to overexpression of inflammation genes [7] in pancreatic acinar cells [8]. Activation of NF-κB is increased in pancreatic cells during AP and exacerbates the inflammatory process. Especially in severe cases of AP, activation of NF-κB leads to excessive release of cytokines. This leads to SIRS and increases tissue damage. NF-κB plays is crucial in the pathogenesis of AP and is being investigated as an important biomarker for prediction in the course of the disease [9].

Adiponectin is a 30-kDa adipocyte-specific protein factor, and its primary function is to improve insulin sensitivity. Furthermore, this adipokine has anti-inflammatory properties through inhibition of macrophage differentiation resulting in protection of many organs [10]. In experimental studies have revealed the role of adiponectin in the pathogenesis and clinical course of AP [11–14].

Serum NF-κB and adiponectin levels are associated with the severity of acute pancreatitis and may serve as potential biomarkers for predicting disease progression. This hypothesis is based on the pro-inflammatory role of NF-κB and the anti-inflammatory, insulin-sensitizing properties of adiponectin. To our knowledge, no human study has evaluated these markers together in AP.

NF-κB was selected due to its central role in mediating inflammatory responses and its known activation in pancreatic acinar cells during AP, contributing to disease severity. Adiponectin was chosen for its anti-inflammatory and insulin-sensitizing effects, which may counteract the inflammatory cascade in AP. Together, these biomarkers represent opposing regulatory mechanisms that may influence the progression and outcome of the disease. However, in the literature review, no study was found that investigated serum NF-κB and serum adiponectin levels together in AP in humans. Therefore, this study aimed to investigate whether circulating NF-κB and adiponectin can offer insights into the progression of the disease and if these markers can be used to predict the severity of AP in patients.

Materials and Methods

Subject groups

This study was approved by the Biruni University Faculty of Medicine Ethics Committee (Date: 12/12/2024, Decision No: 2024-BİAEK/05-04), adhered to the principles outlined in the Declaration of Helsinki. All participants, including both patients and control subjects, were fully briefed about the study, and written informed consent was provided by the patients or their family members. Helsinki declaration in the main text.

A total of 49 patients permitted to the Emergency Department of Gaziosmanpaşa Training and Research Hospital and diagnosed with AP were enrolled in the study. Patients admitted to our emergency surgery outpatient clinic with abdominal pain and diagnosed with AP according to clinical and laboratory findings were prospectively evaluated.

AP is present: The sudden onset of intense upper abdominal pain that may radiate to the back, a marked increase in serum lipase or amylase levels (more than three times the normal range), and radiological evidence that suggests the presence of AP. Patients were diagnosed with AP according to the Revised Atlanta Criteria [15].

The control group consisted of age- and sex-matched healthy individuals who presented to the same institution for routine health check-ups during the same period and had no known chronic diseases or regular medication use.

Inclusion criteria

Patients aged 18 and above presented with abdominal pain, nausea, and vomiting, and met the criteria for acute pancreatitis (clinical signs of AP, amylase-lipase levels at least three times higher than normal, and the presence of at least two radiologic compatibility criteria) were included.

Exlusion criteria

Patients who did not consent to participate, those under 18 years old, and individuals with comorbidities (such as diabetes, renal failure, heart failure, advanced liver failure, recent myocardial infarction or stroke, major surgery within the last 6 months, or pregnancy) were excluded.

Patients' complaints, diseases, presence of exclusion criteria, duration/when complaints started, laboratory results and vital signs were documented on data collection forms before hospitalization. All patients diagnosed were admitted to hospital.

According to the post-hoc power analysis performed using G*Power 3.1.9.2, the statistical power of the independent samples t-test comparing the two groups (n_1=49, n_2=49) was calculated as 95% (1–β=0.95) at a significance level of α=0.05, assuming an effect size of Cohen's d=0.7.

Sample collection and measurements

For patients who presented to the emergency department and met the criteria for AP, antecubital venous access was established, and blood samples were collected for hemogram

and biochemistry. Imaging (hepatobiliary ultrasonography and abdominal computed tomography) was performed on patients with a more than three-fold increase in amylase/lipase levels. Blood samples were centrifuged at 4000 rpm for 10 minutes at 4°C. Biochemical tests were performed right away, and serum aliquots for additional parameters were stored at -80°C for later analysis.

Measurement of serum Nuclear Factor Kappa B (NF-κB) levels

Serum NF-κB levels sandwich human enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (MyBioSource, Cat. No: MBS724341, MyBioSource, Inc., San Diego, CA, USA). Optical density was read with a microtitre plate photometer at 450 nm. The concentration was determined by interpolation from calibration curves prepared with standard samples provided by the manufacturer. The intra-assay and inter-assay coefficients of variation for NF-κB were <10% and <10%, respectively.

Measurement of serum Adiponectin levels

Serum adiponectin levels competitive-ELISA Kit according to the manufacturer's instructions (Quantikine® Human Adiponectin, R&D Systems, Inc., Minneapolis, MN). with detection limit of, respectively, 0.246 ng/mL. Optical density was read with a microtitre plate photometer at 450 nm. The concentration was determined by interpolation from calibration curves prepared with standard samples provided by the manufacturer. The intra-assay and inter-assay coefficients of variation for adiponectin were <10% and <10%, respectively.

The complete blood count (CBC) was recorded with an automatic hematology analyzer (Sysmex, Sysmex XN-1000, Norderstedt, Germany). Biochemical parameters were determined using the enzymatic methods (COBAS 8000, ROCHE-2007, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using SPSS v.26 (IBM Corp., Armonk, NY, USA). Categorical variables were expressed as counts (n) and percentages (%), while continuous variables were presented as mean±standard deviation. Data distribution normality was evaluated using the Kolmogorov-Smirnov test, Q-Q plots, and histograms. The Pearson chi-square test was used for comparing categorical variables, while the Mann-Whitney U test and Student's T-test were applied for comparisons between two independent groups. Spearman's correlation test assessed relationships among numerical variables. Laboratory parameters were categorized based on Receiver Operating Characteristic (ROC) curve analysis, with the optimal cutoff point determined by maximizing sensitivity and specificity. The Youden index was used to identify the best cutoff points, and the area under the curve (AUC) was reported. Confidence intervals were calculated using the Bootstrap method. (Bootstrap method performs multiple resampling (bootstrapping) to evaluate the performance of the model, calculates AUROC for each sample and then gives confidence intervals). Multivariate logistic regression (Enter and Forward: LR) analysis was performed to identify risk factors associated with AP. The goodness-of-fit of the model was evaluated using the Hosmer-Lemeshow test and highly correlated variables are not included in the model. The significance level for statistical tests was set at $p<0.05$.

Results

The sociodemographic and clinical characteristics of participants in both the patient and control groups are shown in Table 1. In this study, 49 age- and sex-matched patients with acute pancreatitis (AP) were compared with 49 healthy controls in terms of sociodemographic and clinical features. The BMI of patients in the AP group was significantly higher than that of the healthy controls ($p<0.001$). The health control group had no comorbidities, and none of the participants in either group were smokers or alcohol users. Amylase and lipase levels were both significantly higher in the AP group compared to the health controls ($p<0.001$ for both). Adiponectin levels were lower in the AP group, while NF-κB levels were significantly higher in the AP group ($p<0.001$ for both).

In the patient group, a strong negative correlation was observed between adiponectin and NF-κB levels ($r=-0.865$, $p<0.05$) (Table 2). No significant correlation was found between adiponectin and amylase levels, but a weak positive correlation was observed between NF-κB and amylase levels ($r=0.408$, $p<0.01$). Very weak correlations were found between lipase and adiponectin ($r=-0.290$) and between lipase and NF-κB ($r=0.280$), both of which were statistically significant ($p<0.05$).

Adiponectin and NF-KB levels were not significantly different between genders in the patient and healthy groups ($p>0.05$). Although Adiponectin levels were higher females in both groups, this difference was not statistically significant ($p=0.164$; p:0.440). In addition, no significant correlation was found between Adiponectin, NF-κB levels with age or BMI levels in both groups (Table 3 and Fig. 1).

Regression analysis was performed to evaluate the potential factors associated with the development of AP. The results indicated that none of the examined variables emerged as independent risk factors for the condition (Table 4). Although some parameters showed significant associations in univariate analysis, they did not retain their significance in the multivariate model.

In Figure 2 and Table 5, the performance of clinical parameters in the diagnosis of AP is shown in ROC curves. The Area Under the Curve (AUC) of adiponectin was 0.000 (95% CI:0.000–0.000), the AUC of NF-KB was 0.000 (95% CI:1.000–1.000), the AUC of amylase was 0.990 (95% CI:0.978–1.000), and the AUC of lipase was 1.000 (95% CI:1.000–1.000). The optimum cut-off point of adiponectin was 3.4 with a sensitivity of 1.000 and specificity of 1.000; the optimum cut-off point of NF-κB was 1.8 with a sensitivity of 1.000 and specificity of 0.96; the optimum cut-off point of amylase was 95.3 with a sensitivity of 0.96 and specificity of 1.000; the optimum cut-off point of lipase was 60.2 with a sensitivity of 0.96 and specificity of 1.000.

Table 1. Sociodemographic and clinical characteristics of patient and control groups

Characteristic	Healthy group (n=49)	Acute pancreatitis group (n=49)	p
Age	66.2±10.4	64.9±11.7	0.572
Gender, n (%)			1.000
Male	23 (46.9)	23 (46.9)	
Female	26 (53.1)	26 (53.1)	
BMI	24.0±2.1	27.1±4.1	**<0.001**
Comorbidity (+)	0(0.0)	25(51.0)	**<0.001**
Diabetes (+)	0(0.0)	34(69.4)	**<0.001**
Hypertension (+)	0(0.0)	25(51.0)	**<0.001**
Fasting blood glucose	80.0±10.2	136.3±46.9	**<0.001**
Amylase (U/L)	65.1±15.1	383.1±215.8	**<0.001**
Lipase (U/L)	34.3±13.4	119.8±55.2	**<0.001**
T. cholesterol (mg/dL)	169.3±13.9	214.7±16.8	**<0.001**
LDL (mg/dL)	101.8±11.0	149.1±10.3	**<0.001**
HDL (mg/dL)	45.9±6.8	39.3±9.4	**<0.001**
Triglyceride (mg/dL)	108.4±22.6	131.7±41.6	**0.003**
VLDL (mg/dL)	21.7±4.5	26.3±8.3	**0.003**
AST (U/L)	21.4±8.4	38.4±27.7	**0.003**
ALT (U/L)	26.2±8.5	35.2±18.8	0.052
LDH (U/L)	108.9±29.3	135.4±69.4	0.187
WBC (10^9/L)	7.9±1.1	10.0±2.6	**<0.001**
CRP (mg/L)	2.8±1.1	67.8±36.3	**<0.001**
Adinopectin (ug/L)	8.1±2.1	0.9±0.4	**<0.001**
NF-κB (ng/L)	0.9±0.4	6.1±0.9	**<0.001**

Student T test, Pearson chi-square Test, Mann-Whitney U test. BMI: Body mass index; T. cholesterol: Total cholesterol; LDL: Low density lipoprotein; HDL: High density lipoprotein; VLDL: Very low density; AST: Aspartate transaminase; ALT: Alanine transaminase; LDH: Lactate dehydrogenase; WBC: White blood cell; CRP: C-reactive protein; NF-κB: Nuclear factor kappa B.

Table 2. Correlation between adiponectin and NF-κB levels and other parameters in patient group (n=49)

	Adinopectin	NF-κB	CRP	Amylase	Lipase	WBC
Adinopectin (ug/L)	.	-0.865**	-0.876**	-0.263	-0.290*	-0.464**
NF-κB (ng/L)	-0.865**	.	0.960**	0.408**	0.282*	0.455**

Spearman Correlation Test; <0.25 very weak; 0.26–0.49 weak; 0.50–0.69 moderate; 0.70–0.89 high; 0.90–1.0 very high correlation. *: p<0.05; **: p<0.01. NF-κB, nuclear factor kappa B; CRP, C-reactive protein; WBC: White blood cell.

Table 3. Evaluation of adiponectin and NF-κB levels by gender

Group	Parameters	Gender		p
		Male	Female	
Acute pancreatitis group (n=49)	Adiponectin (ug/L)	0.8±0.4	1.0±0.4	0.164
	NF-KB (ng/L)	6.4±0.9	6.0±0.9	0.078
	Amylase (U/L)	413.3±200.1	356.3±229.3	0.200
	Lipase(U/L)	120.1±60.6	119.6±51.0	0.630
Healthy group (n=49)	Adiponectin (ug/L)	7.8±2.1	8.3±2.2	0.440
	NF-KB (ng/L)	0.9±0.4	0.9±0.3	0.976
	Amylase (U/L)	65.3±16.0	65.0±14.5	0.976
	Lipase(U/L)	34.1±15.9	34.5±11.1	0.801

Mann-Whitney U test. NF-κB: Nuclear factor kappa B.

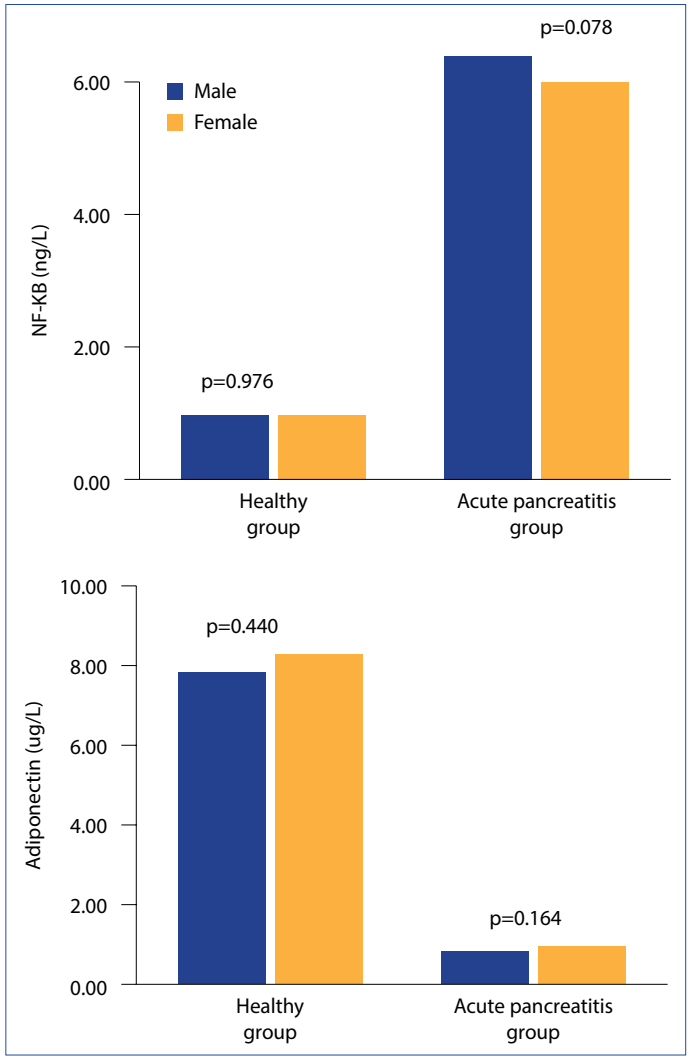

Figure 1. Evaluation of adiponectin and NF-κB levels by gender.
NF-κB: Nuclear factor kappa B.

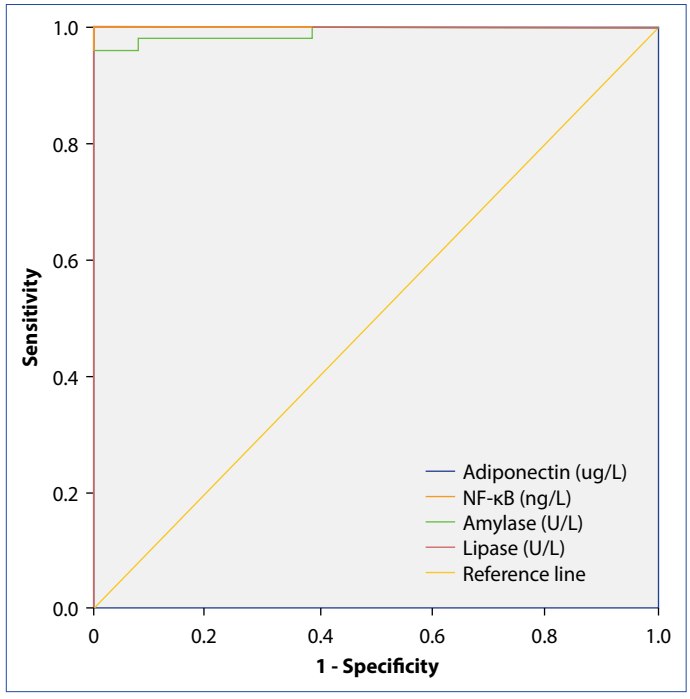

Figure 2. ROC curves evaluation of clinical parameters in the diagnosis of acute pancreatitis.
ROC: Receiver operating characteristic.

Discussion

AP is a gastrointestinal disorder associated with a relatively high mortality rate, ranging from 3% to 17%, and is a common reason for emergency department visits. The disease is frequently diagnosed in emergency settings due to its sudden onset, presenting symptoms such as nausea, vomiting, abdominal bloating, fever, shortness of breath, irritability, and changes in consciousness. Other signs may include fever, low oxygen levels, rapid breathing, elevated heart rate, low blood pressure, abdominal tenderness, bowel obstruction, and/or decreased urine output. Mortality and morbidity risk are influenced by factors like comorbid conditions and complications. Timely treatment initiation plays a crucial role in determining the prognosis. The growing use of rapid diagnostic tools has spurred research into new and faster diagnostic markers [16]. In present study, lower adiponectin levels and elevated NF-κB levels were observed in the AP group. A strong negative correlation between adiponectin and NF-κB was noted. While no significant correlation was found between adiponectin and amylase levels, a weak positive correlation was seen between NF-κB and amylase. We propose that high NF-κB levels and low adiponectin levels may serve as indicators of AP severity.

Although the precise pathogenesis of AP remains unclear, it has been suggested that the main mechanism involves a pathological process leading to autodigestion of pancreatic tissue. This occurs through the early activation of zymogens (digestive enzyme precursors) that are normally inactive within pancreatic acinar cells, ducts, and interstitial spaces [17]. Recent studies have also highlighted that severe pancreatic injury, and the systemic inflammatory response contribute to AP pathogenesis via NF-κB activation [18, 19]. The anti-inflammatory and protective effects of HTD4010 in HTG-AP are at least partially mediated through suppression of the TLR4/NF-κB axis, making this signaling pathway a promising therapeutic target for reducing disease severity in acute pancreatitis [20]. NF-κB signaling pathway as a key mechanism modulated by acinar cell-derived exosomes in the context of AP. NF-κB is well-established as a central mediator of inflammatory responses, and its activation plays a critical role in the progression of AP by promoting the expression of pro-inflammatory cytokines such as IL-6 and TNF-α [21]. Exosomes derived from healthy acinar cells were shown to have anti-inflammatory and cytoprotective effects, both *in vitro* and *in vivo*. Importantly, these exosomes suppressed NF-κB signaling activity, which corresponded with reduced apoptosis, lower ROS production, and decreased levels of systemic inflammatory markers in AP model rats. This suggests that exosomes exert their protective function by interfering with NF-κB-driven transcriptional responses that contribute to acinar cell damage and systemic

Table 4. Evaluation of risk factors associated with acute pancreatitis

Variables	Multivariate-enter method		Multivariate-forward: LR method	
	OR (95% CI)	p	OR (95% CI)	p
Amylase (U/L)	1.112 (0.951–1.299)	0.183	1.149 (0.996–1.324)	0.056
Body mass index	1.624 (0.919–2.870)	0.095	–	–
Hypertension	5.595 (0.000–12.460)	0.999	–	–
Triglyceride (mg/dL)	1.005 (0.958–1.054)	0.844	–	–

Forward LR and Enter methods were used for logistic regression analysis. Enter model: Hosmer Lemeshow test p=0.757, Cox & Snell R^2=0.714 Nagelkerke R^2=0.952, -2 Log Likelihood=13.173; forward LR model: Hosmer Lemeshow test p=0.803, Cox & Snell R^2=0.703 Nagelkerke R^2=0.937, -2 Log Likelihood=16.844. OR: Odds ratio; CI: Confidence interval.

Table 5. Evaluation of AUC and optimum cut-off point of clinical parameters

Parametre	AUC (%95 CI)	p	Cut-off	Sensitivity (%)	Specificity (%)
Adiponectin (ug/L)	0.000 (0.000–0.000)	<0.001	3.4	1.000	1.000
NF-KB (ng/L)	1.000 (1.000–1.000)	<0.001	1.8	1.000	0.960
Amylase (U/L)	0.990 (0.978–1.000)	<0.001	95.3	0.960	1.000
Lipase (U/L)	1.000 (1.000–1.000)	<0.001	60.2	0.960	1.000

AUC: Area under the curve; CI: Confidence interval.

inflammation. These findings support the idea that targeting NF-κB signaling via acinar cell-derived exosomes may offer a novel therapeutic strategy for reducing inflammation and tissue injury in AP. Moreover, this mechanism reinforces the role of intercellular communication—via exosomes—as a regulator of disease severity in pancreatitis [21]. The NF-κB pathway is among the most studied inflammatory signaling pathways in acinar cells and is associated with more severe forms of AP [22]. Results of Yu et al. [23] demonstrate that NF-κB is activated in response to cerulein via NADPH oxidase-derived reactive oxygen species and that this activation is essential for the induction of PAP-1 gene expression in pancreatic acinar cells. Furthermore, recombinant adiponectin has been shown to inhibit inflammation via the NF-κB pathway in AP [13]. Our findings align with the idea that adiponectin exerts protective effects by reducing NF-κB activity and pro-inflammatory cytokine responses in cerulein-induced AP, offering hope for the development of therapies targeting AP. As increasing adiponectin pharmacologically has shown promise in obesity treatment, it is possible that adiponectin may play a significant role in AP treatment in the future.

Recent studies have emphasized the role of adiponectin as an important regulator of inflammation, demonstrating its potential as a biomarker for assessing disease severity in inflammatory conditions such as AP [24]. Consistent with this, our study found higher serum NF-κB activity in the AP group, suggesting that inhibiting the NF-κB pathway could mitigate the early inflammatory response and offer potential new therapeutic approaches for AP. According to the results of this meta-analysis, adiponectin levels were not significantly different between patients with severe acute pancreatitis (SAP) and those with mild acute pancreatitis (MAP). Specifically, the standardized mean difference (SMD) was 0.11 (95% CI: -0.17 to 0.40, p=0.425), indicating no statistically significant association. Despite adiponectin's known anti-inflammatory properties, these findings suggest that it does not play a major role in the pathogenesis or severity differentiation of SAP. It also implies that adiponectin is unlikely to serve as a reliable biomarker for predicting or diagnosing severe forms of acute pancreatitis in clinical settings. While adiponectin is biologically active in inflammation regulation, its levels do not appear to be significantly altered in SAP, and thus it holds limited potential as a diagnostic or prognostic marker for this condition. Further research with larger, more homogenous patient populations may be necessary to clarify any potential role in specific subgroups [10]. Our study confirmed lower adiponectin levels in the AP group and observed a strong negative correlation between adiponectin and NF-κB levels. No significant correlation was found between adiponectin and amylase levels, reinforcing adiponectin's anti-inflammatory role. Adiponectin, an adipocyte-derived cytokine, is recognized for its anti-inflammatory and insulin-sensitizing effects, which are particularly important in inflammatory diseases such as AP [25]. Wos-Wroniewicz et al. [11] reported adiponectin in AP. Experimental studies have shown that adiponectin can inhibit NF-κB activation and downregulate pro-inflammatory cytokines like TNF-α and IL-6, thereby mitigating pancreatic inflammation [11, 13, 26]. These findings highlight adiponectin's modulatory role on the NF-κB pathway, a key mediator of inflammatory signaling in AP [27]. Adiponectin levels were significantly reduced in obese rats compared to the normal group, which is consistent with the known inverse relationship between adiponectin and obesity-related inflammation. Importantly, treatment with Sheng-jiang powder (SJP) re-

sulted in significantly increased serum adiponectin levels in the SJP-treated group (HSG) compared to the untreated obese group (HLG) [28]. This suggests that SJP may exert its protective effects against obesity-induced pancreatic inflammation partly by restoring or enhancing adiponectin levels. Given adiponectin's role in activating the AMPK signaling pathway, which is known to have anti-inflammatory and metabolic regulatory effects, this finding highlight adiponectin as a key mediator in the therapeutic mechanism of SJP. This supports the potential therapeutic significance of adiponectin in AP.

The NF-κB pathway as a central mediator in the inflammatory response associated with AP. NF-κB plays a pivotal role in the transcription of pro-inflammatory cytokines and chemokines, contributing to the development and progression of pancreatic injury and systemic complications. Natural plant-derived compounds can exert anti-inflammatory effects in AP by inhibiting the NF-κB signaling pathway. By blocking NF-κB activation, these phytochemicals reduce the production of inflammatory mediators, thus alleviating the severity of the disease. Targeting NF-κB with phytochemicals represents a promising therapeutic strategy for managing AP. These findings support continued research into natural compounds that can modulate this key inflammatory pathway and potentially lead to the development of effective treatments for AP [29]. Adiponectin is known to inhibit the ROS/NF-κB/NLRP3 inflammatory pathway, which may help differentiate pancreatic cancer (PC) from chronic pancreatitis (CP) in patients with elevated CA-19-9 levels, suggesting a potential diagnostic role for adiponectin in distinguishing CP from PC [30, 31].

Although adiponectin levels were numerically higher in females compared to males in both the acute pancreatitis and healthy control groups, the differences were not statistically significant. Similarly, NF-κB levels did not show a statistically significant difference between genders. These findings are consistent with previous studies suggesting that while adiponectin levels tend to be higher in females due to hormonal influences—particularly estrogen—this difference may not always reach statistical significance in small or moderate-sized study populations [32]. The lack of significance in our study may be attributed to the limited sample size and the cross-sectional design.

The ROC analysis demonstrated that NF-κB and lipase levels had perfect diagnostic performance in distinguishing patients from healthy individuals, with an AUC of 1.000, indicating excellent discriminatory ability. Similarly, amylase also showed near-perfect performance with an AUC of 0.990. These findings suggest that these biomarkers could serve as highly reliable indicators in the clinical differentiation between patient and control groups. In contrast, the adiponectin value, despite reporting 100% sensitivity and specificity, showed an AUC of 0.000, which is inconsistent and likely due to a data recording or analytical error. Overall, the high AUC values and corresponding sensitivity/specificity metrics support the potential diagnostic utility of these parameters, although external validation and larger sample sizes are necessary to confirm these preliminary results. Although weak correlations were observed between NF-κB and amylase, as well as between these biomarkers and lipase, these findings do not undermine the potential clinical relevance of NF-κB and adiponectin. Unlike amylase and lipase, which primarily reflect enzymatic activity, NF-κB and adiponectin provide insights into the inflammatory and metabolic dimensions of AP. Their combined evaluation with traditional markers may enhance disease characterization and should be further investigated in longitudinal studies. In this study, no variable was identified as an independent risk factor for AP in multivariate analysis. Although amylase and BMI showed borderline significance, they did not retain significance in the final models. Both regression models demonstrated good fit, but the lack of significant predictors may reflect sample size limitations. Although this study investigated the potential impact of various clinical and biochemical parameters on the development of AP, no statistically significant independent risk factor was identified. Larger-scale and prospective studies are needed to validate these findings. Additionally, exploring potential interactions and unmeasured confounders may contribute to a better understanding of the pathogenesis of AP.

Limitations of the study

This study has several limitations that should be acknowledged. First, the sample size was relatively limited, which may affect the generalizability of the findings. Larger, multicenter studies are needed to validate our results across more diverse patient populations. Second, insulin levels were not measured, which is a notable limitation given the known association between adiponectin, insulin sensitivity, and metabolic status. Inclusion of insulin and HOMA-IR values could have provided deeper insight into the relationship between metabolic regulation and inflammation in acute pancreatitis. Third, the study design was cross-sectional, preventing us from assessing changes in NF-κB and adiponectin levels over time or establishing causal relationships. Longitudinal studies would allow better evaluation of these markers throughout the disease course. Lastly, although NF-κB and adiponectin were evaluated as potential biomarkers, other relevant inflammatory and metabolic markers such as IL-6, TNF-α, CRP, or leptin were not included, which could have enriched the interpretation of the inflammatory pathways involved in disease severity. Despite these limitations, our findings contribute to the growing body of evidence supporting the role of inflammatory and metabolic biomarkers in acute pancreatitis and highlight areas for future investigation.

Conclusion

Our study aims to guide future research on the use of elevated serum NF-κB and decreased serum adiponectin levels—either alone or in combination with existing scoring systems—in evaluating disease severity in AP. Despite significant research, it remains unclear which scoring systems or laboratory parameters are most effective in assessing the severity of AP. Therefore, further clinical and experimental studies, particularly multicenter trials with larger patient populations, are necessary to identify reliable markers for disease assessment in AP.

Ethics Committee Approval: The study was approved by the Biruni University Faculty of Medicine Ethics Committee (no: 2024-BİAEK/05-04, date: 12/12/2024).

Informed Consent: Informed consent was obtained from all participants.

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Technical Report

Pre- and post-centrifugation stability of total and free prostate specific antigen samples at room temperature storage conditions

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Abstract

Objectives: Considering the recommendations of literature, it was important to note the potential for differences in pre- and post-analytical storage conditions at room temperature between total and free prostate-specific antigen. The aim of our study was to establish whether it would be appropriate to align the pre- and post-analytical times for the determination of free prostate-specific antigen (fPSA) with those for total prostate-specific antigen (tPSA).

Methods: Two blood samples were taken from 48 male patients aged 60 to 84. One specimen was centrifuged within one hour of collection. Each sample was tested immediately for total and free PSA. The second blood sample was kept at room temperature for 12 hours before being tested and then reanalyzed 24 hours after blood sampling. Serum specimens were analyzed on the Roche Cobas E801.

Results: There were no notable alterations in any PSA forms (p=0.866 and 0.971) or calculated ratios (Kappa=1) for the blood sample that was stored at room temperature for 12 hours prior to processing. Furthermore, all forms of PSA demonstrated stability (p=0.956 and 0.901), and fPSA/tPSA ratios showed good agreement in serum for up to 24 hours at room temperature (Kappa=1).

Conclusion: It would be beneficial to extend the pre- and post-analysis times of fPSA to align them with those of tPSA. Following the elevated tPSA discovery, investigating fPSA could be more streamlined, offering an improved patient management solution.

Keywords: fPSA/tPSA ratios, free prostate-specific antigen, *in vitro* stability, total prostate specific antigenstorage

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Prostate-specific antigen (PSA) is a glycoprotein with a molecular weight of 30,000–34,000 Dalton. It has a close structural relationship to the glandular kallikreins and functions as a serine proteinase [1]. In the blood, the proteolytic activity of PSA is inhibited by the formation of irreversible complexes linked to inhibitors such as alpha-1-antichymotrypsin, alpha-2-macroglobulin and other proteins of the acute phase of inflammation [2]. Furthermore, PSA is present in the bloodstream in a free form (fPSA) that has lost its proteolytic activity [3]. In 1991, JE Oesterling [4] showed that the total PSA (tPSA) assay lacked the necessary sensitivity and specificity to be an ideal tool for screening or early diagnosis. The fPSA/tPSA ratio has been shown in several studies to improve sensitivity and specificity in patients with tPSA levels in the grey zone of 4–10 ng/mL [5–8]. It is important to note that pre- and post-analytical factors related to sample handling and storage can affect the values of all the molecular forms of PSA [9]. If the tPSA is sufficiently stable to allow whole blood samples to remain at room temperature for 24 hours before serum separation [10], the level of stability of f-PSA varies between 3 and 8 hours [11]. Some authors have indicated that there is a 5% reduction in fPSA after whole blood is stored for 8 hours at room temper-

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ature, with an 8% decrease observed after 24 hours [12]. It is important to note that the accuracy of the biomarkers fPSA/tPSA ratio is contingent upon the correct application of stability conditions. The objective of the present study was to examine the impact of storage at room temperature on free and total prostate-specific antigen (PSA) levels in blood samples collected 12 hours ago and in serum separated from clotted blood stored at room temperature for varying periods.

Materials and Methods

Ethical approval

The laboratory investigations were conducted in accordance with the EU General Data Protection Regulation (EU Regulation 2016/679 and Directive 95/46/EC) and French data protection legislation (Law 78–17 of 1978, as amended by Decree 2019–536 of May 29, 2019). In accordance with Article 6 of the European Parliament and Council Directive 95/46/EC of 24 October 1995 and Article 2 of the French Data Protection Act of 6 January 1978 and Decree 2019–536 of 29 May 2019, a review by an ethics committee is not required for the secondary use of samples collected for healthcare purposes. In such cases, the use of human biological material for medical or scientific purposes other than those for which it was originally collected is permitted (article L.1211-2 of the French Public Health Code). The procedures followed were in accordance with the French Public Health Code. The database is registered with the French National Commission on Informatics and Liberty (CNIL) under record No. 2073511v0.

Sample size

In quantitative studies, the sample size is a critical factor in obtaining reliable data. The final size of the sample will depend on how precise (accurate) it needs to be, what the budget is, and all the other practical issues.

The following formula was used to calculate the required sample size for our study:

$s = z^2 \times p (1 - p) / m^2$, with:

- "s" was the sample size retained.
- "z" was the confidence level according to reduced centered normal distribution for 99% confidence (z=2.575).
- "p" was the estimated proportion f-PSA analyzed in the laboratory the characteristic (2% or 0.02).
- "m" was the margin of error (we might want to know the true proportion to within 5% or 0.05).

According to this formula, the size of the population tested should be approximately 50 patients.

Subjects and collection tubes

Two 5ml coagulated blood samples were taken (by single venipuncture) from 48 male patients aged 60 to 84, who were attending the laboratory for a blood test. All samples were collected using BD Vacutainer® SST™ II (ref 367957) tubes with Gel and Clot Activator.

One of the specimens (Sample "1") was centrifuged within 1 hour upon collection (at 2000 g for 10 min) and the serum fraction separated. Each sample "1" was assayed immediately for tPSA and fPSA.

The second blood specimen (Sample 2) was left undisturbed on the laboratory bench at room temperature for 12 hours prior to processing. Following storage, the sample was centrifuged at 2000g for 10 minutes, after which the serum fraction was assayed immediately for tPSA and fPSA. The stability of total and free PSA in coagulated blood during a 12-hour period at room temperature (15°–25°C) was evaluated by comparing sample 1 with sample 2, designated "time-12h." To evaluate the stability of tPSA and fPSA in serum at room temperature, each serum fraction of sample 2 was reanalyzed 24 hours after the blood collection. The stability of total and free PSA in serum at room temperature (15°–25°C) was assessed by comparing sample 1 with sample 2, designated "time-24h".

Instruments

The total and free PSA levels were determined using the Roche Cobas E801 system (Roche Diagnostics, Mannheim, Germany). The total and free PSA were determined using the ECLIA method. The expanded uncertainty for the total and free PSA were estimated at 12.4% (with a 95% confidence interval between 8.8% and 21.1%) and 8.6% (with a 95% confidence interval between 6.0% and 15.1%), respectively. The samples were analyzed in the different runs using the same instrument which had been verified according to the accreditation criteria of ISO 15189 [13].

Statistical analysis

The conformity of the numerical values to a normal distribution was evaluated using a Shapiro–Wilk test, which demonstrated that the distribution of results was non-parametric. The comparison between populations was evaluated using a Mann–Whitney test ($p<0.05$ was considered significantly different). To illustrate the impact of the results according to the methods used, boxplots have been used.

The analytical agreements between sample 1 and sample 2 (time-12h and time-24h) were analysed using a scatter plot with a Passing-Bablok regression analysis. The correlations were evaluated using the Pearson correlation test, with a p-value of less than 0.05 considered statistically significant. In the regression analysis, the limits of agreement were plotted according to the desirable total error formula developed by Callum G. Frazer [14], using the biological variation of total and free PSA defined by Carobene et al. [15]. The acceptable accuracy for tPSA and fPSA should fall within the total allowable error range of ±15.2% and ±14.0%, respectively.

Agreement results versus fPSA/tPSA ratio

For all samples with tPSA levels of 2.6 ng/mL or above (this limit accounts for the margin of error inherent to the measurement process at a value of 3 ng/mL), the results of the pre- and post-centrifugation phases were evaluated in comparison to the

Table 1. Statistical description of the tPSA and fPSA results according to different time periods.

	tPSA					fPSA				
	Sample "1" (time <1h)	Sample "2" designated "time-12h"	Sample "2" designated "time-24h"	Mann-Whitney test "time<1h" versus "time-12h"	Mann-Whitney test "time<1h" versus "time-24h"	Sample "1" (time <1h)	Sample "2" designated "time-12h"	Sample "2" designated "time-24h"	Mann-Whitney test "time<1h" versus "time-12h"	Mann-Whitney test "time<1h" versus "time-24h"
Nb. specimens	48	48	48	p=0.866	p=0.956	48	48	48	p=0.971	p=0.901
Minimum	0.014	0.014	0.014			0.020	0.020	0.020		
Maximum	25.7	25.2	25.2			4.670	4.300	4.230		
1st quartile	0.401	0.400	0.411			0.155	0.158	0.150		
Médian	0.900	0.922	0.910			0.260	0.260	0.255		
3rd quartile	2.855	2.9575	2.9725			0.473	0.460	0.455		
Mean	2.680	2.717	2.715			0.507	0.496	0.491		
Variance (n-1)	24.374	24.195	24.842			0.651	0.580	0.576		
SD (n-1)	4.937	4.918	4.984			0.807	0.761	0.759		

tPSA: Total prostate-specific antigen; fPSA: Free prostate-specific antigen; SD: Standard deviation

Table 2. The agreement of fPSA/tPSA ratio using a threshold of 0.23 according to different time periods

	fPSA/tPSA ratio (time-12h)		fPSA/tPSA ratio (time-24h)	
	<0.23	≥0.23	<0.23	≥0.23
fPSA/tPSA ratio (time <1h)				
<0.23	12	0	12	0
≥0.23	0	3	0	3
Kappa [95% CI]	1.00 (1.00–1.00)		1.00 (1.00–1.00)	

tPSA: Total prostate-specific antigen; fPSA: Free prostate-specific antigen; CI: Confidence interval.

fPSA/tPSA ratio using a pathological decision threshold of 0.23, as defined by de la Taille et al. [16]. The kappa coefficients with a 95% CI were calculated to estimate the agreement of evidence and recommendation levels between all paired samples [17]. According to Landis and Koch [18], kappa coefficients can be interpreted as one of the following six degrees of agreement: Poor (κ<0), slight (0.01–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), and almost perfect (0.81–1.00).

Results

The study cohort comprised 48 patients aged between 60 and 84 years, median age 74 (1st quartile 68, 3rd quartile 77). Table 1 provides a statistical overview of the results. The median values for tPSA and fPSA for the population of specimens (Sample 1) that were centrifuged and analyzed within one hour of collection were 0.900 ng/mL and 0.260 ng/mL, respectively. A total of 15 patients from the 48-patient cohort presented with total prostate-specific antigen (tPSA) levels above 2.6 ng/mL. These subjects were included for the purpose of evaluating the Kappa concordance rate for the fPSA/tPSA proportion.

Effect of delayed centrifugation 12 hours after collection

The median values for tPSA and fPSA in the specimens (sample 2, designated "time-12h.") left undisturbed on the laboratory bench at room temperature for 12 hours prior to processing and analysis were 0.922 ng/mL and 0.260 ng/mL, respectively. The data showed no statistically significant differences for tPSA and fPSA with p-values of 0.866 and 0.971, respectively. The box plot showed a notable degree of convergence between the two measurement periods (Fig. 1a, b). For tPSA, the Pearson correlation coefficient (r) was significant and strong (r=1.000 [p<0.001; 95% confidence interval [CI]: 0.992–1.007]). The regression slope was 0.996 (p<0.001; 95% CI: 0.989–1.003) and the y-intercept was 0.048 (p<0.001; 95% CI: 0.006–0.089). Regarding fPSA, the Pearson correlation coefficient (r) was significant and strong (r=0.999 [p<0.001; 95% confidence interval [CI]: 0.987–1.011]). The regression slope was 0.943 (p<0.001; 95% CI: 0.932–0.954) and the y-intercept was 0.018 (p<0.001; 95% CI: 0.008–0.029). The regression analysis revealed no analytical discordance within the allowable

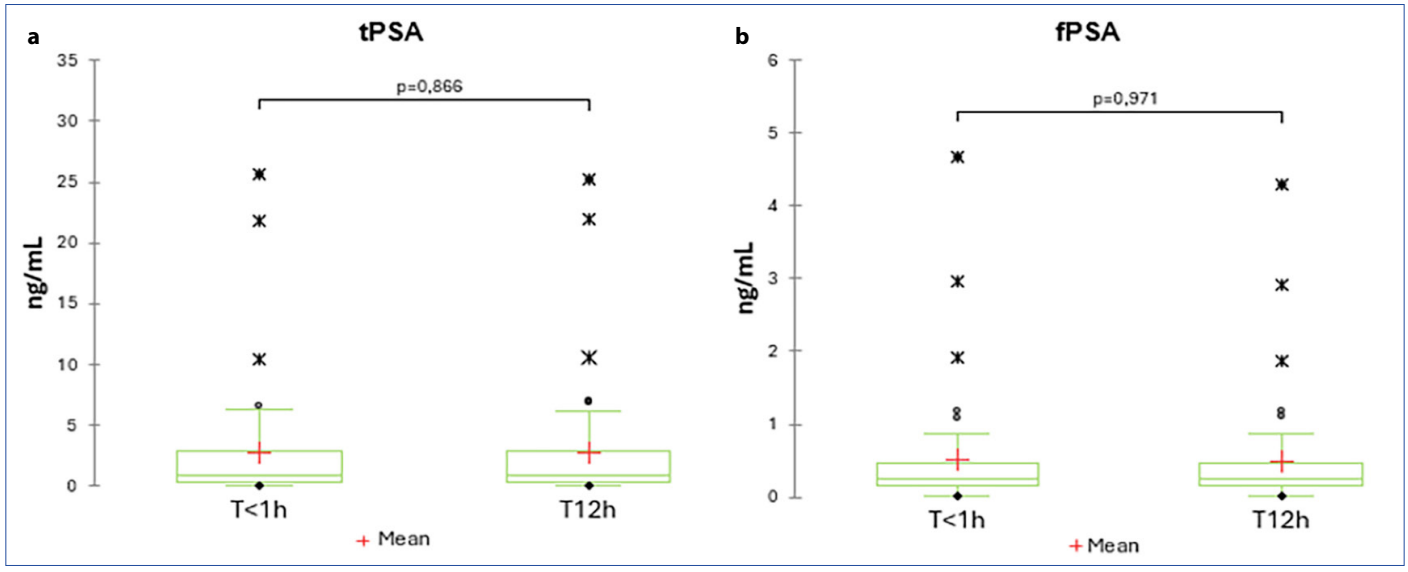

Figure 1. Boxplots of tPSA and fPSA results between samples analyzed within an hour of collection and samples kept for 12 hours before centrifugation.

tPSA: Total prostate-specific antigen; fPSA: Free prostate-specific antigen.

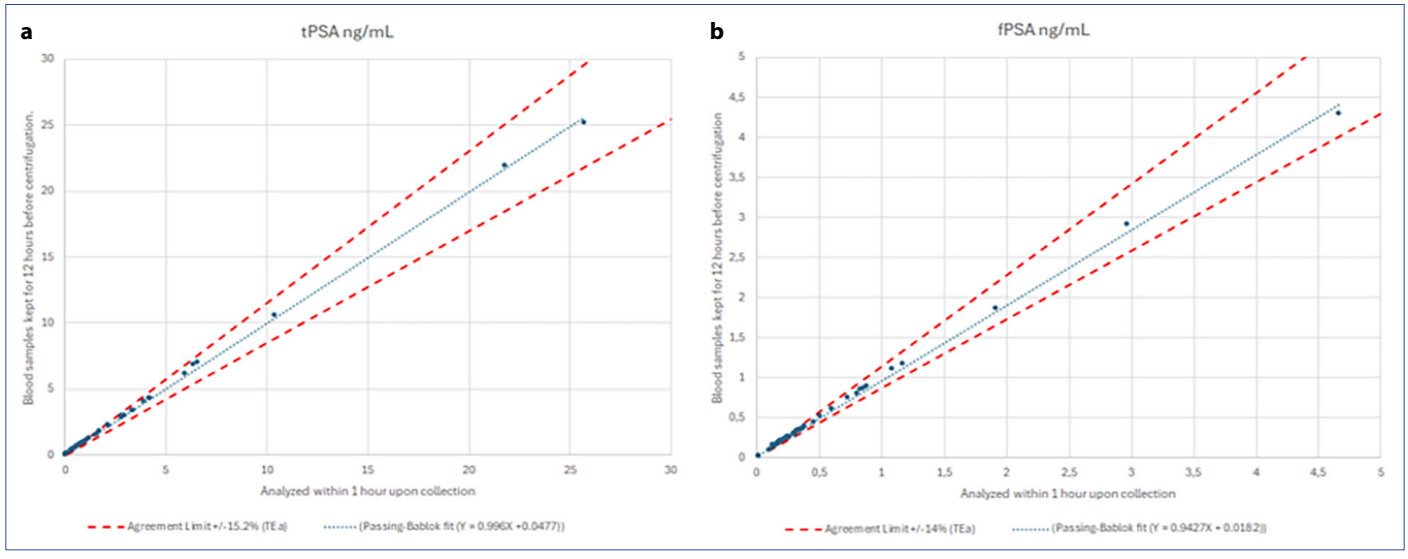

Figure 2. Comparison of tPSA and fPSA results between samples analyzed within an hour of collection and samples kept for 12 hours before centrifugation.

total error range (Fig. 2a, b). An interpretation based on the fPSA/tPSA ratio with a decision threshold of 0.23 yielded perfect agreement (Kappa=1.00) and is detailed in Table 2.

Effect of stability 24 hours after collection in serum

The median values for tPSA and fPSA in the specimens (sample 2, designated "time-24h") that were left undisturbed on the laboratory bench at room temperature for 12 hours before centrifugation and reanalyzed 24 hours after the sample collection were 0.910 and 0.255, respectively. The data showed no statistically significant differences for tPSA and fPSA with p-values of 0.956 and 0.901, respectively. The box plot showed a notable degree of convergence between the two measurement periods (Fig. 3a, b). For tPSA, the Pearson correlation coefficient (r) was significant and strong (r=0.999 [p<0.001; 95% confidence interval [CI]: 0.990–1.009]). The regression slope was 1.009 (p<0.001; 95% CI: 0.999–1.019) and the y-intercept was 0.011 (p<0.001; 95% CI: -0.044–0.065). Regarding fPSA, the Pearson correlation coefficient (r) was significant and strong (r=0.998 [p<0.001; 95% confidence interval [CI]: 0.981–1.016]). The regression slope was 0.939 (p<0.001; 95% CI: 0.922–0.955) and the y-intercept was 0.015 (p<0.001; 95% CI: 0.000–0.031). The regression analysis revealed no analytical discordance within the allowable total error range (Fig. 4a, b). An analysis based on the fPSA/tPSA ratio with a decision threshold of 0.23 resulted in perfect agreement (Kappa=1.00), as shown in Table 2.

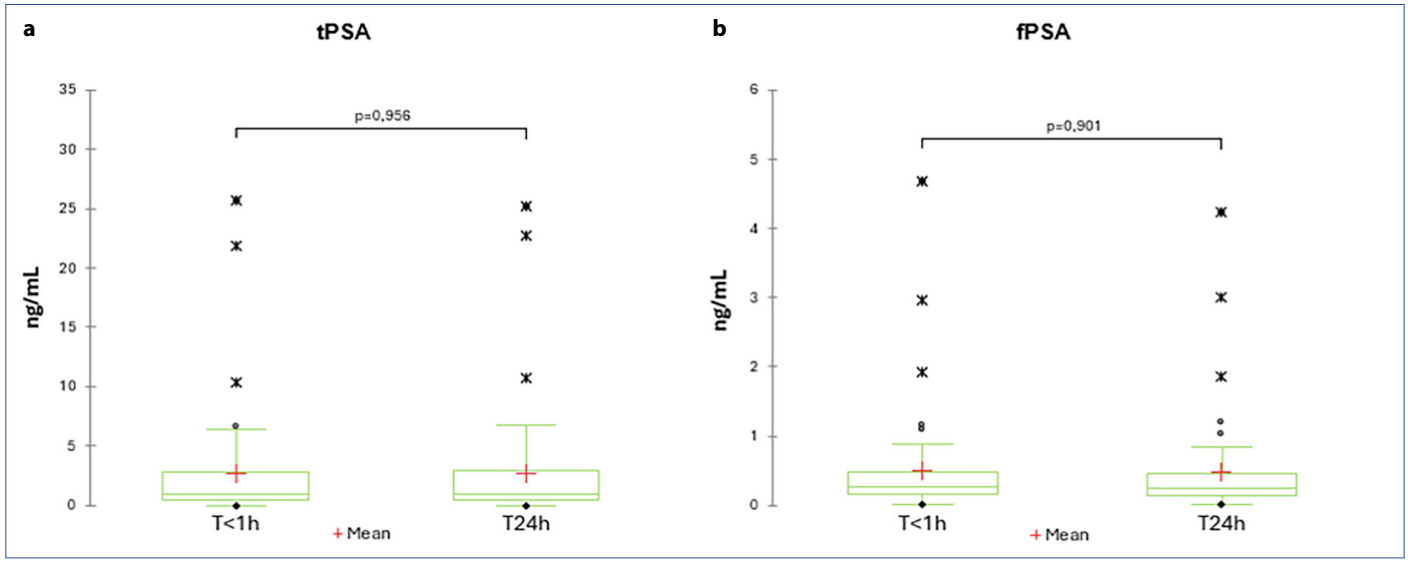

Figure 3. Boxplots of tPSA and fPSA results between samples analyzed within an hour of collection and samples kept for 12 hours before centrifugation and reanalyzed 24 hours after venipuncture.

tPSA: Total prostate-specific antigen; fPSA: Free prostate-specific antigen.

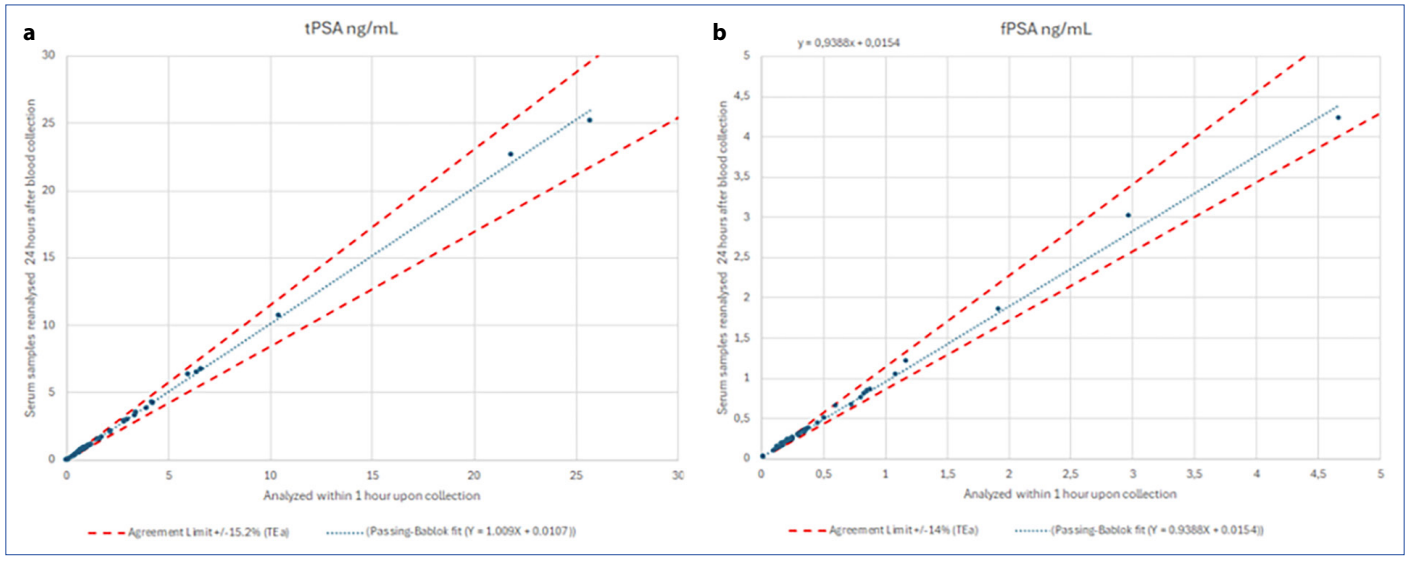

Figure 4. Comparison of tPSA and fPSA results between samples analyzed within an hour of collection and samples kept for 12 hours before centrifugation and reanalyzed 24 hours after venipuncture.

Discussion

Several studies have investigated the short- and long-term stability of tPSA and fPSA assays since their introduction in the late 1980s [19–21]. In line with the results of this study, other reports have shown that tPSA levels are relatively stable when stored at room temperature for a period of 24 hours [22, 23]. Regarding fPSA stability at room temperature, there is a lack of consensus in the literature on this topic, as the findings of different studies vary considerably. Piironen et al. [24] and Jung et al. [12] showed the rapid loss of fPSA in the presence of clotted blood cells could be due to complex formation with α2-macroglobulin (PSA-AMG), which occurs faster than the complex formation with α1-antichymotrypsin (PSA-ACT). In accordance with the recommendations of these authors, serum preparation should be conducted promptly, within five hours of venipuncture, and fPSA analysis should be completed within eight hours to ensure optimal stability of PSA variants and eliminate pre-analytical variables as sources of variability. Kumari et al. [23] reported excellent stability *in vitro* of fPSA at room temperature. The authors reported that clotted blood stored at room temperature for 24 hours showed no decline in fPSA. This finding is at odds with the results of other studies, which have demonstrated a decrease in free PSA when separation is delayed [12, 24].

The objective of our study was to gain a comprehensive understanding of the room temperature stability characteristics

of free and total PSA from a pre- and post-analytical perspective. Our study corroborates the findings of previous research, confirming the stability of tPSA. Furthermore, it offers valuable insights into the routine use of fPSA. Whole blood samples may be kept at room temperature for up to 12 hours before processing. The fPSA results of subsequent assays on serum samples stored at room temperature remain unaffected until 24 hours post-blood collection.

Since 2010, the number of laboratories in France conducting these analyses has decreased significantly. It is standard practice for blood samples to be collected at a peripheral laboratory or a healthcare practitioner's office, which are geographically distant from the technical laboratory responsible for analyzing the samples. This necessitates the transportation of samples, resulting in a notable increase in the time required for the pre-analysis phase. Our results will ensure that samples are handled correctly, both in the field and in the laboratory, and guarantee their accurate evaluation.

According to scientific guidelines [25–27], it is not recommended that free PSA be used as a first-line test. The free PSA/total PSA ratio is a useful indicator for assessing the risk of prostate cancer in patients with tPSA levels between 4 and 10 ng/mL. Considering this, it would be beneficial to extend the pre- and post-analysis times of fPSA to align them with those of tPSA. Following the elevated tPSA discovery, investigating fPSA could be more streamlined, offering an improved patient management solution.

Nevertheless, it was observed that most of the results of t-PSA falling within the range of 0.4 and 2.9 ng/mL were a limiting factor. Further investigations should be conducted to examine a greater number of patients with tPSA 4–10 ng/mL and also more patients with tPSA above 10 ng/mL.

Conclusion

Regarding the stability of free PSA, our results indicate that samples may be stored on the clot at room temperature for up to 12 hours, with the option of reanalysis up to 24 hours after blood collection.

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Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Letter to the Editor

Advancing metabonomics in understanding immunological mechanisms of recurrent spontaneous abortion

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Dear Editor,

We read with great interest the recent paper by Devi et al. [1] on "Immunological insights into recurrent spontaneous abortions: The role of GATA3 and cytokine expression in maternal and placental tissues.". We are writing this article to highlight our interest in understanding the recent developments in metabonomics and its potential to elucidate the immunological mechanisms underlying recurrent spontaneous abortion (RSA). Despite comprehensive research, it remains unclear which pathophysiological factors precisely contribute to RSA, especially in terms of maternal-fetal immune tolerance and dysregulated metabolic pathways. Recent advances in metabonomics provide a promising approach to bridge these gaps by suggesting a comprehensive biochemical perspective of immunometabolic interactions involved in RSA [1–3].

Several studies have highlighted the potential role of metabolic reprogramming in immune cells, particularly T-helper (Th) cells, regulatory T cells (Tregs), and natural killer (NK) cells, which are critical for pregnancy maintenance [3, 4]. Metabonomics-based analyses have determined alterations in lipid metabolism, amino acid pathways, and oxidative stress indicators in women with RSA. This demonstrates that metabolic imbalances may make a contribution in immune dysfunction. Moreover, metabolomic profiling has revealed distinct biomarkers that distinguish successful pregnancies from those complicated by RSA, providing opportunities for potential diagnostic and therapeutic advances [2, 5].

Despite these promising findings, further research involving the integration of metabonomics data with immunophenotyping and transcriptomic analyses is required to elucidate causal relationships between metabolic changes and immune dysfunction in RSA. The application of high-throughput metabolomic technologies, such as nuclear magnetic resonance (NMR) and mass spectrometry (MS)-based approaches, will play a crucial role in the identification of new metabolic markers associated with pregnancy loss. In addition, longitudinal studies focusing on metabolic changes during early pregnancy could provide important information on early predictive markers for RSA.

Given the increasing interest in immunometabolism and reproductive immunology, we believe that a specific focus on metabonomics in RSA research would significantly enhance our understanding of its pathogenesis and inform targeted therapeutic strategies. We encourage the International Journal of Medical Biochemistry to further explore this emerging field, potentially through reviews or special issues, to foster interdisciplinary collaboration and accelerate translational applications.

Thank you for considering this perspective. We look forward to the journal's continued contributions to this vital area of research.

Sincerely.

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