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AIM AND SCOPE

International Journal of Medical Biochemistry (IJMB) is a double-blind peer-reviewed, open-access journal of the Association of Clinical Biochemistry Specialists Türkiye.

International Journal of Medical Biochemistry (IJMB) publishes articles relating to clinical and experimental chemistry, molecular biology, genetics, therapeutic drug monitoring, toxicology, immunology, hematology and laboratory medicine with the focus on analytical and clinical investigation of laboratory tests used for diagnosis, prognosis, and monitoring of diseases.

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Authors are responsible for the accuracy of data. The Editorial Board of the International Journal of Medical Biochemistry and the Publisher adheres to the principles of the International Council of Medical Journal Editors (ICMJE), the World Association of Medical Editors (WAME), the Council of Science Editors (CSE), the Committee on Publication Ethics (COPE), the US National Library of Medicine (NLM), the World Medical Association (WMA) and the European Association of Science Editors (EASE).

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JOURNAL FREQUENCY

The International Journal of Medical Biochemistry published three issues per year from its establishment until 2024. The journal has started publishing four issues per year since 2025. The publication months are January, April, July, and October.

ABSTRACTING AND INDEXING

International Journal of Medical Biochemistry is indexed in TUBITAK TR Index (2019), CNKI (2019), TurkMedline (2019), Open Ukrainian Citation Index (2019), ProQuest (2019), CABI (2021), CEEAS (2021), EBSCO (2022), CAS (American Chemical Society) (2022), Directory of Open Access Journals - DOAJ (2022), Scopus (2023), Gale Cengage (2023), Sherpa Romeo (2024), ASCI (2024) and Web of Science - ESCI (2025), EZB (2025), OpenAlex (2025), Sudoc (2025), ZDB (2025).

ABBREVIATION

Int J Med Biochem

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Submission Fee

As of January 1, 2026, authors are required to pay a submission fee of USD 120 (including VAT) at the time of manuscript submission to the International Journal of Medical Biochemistry (IJMB). The submission process will not be initiated for manuscripts for which the submission fee has not been paid. This fee is charged to contribute to the costs of editorial handling. The submission fee is applied during the manuscript submission process and is non-refundable, regardless of the final editorial decision (acceptance or rejection). Kindly note that any bank transaction fees must be covered by the author.

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The abstracts and full texts of published articles can be accessed free of charge at www.internationalbiochemistry.com.

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International Journal of Medical Biochemistry publishes articles relating to clinical chemistry, molecular biology and genetics, therapeutic drug monitoring and toxicology, hematology, immunology and laboratory medicine with the focus on analytical and clinical investigation of laboratory tests used for diagnosis, prognosis, treatment and therapy, and monitoring of disease. The official language of the Journal is English. The journal will be published 4 times a year in print and electronically.

Main Topics

- Clinical Biochemistry
- Molecular Biology
- Clinical Haematology
- Clinical Immunology
- Drug Monitoring and Analysis
- Diagnostic Biomarkers
- Disease-Oriented Topics (Cardiovascular Disease, Cancers, Diabetes, Obesity, Genetic Disorders, Neurodegenerative Disease e.g..)
- Pediatric Biochemistry
- Inherited Metabolic Disorders
- Newborn Screening: Congenital and Genetic Disorders
- New Reagents, Instrumentation, Technologies and Methodologies
- Laboratory Medicine; Quality, Safety, translational laboratory
- Clinical Metrology

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The primary conditions for the acceptance of manuscripts for publication are originality, scientific value and citation potential.

The manuscripts should be prepared in accordance with ICMJE Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (updated in May 2022 - https://www.icmje.org/recommendations/).

Authors are required to prepare manuscripts in accordance with the international guidelines* below.

Randomized Controlled Trial **	CONSORT (Consolidated Standards of Reporting Trials)
Non-Randomized Trial **	TREND (Transparent Reporting of Evaluations with Non-randomised Designs)
Trial Protocol	SPIRIT (Standard Protocol Items Recommendations for Interventional Trials)
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Systematic Reviews and Meta-Analyses	PRISMA (the Preferred Reporting Items for Systematic Reviews and Meta-Analyses)
Systematic Reviews and Meta-Analyses Protocol	PRISMA-P (the Preferred Reporting Items for Systematic Reviews and Meta-Analyses - Protocol)
Qualitative Research	-SRQR (the Standards for Reporting Qualitative Research) -COREQ (COnsolidated criteria for REporting Qualitative research: interviews and focus groups
Methodological Studies (Translating and Adapting Tests)	ITC (International Test Commission) Guidelines for Translating and Adapting Tests
Methodological Studies (Developing Tests)	COSMIN (COnsensus based Standarts fort the selection of Health Measurement Instruments- Study Design for Patient-reported outcome measurement instruments)
Case Report	CARE (CAse REport)

* Enhancing the QUAlity and Transparency of Health Research (equator network).
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EDITORIAL

The changing role of medical biochemistry

Medical biochemistry continues to grow as a field that connects laboratory findings with clinical decision-making. The articles in this issue reflect that broad and changing role. They address important topics such as neurodegeneration, diabetes, inflammation, hematologic disease, colorectal cancer screening, diabetic retinopathy, cardiometabolic risk, and experimental cancer research. Together, they show how laboratory medicine supports both better understanding of disease and better patient care.

A common message in this issue is that laboratory medicine is no longer limited to measuring analytes. It also helps explain disease mechanisms, evaluate risk, compare methods, and improve the clinical value of test results. Some studies focus on biomarkers and pathophysiology, while others highlight analytical performance and test agreement. This balance is crucial for the field's future.

As the scope of medical biochemistry expands, the need for scientific rigor, clear interpretation, and clinical relevance becomes even greater. We hope the studies in this issue will contribute to ongoing research, encourage collaboration, and support the continued development of laboratory medicine.

Prof. Dildar Konukoglu, MD.

Editor-in-Chief

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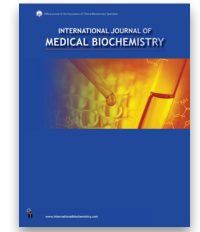

Research Article

Plasma free amino acids in Parkinson's disease: An exploratory case–control study

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Abstract

Objectives: Parkinson's disease (PD) is associated with systemic metabolic alterations; however, reproducibility and methodological standardization remain ongoing challenges in metabolomics research. This exploratory case–control study aimed to evaluate whether targeted plasma free amino acid profiling reveals statistically robust differences between PD patients and healthy controls.

Methods: Forty-three patients with PD and 43 age- and sex-matched healthy controls were included. Plasma free amino acids were quantified using a targeted triple quadrupole LC–MS/MS platform with Appendix 1 isotope-labeled internal standards. Between-group comparisons were performed with appropriate statistical tests. False discovery rate (FDR) correction and effect size (Cohen's d) calculations were applied. Compound-based KEGG pathway enrichment analysis was conducted using FDR-significant metabolites. ROC analyses were performed for signal strength assessment only.

Results: After FDR correction, alanine, arginine, aspartic acid, proline, taurine, threonine, and phenylalanine/tyrosine-related ratios remained significant, with moderate-to-large effect sizes. Compound-based KEGG enrichment demonstrated significant clustering within interconnected amino acid metabolism pathways, including arginine and proline metabolism, taurine and hypotaurine metabolism, glycine, serine and threonine metabolism, and alanine, aspartate and glutamate metabolism (pathway-level FDR <0.05). Exploratory ROC analyses showed moderate signal strength for proline (AUC=0.794), taurine (AUC=0.792), and threonine (AUC=0.780).

Conclusion: Targeted plasma amino acid profiling revealed coordinated systemic alterations in amino acid metabolism in PD within a statistically disciplined analytical framework. These findings reflect peripheral metabolic variation and should be interpreted as exploratory and hypothesis-generating. The study primarily contributes an analytically validated and FDR-corrected dataset to the discussion on methodological rigor in PD metabolomics, rather than evidence of diagnostic or mechanistic inference. Validation in longitudinal, clinically well-characterized cohorts is required.

Keywords: Alanine, amino acids, arginine, aspartic acid, free amino acids, LC–MS/MS, phenylalanine, plasma, proline, targeted metabolomics, taurine, threonine

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Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized primarily by the loss of dopaminergic neurons in the substantia nigra and the presence of intracellular protein aggregates, leading to a wide range of motor and non-motor symptoms [1, 2]. The disease predominantly affects older individuals, with a prevalence of approximately 1% in those over 60 years of age, increasing to nearly 5% in individuals older than 85 years [3]. Despite extensive research efforts, the pathophysiological mechanisms underlying PD have not been fully elucidated, and no disease-modifying therapy is currently available to halt or slow disease progression [2, 4].

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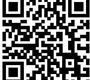

Although PD has traditionally been regarded as a central nervous system disorder, accumulating evidence indicates that it is a multisystem disease involving widespread metabolic and biochemical alterations beyond the brain [4, 5]. In this context, peripheral biofluids such as plasma have gained increasing attention as accessible matrices for exploring disease-associated metabolic alterations. Metabolomics-based approaches, in particular, have provided valuable insights into systemic metabolic disturbances associated with PD [5, 6].

Amino acids play critical roles in neurotransmitter synthesis, energy metabolism, nitrogen balance, and cellular signaling. Under physiological conditions, plasma amino acid concentrations are tightly regulated through coordinated anabolic and catabolic processes. Disruptions in amino acid homeostasis may therefore reflect altered metabolic pathways associated with neurodegeneration [7]. Several studies have reported significant alterations in amino acid levels in plasma and cerebrospinal fluid (CSF) of patients with PD compared with healthy controls, suggesting a link between amino acid metabolism and disease-related processes [6, 8].

Targeted and untargeted metabolomic investigations have demonstrated changes in multiple amino acids in PD, including alterations in alanine, arginine, glutamate, taurine, tyrosine, and threonine, as well as broader disturbances in nitrogen and energy metabolism [8–10]. These alterations have been reported in the context of several mechanisms proposed in Parkinson's disease, including mitochondrial dysfunction, oxidative stress, altered neurotransmission, and changes in protein metabolism [4, 11, 12]. However, reported findings remain heterogeneous across studies, likely due to differences in analytical platforms, biological matrices, disease stage, medication status, and clinical heterogeneity of patient populations [5, 6].

More recent studies have suggested that metabolomic patterns, including alterations in amino acids, may support future biomarker-oriented research in Parkinson's disease, particularly when combined with standardized clinical phenotyping and complementary biological matrices (e.g., cerebrospinal fluid) [11–13]. Nevertheless, the clinical relevance and reproducibility of plasma amino acid alterations require further investigation in well-characterized cohorts.

In this study, we aimed to investigate plasma amino acid alterations in patients with Parkinson's disease using a targeted LC–MS/MS approach. The objective was to provide a statistically controlled descriptive dataset of circulating amino acids in PD within an exploratory framework.

Materials and Methods

This study was a clinical case-control study. The research was conducted in a multidisciplinary manner between Sivas Cumhuriyet University Faculty of Medicine Neurology Clinic and Department of Medical Biochemistry. The plasma free amino acid concentrations were measured in Istanbul Ahenk laboratories. A priori power analysis (α=0.05, 1–β=0.80) indicated that 43 participants per group would provide 80.7% statistical power. Patients were diagnosed by experienced neurologists, and atypical parkinsonism was excluded clinically. The sample of the study was composed of patients clinically diagnosed with Parkinson's disease by experienced neurologists at Sivas Cumhuriyet University Faculty of Medicine Neurology Clinic between 01.12.2020 and 01.07.2021. Patients were recruited without any discrimination in terms of age and gender. All patients were evaluated by experienced neurologists at the Neurology Outpatient Clinic and diagnosed with Parkinson's disease based on clinical judgment, including characteristic motor symptoms and neurological examination findings. Patients with secondary parkinsonism, atypical parkinsonian syndromes, or other neurodegenerative diseases were excluded based on clinical assessment and medical history. Standardized diagnostic criteria (e.g., MDS or UK Brain Bank) were not formally documented in the dataset. Detailed clinical characteristics such as disease duration, disease stage, motor severity scores (e.g., UPDRS or Hoehn–Yahr), and dopaminergic treatment status were not systematically recorded and were therefore not included in the analysis.

Exclusion criteria

Participants with a history of major psychiatric or neurological disorders, head trauma, acute or chronic systemic diseases (including diabetes mellitus, chronic renal failure, malignancy, and hematological disorders), or secondary parkinsonism were excluded. In addition, individuals with neurodegenerative diseases other than Parkinson's disease or those using medications that may affect cognitive function were not included.

Ethics

Those who underwent anamnesis and physical examination and volunteers who signed the consent form created by the decision of Sivas Cumhuriyet University Faculty of Medicine Ethics Committee dated 04.09.2019 and numbered 2019-09/01 were included in the study. The study was conducted in accordance with the Helsinki Declaration.

Taking and storing samples

Blood samples were collected under routine clinical conditions. All samples were obtained after an overnight fasting period of at least 12 hours; however, dietary protein intake, circadian variation, and recent physical activity could not be fully standardized. After overnight fasting, venous blood (~10 mL) was collected into EDTA tubes. Samples were kept at room temperature for 5–10 minutes and centrifuged at 4000 rpm for 5 minutes. The plasma supernatant was aliquoted and stored at –80°C until LC–MS/MS analysis.

Free amino acid measurement method

Plasma free amino acids were quantified using a targeted LC–MS/MS approach. When the required number of patients for the study was reached, all samples were removed from

−80˚C and brought to room temperature. Samples were measured at once on an LC-MS/MS device using Jasem brand kits, using 20 standard amino acids and their metabolites. Analyses of plasma samples were performed on the Agilent HPLC system, consisting of a binary pump (G4220A), column chamber (G1316C), and autosampler (G4226A) coupled with a 6460 triple quadrupole mass spectrometer (6460A, Agilent Technologies, Santa Clara, CA, USA). CE-IVD (In vitro diagnostic) certified and validated Jasem Quantitative Amino Acids LC-MS/MS Analysis Kit was used for the measurement of free amino acid concentrations (Sem Laboratory). Mass spectrometry of the kit analytical method: Drying gas temperature: 150˚C, Drying gas flow: 10 L/min, Nebulizer pressure: 40 psi, Sheath gas temperature: 400˚C, Sheath gas flow: 10 L/min, Capillary voltage: 2000V. Appendix 1 isotope-labeled internal standards were used for each analyte. Calibration curves were generated using multi-point standard solutions, and quality control samples were analyzed at regular intervals to ensure analytical accuracy and precision.

Statistical analysis

The obtained data were uploaded to the SPSS 22.0 package program. In the analysis of the data, frequency tables of categorical data and descriptive statistics for quantitative variables were calculated to evaluate the data set. To evaluate quantitative variables with the appropriate test, a normality test was applied using the Shapiro-Wilk test. For difference analysis of quantitative variables suitable for normal distribution, an Independent samples t-test was applied for two-category comparisons. For difference analysis of quantitative variables that do not comply with normal distribution, the Mann-Whitney U test was applied for two-category comparisons, and the Kruskal-Wallis H test was applied for comparisons involving more than two categories. If a statistically significant difference was found as a result of multiple comparisons, the Mann-Whitney U test was used to investigate which two groups caused the difference. Analysis results were interpreted at the 5% significance level. ROC analysis was performed in an exploratory manner to evaluate the discriminatory performance of selected amino acids. Age was compared using an independent samples t-test. To account for multiple comparisons across amino acids, p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) procedure. Adjusted p-values (q-values) <0.05 were considered statistically significant. In addition to p-values, effect sizes were calculated to support the interpretation of the magnitude of group differences. Cohen's d was reported for normally distributed variables, while rank-biserial correlation (r) was used for non-normally distributed comparisons. Effect sizes were interpreted as small (0.2), medium (0.5), and large (0.8) for Cohen's d.

Results

In the study, the age range of a total of 86 volunteers, 43 patients and 43 healthy individuals, was calculated as 32–88 years. There were 18 (42.85%) women and 25 (57.15%) men in the control group (n=43), and 21 (48.83%) women and 22 (51.17%) men in the patient group. Age was compared using the independent samples t-test, and gender distribution was evaluated using the chi-square test. No statistically significant differences were found between the patient and control groups for age (p=0.295) or gender (p=0.790). Because multiple amino acids were tested simultaneously, p-values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) procedure; metabolites that remained significant after adjustment were classified as FDR-significant, whereas those significant only at the nominal level ($p<0.05$) were reported as nominal findings. Results were interpreted as statistically significant at an FDR-adjusted q-value <0.05. Findings that were significant only at the nominal level ($p<0.05$) but not after FDR correction were reported as nominally significant. Although several amino acids showed nominal statistical differences between groups, effect size estimates indicated that only a subset of these differences reached moderate-to-large magnitude, supporting cautious interpretation of biological relevance. Effect sizes were calculated to quantify the magnitude of group differences. Cohen's d was used for normally distributed variables, whereas rank-biserial correlation was reported for non-normally distributed variables. KEGG compound-based pathway enrichment analysis identified clustering within amino acid metabolism–related pathways, including arginine and proline metabolism and alanine, aspartate, and glutamate metabolism. KEGG compound-based pathway enrichment analysis was performed using FDR-significant metabolites (alanine, arginine, aspartate, proline, taurine, and threonine). Significant clustering was observed within amino acid metabolism–related pathways. After pathway-level FDR correction, the following pathways remained statistically significant: arginine and proline metabolism (FDR=0.031), taurine and hypotaurine metabolism (FDR=0.031), glycine, serine, and threonine metabolism (FDR=0.031), alanine, aspartate, and glutamate metabolism (FDR=0.031), and arginine biosynthesis (FDR=0.031). These results indicate coordinated alterations within interconnected amino acid metabolic networks.

Plasma amino acid levels

In our study, a normality test was applied to numerical variables. As a result of the normality test performed with the Shapiro-Wilk test, the suitability of all variables included in the analysis to normal distribution was calculated. Serine, valine, asparagine, methionine, histidine, phenylalanine, tryptophan, glycine, isoleucine, lysine, glutamine, leucine, and β-alanine measurements had a statistically normal distribution ($p>0.05$). Other variables (alanine, proline, threonine, tyrosine, aspartic acid, glutamic acid, arginine, phenylalanine/tyrosine) did not show a statistically normal distribution ($p<0.05$) (Table 1). After adjustment for multiple comparisons using the Benjamini–Hochberg FDR method, seven variables remained significantly different between PD patients and

Table 1. Plasma free amino acid concentrations in Parkinson's disease patients and controls (µmol/L)

Variable	Control (n=43) Mean±SE (min–max)	Patient (n=43) Mean±SE (min–max)	p	FDR q-value	Cohen's d
Alanine*	499.68±18.07 (246.04–782.10)	578.16±21.04 (254.38–1075.27)	0.006	0.017	0.60
Arginine*	120.39±10.39 (8.64–265.51)	83.03±3.74 (34.91–149.63)	0.001	0.005	-0.72
Asparagine	61.94±2.94 (31.10–122.29)	62.58±1.91 (29.17–87.02)	0.681	0.712	0.05
Aspartic acid*	60.23±5.76 (11.31–202.38)	34.29±2.36 (7.35–82.17)	<0.001	<0.001	-0.90
β-alanine	4.12±3.13 (0.30–4.93)	2.59±0.09 (0.52–2.87)	0.554	0.607	-0.13
Phenylalanine	110.54±5.42 (55.73–223.40)	99.89±2.95 (57.02–148.68)	0.089	0.135	-0.38
Glycine	351.83±16.52 (180.64–653.40)	372.93±17.33 (161.13–669.19)	0.383	0.466	0.19
Glutamic acid	287.54±29.61 (102.38–873.32)	216.67±11.87 (99.98–473.89)	0.030	0.069	-0.48
Glutamine	516.54±27.10 (19.53–791.39)	476.50±28.11 (31.62–781.65)	0.310	0.407	-0.23
Histidine	125.92±3.35 (67.61–166.98)	132.79±2.56 (94.13–206.96)	0.108	0.155	0.34
Isoleucine	135.28±4.70 (75.18–190.68)	145.29±3.67 (98.77–202.31)	0.184	0.256	0.27
Lysine	148.06±6.08 (84.42–211.66)	156.58±4.53 (87.86–248.61)	0.244	0.337	0.22
Leucine	134.41±3.58 (75.18–190.68)	145.28±4.66 (98.77–202.31)	0.508	0.571	0.15
Methionine	34.60±1.21 (17.97–54.69)	36.54±1.01 (19.47–52.64)	0.218	0.320	0.22
Proline*	230.96±10.54 (103.47–404.83)	329.58±14.67 (155.14–560.12)	<0.001	<0.001	1.20
Serine	219.67±8.24 (112.92–339.73)	200.27±7.65 (143.10–388.88)	0.088	0.135	-0.37
Taurine*	120.34±8.89 (47.79–293.65)	189.79±9.61 (47.82–338.74)	<0.001	<0.001	1.13
Tyrosine	82.68±4.13 (47.52–187.71)	93.35±3.52 (54.93–166.72)	0.047	0.093	0.42
Threonine*	164.44±6.31 (91.97–274.10)	209.28±7.13 (73.04–316.90)	<0.001	<0.001	1.00
Tryptophan	69.77±2.42 (39.60–103.27)	74.36±2.69 (27.74–118.55)	0.210	0.320	0.22
Valine	267.46±10.14 (110.39–400.82)	267.90±9.52 (131.20–396.76)	0.970	0.970	0.01
Phenylalanine/tyrosine*	1.345±0.070 (0.420–2.460)	1.110±0.038 (0.620–1.950)	0.002	0.009	-0.66
Tyrosine/phenylalanine*	0.747±0.050 (0.500–2.250)	0.935±0.045 (0.510–1.740)	0.003	0.010	0.64

*: Non-normally distributed variables analyzed using non-parametric tests. Values are presented as mean±standard error (SE) and minimum–maximum (min–max). p-values are from group comparisons; q-values indicate false discovery rate (FDR)-adjusted significance. Effect size is reported as Cohen's d.

controls: alanine (higher in PD), arginine (lower in PD), aspartic acid (lower in PD), proline (higher in PD), taurine (higher in PD), threonine (higher in PD), as well as the phenylalanine/tyrosine and tyrosine/phenylalanine ratios (both significantly altered). Other amino acids did not show statistically significant differences after FDR correction and were considered nominal. Glutamic acid showed a nominal difference between groups (p=0.030); however, this association did not remain statistically significant after false discovery rate (FDR) correction and was therefore interpreted as exploratory.

ROC analysis of selected amino acids

Exploratory ROC curve analyses were performed for amino acids showing nominal between-group differences to assess their discriminatory performance. Proline (AUC=0.794, 95% CI: 0.703–0.884), taurine (AUC=0.792, 95% CI: 0.697–0.886), and threonine (AUC=0.780, 95% CI: 0.685–0.876) demonstrated moderate discrimination. Alanine (AUC=0.664, 95% CI: 0.552–0.777) and tyrosine (AUC=0.621, 95% CI: 0.506–0.736) showed limited discrimination, while glutamic acid showed poor discrimination (AUC=0.558, 95% CI: 0.436–0.681). Amino acids with AUC<0.5 (aspartic acid and arginine) were considered to show inverse discrimination and were not interpreted as clinically meaningful. These ROC analyses were hypothesis-generating only and were not intended to establish diagnostic validity, particularly in the absence of external validation (Table 2). Amino acids with AUC<0.5 (aspartic acid and arginine) were considered to show inverse discrimination and are presented in Appendix 1.

Discussion

This study provides an exploratory cross-sectional comparison of plasma free amino acid concentrations between patients with Parkinson's disease (PD) and healthy controls. The findings should be interpreted as hypothesis-generating, as detailed clinical phenotyping (e.g., disease duration, severity scales, dopaminergic treatment status) and comprehensive pre-analytical standardization were not fully available. Therefore, the observed alterations cannot be interpreted as disease-specific metabolic signatures or as evidence of diagnostic utility.

Parkinson's disease is a progressive neurodegenerative disorder characterized by the degeneration of dopaminergic neurons [2]. Although amino acids are capable of crossing the blood–brain barrier [11], and plasma–CSF concentration ratios have been described, plasma amino acid levels do not directly reflect central neurotransmitter concentrations.

Table 2. ROC curve analysis of plasma amino acids for exploratory discrimination of Parkinson's disease

Amino acid	Cut-off (µmol/L)	Sensitivity	Specificity	p	AUC	95% CI	
						Lower	Upper
Alanine	652.86	0.191	0.045	0.007	0.664	0.552	0.777
Proline	380.09	0.298	0.023	<0.001	0.794	0.703	0.884
Threonine	258.67	0.191	0.023	<0.001	0.780	0.685	0.876
Glutamic acid	157.57	0.744	0.787	0.030	0.558	0.436	0.681
Tyrosine	133.03	0.085	0.023	0.047	0.621	0.506	0.736
Taurine	259.54	0.149	0.023	<0.001	0.792	0.697	0.886

Cut-off values are presented in µmol/L. ROC analyses were performed for exploratory purposes only and do not indicate diagnostic validity. CI: confidence interval; AUC: Area under the curve.

Cerebrospinal fluid (CSF) amino acid measurements may provide more direct insight into neurochemical alterations; however, CSF sampling was not feasible in the present cohort due to ethical and participant-related constraints. Consequently, mechanistic interpretations at the central nervous system level remain limited.

Several previous metabolomic investigations have reported altered amino acid profiles in PD. Decreased plasma levels of alanine, aspartic acid, arginine, phenylalanine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, serine, taurine, tyrosine, threonine, tryptophan, and valine, along with increased glutamine, have been described in certain cohorts [8–10, 13]. However, findings across studies are inconsistent. Previous studies have reported heterogeneous alanine-related findings in Parkinson's disease, including stage-related serum differences and inconsistent blood and cerebrospinal fluid (CSF) results [14–19]. For instance, Molina et al. [17] reported decreased CSF alanine levels and reduced CSF/plasma ratios, while the reduction in plasma alanine did not reach statistical significance. In our cohort, plasma alanine levels were significantly increased (p=0.006). These discrepancies across studies may reflect differences in analytical platforms, biological matrices, cohort characteristics, medication status, and disease heterogeneity. Disturbances in aromatic amino acid metabolism, including tyrosine- and phenylalanine-related changes, have also been reported in PD [20, 21]. In our study, tyrosine-related alterations were observed, although peripheral measurements cannot be assumed to reflect dopaminergic neurotransmission directly. Similarly, previous studies have described inconsistent findings regarding aspartate and glutamate levels in PD, with both increases and decreases reported across plasma/serum and CSF studies [15, 16, 18, 19]. In the present analysis, plasma aspartic acid and glutamic acid levels were significantly decreased. Given the absence of CSF or functional neurochemical data, these alterations should be interpreted as peripheral metabolic variation rather than central excitotoxic mechanisms.

Arginine metabolism has also been implicated in neurodegenerative processes. Prior studies and meta-analytic evidence suggest that arginine-related alterations may occur in Parkinson's disease, although findings vary across biological matrices and cohorts [14, 19]. In line with these observations, plasma arginine levels were significantly decreased in our cohort (p=0.001). Taurine alterations have been variably reported; Engelborghs et al. [22] reported lower CSF taurine levels in PD, whereas in our cohort taurine levels were significantly increased. These divergent findings further underscore the heterogeneity of amino acid alterations in PD populations. In a previous study, reduced plasma levels of aspartate (Asp), glutamate (Glu), and taurine (Tau) were reported in early Parkinson's disease, and ROC analyses suggested potential discriminatory value for these amino acid neurotransmitters, with AUC values of 0.871, 0.882, and 0.845, respectively [15]. In our study, corresponding AUC values were lower (0.283, 0.558, and 0.792). Several amino acids demonstrated AUC values below 0.5, indicating poor or inverse discriminatory performance. ROC analyses in the present study were conducted solely to assess signal magnitude and should not be interpreted as evidence of diagnostic or biomarker validity, particularly in the absence of external validation cohorts or clinically anchored endpoints. Figura et al. [14] reported stage-related changes in serum threonine levels in PD. In our cohort, threonine levels were significantly elevated (p<0.001). While alterations in threonine and related amino acids may reflect systemic changes in protein or intermediary metabolism, direct mechanistic links to neurodegeneration require validation in studies incorporating CSF, imaging, or tissue-based analyses.

In essential tremor, altered plasma aspartate, glutamate, and taurine levels have also been described [23]. β-alanine is biochemically related to inhibitory neurotransmission and amino acid metabolism [24]. In our cohort, aspartic acid levels were significantly decreased, while β-alanine showed a non-significant reduction. These observations may indicate coordinated alterations within related metabolic pathways; however, without enzymatic, transcriptomic, or central nervous system–specific data, such interpretations remain speculative. Glutamate-related pathways have frequently been discussed in the context of PD pathophysiology. However, since the majority of glutamic acid is utilized within tissues and peripheral concentrations are relatively low, plasma measurements cannot be assumed to mirror synaptic glutamatergic transmission. Therefore, peripheral amino acid alterations should not be extrapolated directly to central neurotransmission [25–27].

Overall, this study adds to the growing body of targeted LC–MS/MS-based investigations evaluating plasma amino acid alterations in PD. The findings provide descriptive evidence of coordinated peripheral metabolic variation. However, given the cross-sectional design, plasma-only measurements, and limited clinical characterization, the results should be regarded as exploratory and hypothesis-generating rather than definitive.

Limitations

This study has several limitations. First, the cross-sectional case–control design precludes causal inference and does not allow longitudinal assessment of metabolic changes. Second, detailed clinical phenotyping including disease duration, diagnostic criteria (e.g., MDS or UK Brain Bank), Hoehn–Yahr stage, UPDRS scores, and dopaminergic treatment status was not retained in the analytical dataset, limiting stratified interpretation. Third, although samples were collected under standardized fasting conditions, additional pre-analytical variables such as dietary protein intake, circadian variation, and recent physical activity were not systematically controlled. Fourth, only plasma samples were analyzed; CSF, urine, or imaging biomarkers were not available. Fifth, the targeted LC–MS/MS platform covered a limited metabolite panel, constraining pathway-level inference. Finally, multiple comparisons were performed and ROC analyses were exploratory; therefore, findings should be interpreted cautiously and require confirmation in independent, clinically well-characterized cohorts before any clinical relevance can be inferred.

Conclusion

In this exploratory cross-sectional case-control study, targeted LC–MS/MS profiling identified statistically significant alterations in selected plasma free amino acids in patients with Parkinson's disease compared with healthy controls. The observed differences were consistent with previously reported metabolic heterogeneity in PD, although the direction and magnitude of specific amino acid changes varied across studies.

Given the absence of detailed clinical phenotyping, standardized diagnostic documentation, and complementary cerebrospinal fluid or functional measurements, the present findings should be interpreted as descriptive and hypothesis-generating. Peripheral plasma amino acid alterations cannot be directly extrapolated to central neurochemical processes, nor do the current results support diagnostic, prognostic, or treatment-guiding application.

The primary contribution of this study is the provision of a targeted, analytically validated plasma amino acid dataset generated under a standardized LC–MS/MS framework. Independent replication in larger, clinically well-characterized, and longitudinal cohorts is required before biological or clinical implications can be established.

Disclosures

Ethics Committee Approval: The study was approved by the Sivas Cumhuriyet University Faculty of Medicine Ethics Committee (no: 2019-09/01, date: 04/09/2019).

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Conflict of Interest Statement: None declared.

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Appendix 1. ROC analyses with inverse discrimination (AUC<0.5)

Amino acid	Cut-off (μmol/L)	Sensitivity	Specificity	p	AUC	95% CI	
						Lower	Upper
Aspartic acid	19.45	0.872	0.818	<0.001	0.283	0.172	0.395
Arginine	54.13	0.936	0.773	0.019	0.357	0.230	0.484

AUC <0.5 indicates inverse discrimination and was not considered clinically meaningful. CI: confidence interval; AUC: Area under the curve.

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Research Article

Leap-2 and irisin in the pathophysiology of Type 2 diabetes mellitus

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Abstract

Objectives: Insulin resistance is one of the main reasons responsible for the pathogenesis of Type 2 Diabetes Mellitus (T2DM). LEAP2 functions as an endogenous antagonist of the ghrelin receptor and is associated with insulin resistance. Irisin is a thermogenic myokine that causes energy expenditure by converting white adipose tissue into brown adipose tissue. Based on this information, we aimed to reveal the possible relationship between Leap2, irisin levels, and insulin resistance in newly diagnosed T2DM patients

Methods: Our study consisted of 82 patients newly diagnosed with T2DM and 74 Healthy control groups who do not use any medication. Leap2 and irisin levels were measured using the enzyme-linked immunosorbent assay method.

Results: Compared to the control group, we found the serum irisin levels significantly lower in the diabetic group. LEAP2 levels were significantly higher in the diabetic group. In the patient group, we found a negative correlation between irisin levels and HOMA-IR and insulin levels and a positive correlation with HDL. On the contrary, we found a positive correlation between LEAP2 levels and HOMA-IR, insulin, and triglyceride levels.

Conclusion: In patients with T2DM, LEAP2 levels are higher and irisin levels are lower than in healthy people. Various molecules have been the target of many studies on maintaining glucous homeostasis, and preventing and improving diabetes mellitus. Therefore, the role of adipomyokines in T2DM and insulin resistance should be further investigated. To our knowledge, this study will be the first report correlating T2DM, LEAP2, and irisin levels and HOMA-IR in humans.

Keywords: Diabetes mellitus, HOMA-IR, irisin, Leap2

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Diabetes mellitus (DM) is responsible for over 3.4 million deaths annually [1]. According to the International Diabetes Federation (IDF), around 589 million people worldwide are currently affected by diabetes, and this number is projected to rise to approximately 784 million by 2045 [2]. The most characteristic feature of DM is hyperglycemia [3].

In patients with type 2 diabetes mellitus (T2DM), there are at least two main pathological mechanisms. The first, known as insulin resistance, involves decreased insulin activity in peripheral tissues and is widely recognized as a primary underlying cause. The second mechanism, β-cell dysfunction, refers to the pancreas's inability to secrete sufficient insulin to compensate for insulin resistance [4]. In general, insulin deficiency or the ineffectiveness of insulin, even at adequate levels, constitutes the core issue in the pathogenesis of the disease. Recent studies have demonstrated a direct relationship between these factors and adipokines [5–9].

Irisin is produced from fibronectin type III domain-containing 5 (FNDC5), a transmembrane protein cleaved by specific proteases. Irisin, a thermogenic protein, promotes energy expenditure by converting white adipose tissue into brown adipose tissue. First identified in muscle tissue, irisin has a molecular weight of 12 kDa and comprises 112 amino acids. It is classified as a myokine secreted by skeletal muscles and is known to of-

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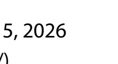

fer protective effects against metabolic diseases when stimulated by regular exercise [10]. While irisin is thought to protect against diabetes and obesity, it is also associated with muscle mass and insulin sensitivity [11, 12]. Irisin has been studied in various conditions, including obesity, T2DM, cardiovascular diseases, chronic renal failure, non-alcoholic steatohepatitis (NASH), and polycystic ovary syndrome (PCOS). Serum irisin levels are reported to be lower in conditions such as cardiovascular disease, T2DM, and NASH compared to healthy controls [13]. Additionally, studies on diabetic mice have shown that increased irisin expression improves glucose tolerance and reduces fasting insulin levels, suggesting its potential as a therapeutic target for T2DM and obesity [10].

Liver-expressed antimicrobial peptide 2 (LEAP2), first identified in 2003, is expressed primarily in the liver and small intestine, with its secretion being suppressed during fasting. LEAP2 acts as an endogenous antagonist of the growth hormone secretagogue receptor (GHSR), which is activated by ghrelin. By inhibiting GHSR, LEAP2 counteracts ghrelin's effects on growth hormone release, appetite stimulation, and glucose elevation during fasting [14]. The evidence that LEAP2 has appetite-suppressing properties and can modulate insulin secretion raises the question of whether this peptide may play a role in diabetes [15].

Interestingly, plasma LEAP2 levels are positively correlated with plasma glucose, HOMA-IR, body mass index (BMI), body fat percentage, serum triglycerides, and the visceral-to-subcutaneous adipose tissue ratio [16, 17]. Given the increasing prevalence of obesity-related metabolic disorders, including T2DM, LEAP2 has garnered attention as a potential regulator of glucose homeostasis and metabolic balance [15].

Based on this information, we conducted the first comparative study of LEAP2 and irisin in T2DM. While irisin has been widely studied, LEAP2 remains a relatively new focus of investigation. The aim of this study is to analyse circulating concentrations of irisin and LEAP2 key regulators of energy balance and glucose metabolism in type 2 diabetes mellitus (T2DM) and to investigate their relationship with insulin resistance. In this way, we seek to clarify the role of LEAP2 and irisin in the pathophysiology of T2DM and to explore their potential value as biomarkers.

Materials and Methods

This study was conducted in collaboration with the Internal Medicine Clinic and Biochemistry Laboratory at Elazig Fethi Sekin City Hospital, with ethical approval obtained from Fırat University Ethics Department. All participants were informed about the study, and written consent was obtained. This study was conducted in accordance with the ethical principles of the Helsinki Declaration. Individuals with any systemic disease (e.g., coronary heart disease, liver disease, acute or chronic renal failure, malignancy), patients younger than 18 years, and those previously diagnosed diabetes were excluded from the study. Detailed histories were taken, including age, height, and weight, for all participants. Our study included 82 patients (42 females, 40 males) newly diagnosed with T2DM and 74 healthy controls (37 females, 37 males) based on the 2021 diagnostic criteria of the International Diabetes Federation (IDF). Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2), and HOMA-IR was calculated using the formula: İnsulin (µU/ml) x glucose (mg/dl) / 405.

Blood samples were collected from both groups after 10 hours of fasting into three tubes: One with gel for biochemistry, one containing EDTA, and one with aprotinin (BD Vacutainer; Becton, Dickenson and Co., Franklin Lakes, NJ, USA). Blood samples taken into biochemistry tubes were centrifuged for 15 minutes at 3500 rpm from serums obtained; glucose, insulin, lipid parameters, EDTA from the tube containing whole blood HbA1c (by HPLC method) were studied on the same day. Blood samples taken into aprotinin-containing tubes were centrifuged at 3500 rpm for 10 minutes and the resulting plasmas were stored at -20°C until the study day to study LEAP2 and irisin.

The glucose and lipid parameters were measured using the AU5800 analyzer (Beckman Coulter, Inc., Miami, FL, USA). Insulin levels were analyzed with the DXI800 (Beckman Coulter, Inc., Miami, FL, USA) immunoassay system, and HbA1c levels were assessed using the Premier HB920 (Trinity Biotech, Ireland) device.

Plasma levels of LEAP2 and irisin were determined using ELISA kits specific for each protein (Sunred, Cat. Number: SRB-T-81190; Sunred, Cat. Number: 201–12–5328, Shanghai, China). Absorbance was measured using a Chromate 4300 Microplate Reader. The minimum detection limit for LEAP2 and irisin was 0.438 pg/ml, 0.157 ng/ml, respectively. The intra-assay and inter-assay coefficients of variation for LEAP2 and irisin measurements were <10% and <12%, respectively.

Statistical analysis was performed using SPSS. Data were expressed as mean±standard deviation. The Shapiro-Wilk test was used to determine the normality of the data distribution. The Chi-square test was employed for the evaluation of categorical data. Normally distributed data were analyzed using the Students t-test, while non-normally distributed variables were compared using the Mann-Whitney U test, with median values provided. A partial correlation analysis was conducted after adjusting for such as age, BMI, and sex to evalute the relationships between the variables. A p-value <0.05 was considered statistically significant.

Results

Laboratory, demographic, and clinical data are summarized in Table 1. There were no significant differences between groups in gender distribution or age. However, in terms of Body Mass Index (BMI), the diabetic group was significantly higher than the control group (p<0.001). Cholesterol, LDL, and triglyceride levels were significantly higher in the diabetic group (p<0.05 for LDL, p<0.001 for others), while HDL levels were higher in

Table 1. Laboratory and demographic data of the groups

	Control n=74 Mean±SD	T2DM n=82 Mean±SD	p
Age* (year)	46.9±7.7	48.1±5.3	>0.05
İrisin (ng/mL)	30.28±14.7	23.12±12.6	**<0.01**
Leap2 (ng/mL)	9.96±4.68	11.13±4.59	**<0.05**
Glucose (mg/dL)	89.6±7.96	190.2±58.3	**<0.001**
Cholesterol* (mg/dL)	188.9±27.9	208.8±32.3	**<0.001**
HDL (mg/dL)	46.65±8.27	41.17±7.69	**<0.001**
LDL* (mg/dL)	116.7±23.8	129.4±28.1	**<0.05**
Triglyceride (mg/dL)	128.7±52.1	190.4±65.3	**<0.001**
Insulin (mIU/L)	8.91±5.36	11.3±5.87	**<0.05**
Hba1C (%)	5.51±0.42	8.4±1.62	**<0.001**
BMI (kg/m^2)	24.62±2.37	28.99±3.07	**<0.001**
HOMA-IR	1.98±1.29	5.64±4.43	**<0.001**

*: Normal distribution according to the Shapiro-Wilk test. HDL: High-density lipoprotein; LDL: Low Density lipoprotein; Hba1C: A hemoglobin A1C test; BMI: Body mass index; HOMA-IR: Homeostasis model assessment-estimated insulin resistance

the control group (p<0.001). Serum glucose and HbA1c levels were found to be significantly higher than the control group (p<0.001). In addition, serum insulin levels were found to be significantly higher in the diabetic group compared to the control group (p<0.05). HOMA-IR values were also significantly higher in the diabetic group (p<0.001).

Regarding the studied biomarkers, serum irisin levels were significantly lower in the diabetic group compared to controls (p<0.01), whereas serum LEAP2 levels were significantly higher in the diabetic group (p<0.05).

A partial correlation analysis was performed controlling for age, BMI, and sex to evaluate the relationships between variables. In the patient group, irisin levels were negatively correlated with HOMA-IR (r=-0.236 p=0.034) and insulin (r=-0.231, p=0.038) levels, and positively correlated with HDL (r=0.343 p=0.002). On the contrary, a positive correlation was found between Leap2 level and HOMA-IR (r=0.422, p=0.000), insulin (r=0.469, p=0.000) and triglyceride (r=0.272, p=0.015) levels (Figs. 1, 2).

In the control group, irisin levels showed a negative non-significant association with HOMA-IR (r=-0.209 p=0.08) and insulin (r=-0.220, p=0.06) levels, and a weak positive correlation with HDL (r=0.234 p=0.049). In contrast, Leap2 levels showed non-significant positive association with HOMA-IR (r=0.203, p=0.09), insulin (r=0.199, p=0.096) and, triglyceride (r=0.182, p=0.12).

Discussion

The global prevalence of type 2 diabetes mellitus has reached epidemic proportions, currently affecting over 400 million individuals, with further increases anticipated in the coming decades [18]. These alarming projections highlight an urgent need for novel preventive and therapeutic strategies to combat the rising burden of T2DM worldwide [19]. Central to the pathophysiology of T2DM is insulin resistance, which disrupts cellular signaling and impairs key metabolic processes. This resistance primarily manifests in skeletal muscle, liver, and adipose tissues, playing a pivotal role in the development of the disease. Exploring the endocrine functions of adipose tissue and the involvement of adipokines in insulin resistance is essential for understanding the underlying mechanisms of diabetes and other chronic metabolic disorders [20]. Among the many signaling molecules derived from adipose tissue, irisin and LEAP2 have emerged as key regulators with potential opposing roles in metabolic balance. Understanding the interplay between these molecules could provide crucial insights into the pathogenesis of T2DM.

Irisin has been shown to enhance glucose uptake by tissues and promote glycogen storage while suppressing gluconeogenesis and glycogenolysis. Additionally, it facilitates fatty acid oxidation, playing a multifaceted role in maintaining glucose homeostasis [21–23]. Through its direct and indirect effects on adipose, muscle, liver, and pancreatic tissues, irisin regulates energy metabolism and improves insulin sensitivity, particularly in response to exercise [24–26]. This effect is mediated via the activation of Peroxisome Proliferator-Activated Receptor Gamma Coactivator-1 Alpha (PGC-1α), whose expression and activity are often reduced in T2DM patients, as evidenced by their low irisin levels [27, 28].

In this study, irisin levels were found to be significantly lower in patients with T2DM compared with the healthy control group. In many studies, irisin levels have been found to be lower in newly diagnosed T2DM compared to the control group [29–32]. In addition, a recent study reported that irisin levels in patients with T2DM, obesity, and hypertension were negatively correlated with fasting insulin and HbA1C levels compared to the control group. Therefore, it has been found to be associated with the risk of metabolic syndrome and

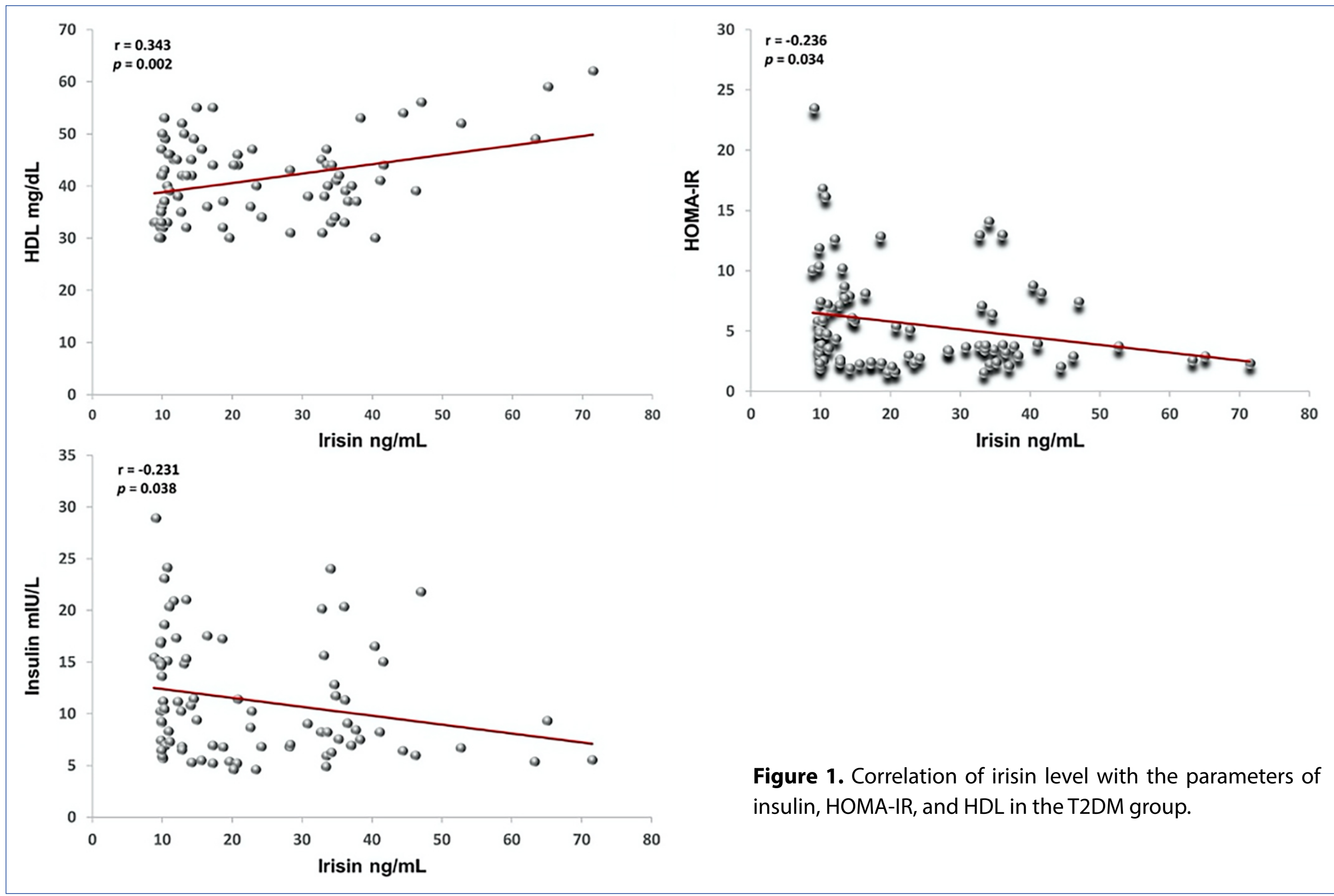

Figure 1. Correlation of irisin level with the parameters of insulin, HOMA-IR, and HDL in the T2DM group.

hyperglycemia in adults. Consistent with these findings, a cross-sectional study published in 2025 reported that irisin levels were lower in individuals with diabetes independent of BMI, and that low irisin was associated with an unfavorable lipid profile (low HDL cholesterol and high triglycerides) and insulin resistance [29, 33]. However, there are also studies reporting positive relationships between irisin and insulin levels, glucose, and HOMA-IR [11, 12, 34, 35]. Interestingly, the reasons for the discrepancy between irisin-HOMA-IR in the literature are not yet understood. In our study, irisin levels were modestly and negatively correlated with HOMA-IR and insulin. In addition, we also obtained a negative correlation with metabolic syndrome parameters consisting of fasting glucose, fasting insulin, HbA1C, triglyceride, and BMI. We believe that this result once again demonstrates the importance of irisin in terms of metabolic syndrome. In contrast, our interpretation regarding studies reporting a positive correlation with irisin is that these discrepancies in the literature are most likely attributable to methodological and analytical variability (including differences in measurement kits/assays and pre-analytical sample handling), genetic differences, and population characteristics (such as diabetes duration, medication use, and physical activity).

This study also demonstrated that LEAP2 levels were significantly higher in patients with T2DM compared with the healthy control group. LEAP2 is expressed in many organs and tissues such as the liver, stomach, duodenum, jejunum, and kidneys. Recent studies have reported that the administration of LEAP2 in mice inhibits ghrelin-induced GHSR activation, thereby blocking the main effects of ghrelin. In contrast, it is thought that the effect of ghrelin increases with the blocking of endogenous LEAP2, thus LEAP2 adjusts the effect of ghrelin *in vivo* [16, 17].

There are very few studies on LEAP2 in the literature, and HOMA-IR comparisons are with obesity [36]. Our study showed that LEAP2 levels were positively correlated with HOMA-IR in T2DM, similar to what has been reported in obesity. The positive correlation observed between LEAP2 and HOMA-IR suggests that this peptide may play an active role in the pathophysiology of insulin resistance. When our study group is examined, we think that the BMI values of our subjects are lower than previous studies and the results cannot be attributed to obesity. In terms of diabetes metabolism, we have general information that it is largely synthesized in the liver, released into the blood and partially eliminated by the kidneys [17]. In addition, we believe that LEAP2 levels are not only associated with obesity but also with insulin resistance, as in our study. Recent studies have shown that LEAP2 is regulated by glucagon and insulin. Johansen et al. [37] reported that LEAP2 concentrations decrease significantly during

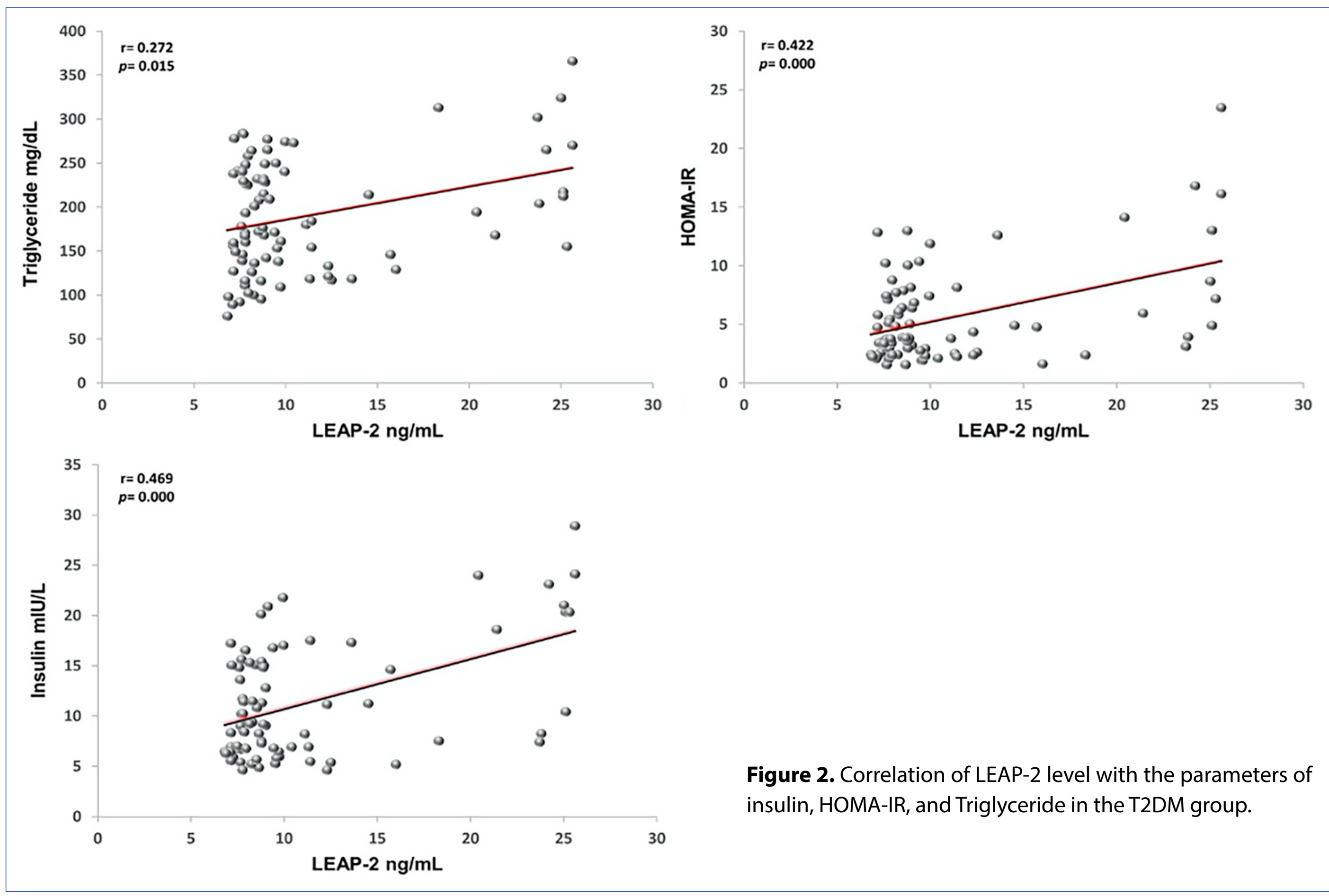

Figure 2. Correlation of LEAP-2 level with the parameters of insulin, HOMA-IR, and Triglyceride in the T2DM group.

glucagon infusion. This finding is relevant for understanding the hormonal control of energy homeostasis in T2DM.

In recent years, the regulatory role of LEAP2 in energy balance, appetite control and glucose metabolism has become increasingly clear. In particular, this peptide has been shown not only to act as a ghrelin antagonist but also as an inverse agonist of the growth hormone secretagogue receptor, thereby suppressing its basal activity [38]. Consistent with previous studies reporting elevated LEAP2 levels in association with insulin resistance and metabolic stress, our study identified increased LEAP2 levels in the T2DM group, which were significantly correlated with HOMA-IR. This association suggests that this peptide may serve not only as a biomarker but also as a candidate molecule involved in metabolic regulator [14]. Furthermore, recent data indicate that insulin increases LEAP2 secretion in the postprandial period, whereas glucagon suppresses it [38]. Thus, the elevated LEAP2 concentrations detected in T2DM may reflect both a response to insulin resistance and the impact of chronic hyperglycaemia on hepatic signalling pathways. Taken together, these observations reinforce the consistency of our findings with the existing literature and position LEAP2 as a potential diagnostic and therapeutic target in T2DM. These findings raise the question of whether LEAP2 is associated with metabolic syndrome, PCOS, and other diabetic phenotypes in subsequent studies. In addition, it is considered that LEAP2 may play a role in the regulation of the anabolic response in the metabolic environment and may modulate the anabolic response in association with irisin. For this purpose, more comprehensively designed studies are needed to more clearly explain the metabolic relationships, pathways, and mechanism of action of this new agent, which is therapeutically promising, especially for Diabetes.

Limitations of the Study

This study has several limitations. First, as a cross-sectional study, it cannot establish a causal relationship between circulating levels of LEAP2, irisin, and T2DM. Second, the sample size of this research is relatively small and is limited to a single-center cohort. Larger, multicenter studies are required to validate these findings and improve their generalizability. Third, genetic, nutritional, and environmental differences specific to the population studied may introduce biases and variability in the results. Future research should include (i) longitudinal and interventional designs, (ii) larger and ethnically diverse cohorts, (iii) standardized analyses for both irisin and LEAP2, and (iv) studies investigating interactions at the receptor and intracellular signaling levels. These factors should be considered in future studies to better understand the influence of these variables on LEAP2 and irisin levels.

Conclusion

In our literature review, we could not find any comparative studies on the relationship between T2DM, LEAP2, irisin levels, and HOMA-IR in humans. To our knowledge, this study will be the first report comparing both adipomyokines and associating them with HOMA-IR.

LEAP2 levels are higher and irisin levels are lower in patients with T2DM than in healthy people. Maintaining glucose homeostasis has been the goal of many studies aimed at preventing and improving diabetes mellitus. In the future, LEAP2 and irisin could become key molecular targets for the development of strategies aimed at preventing T2DM and its comorbidities. However, how irisin and LEAP2-based interventions might be standardised in terms of clinical efficacy remains an open question. Therefore, the role of adipomyokines in T2DM and insulin resistance should be investigated further.

Disclosures

Ethics Committee Approval: The study was approved by the Fırat University Ethics Committee (no: 25, date: 01/08/2019).

Informed Consent: Informed consent was obtained from all participants.

Conflict of Interest Statement: The authors declare that there is no conflict of interest regarding the publication of this article.

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Research Article

Protective effect of boric acid against LPS-induced oxidative damage and inflammation in HaCaT keratinocytes: A simultaneous treatment approach

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Abstract

Objectives: Psoriasis is a chronic inflammatory disease characterized by epidermal dysregulation and increased oxidative stress. This study investigated the protective effects of boric acid (BA) in HaCaT keratinocytes exposed to lipopolysaccharide (LPS)-induced acute inflammatory redox stress using a simultaneous treatment protocol.

Methods: HaCaT cells were assigned to control, LPS (10 µg/mL or 200 ng/mL), BA (20 or 100 µM), and simultaneous treatment (LPS + BA) groups. Cell viability was assessed by CCK-8 assay. Oxidative status was evaluated by malondialdehyde (MDA) and advanced oxidation protein products (AOPP), total sulfhydryl (TSH), and antioxidant parameters [superoxide dismutase (SOD) and catalase (CAT)]. Cell migration was analyzed by a wound healing assay.

Results: LPS exposure did not cause overt cytotoxicity at 24 h but was associated with increased MDA and reduced CAT activity, indicating inflammatory oxidative stress. AOPP levels did not show a marked change under these acute conditions. Simultaneous BA administration maintained keratinocyte viability and attenuated LPS-associated lipid peroxidation, while partially restoring antioxidant defenses and improving wound closure.

Conclusion: BA modulates oxidative stress markers and supports antioxidant defense and migratory capacity in LPS-stimulated keratinocytes. These findings support BA as a candidate redox-modulating compound that warrants validation in immune-competent and in vivo models relevant to psoriasis.

Keywords: Antioxidant defense, boric acid, HaCaT keratinocytes, inflammation, LPS (Lipopolysaccharide), oxidative stress, psoriasis

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Psoriasis is a chronic, systemic inflammatory dermatosis affecting 2–3% of the global population, characterized by a complex interplay of genetic susceptibility, immune dysregulation, and keratinocyte hyperproliferation [1, 2]. Pathogenesis is primarily driven by the aberrant activation of the T-cell/cytokine axis, which triggers a sustained inflammatory cascade within the epidermal microenvironment [3].

Boron is a trace non-metal element for organisms. Boron exists as inorganic salts and organic esters, with boric acid (BA) being its primary form in humans [4]. While inorganic BA is systemically absorbed and excreted unchanged [5], it may exert cytotoxic effects on the microbiota [6]. Conversely, organic boron esters are largely indigestible, with over 95% reaching the colon intact. This highlights their potential as prebiotics that support host-microbiota symbiosis and justify the development of boron-based nutraceuticals targeting the microbiome [4, 6]. BA, a naturally occurring bioactive compound, exhibits significant antioxidant and anti-inflammatory proper-

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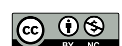

ties. Recent evidence suggests that boron can modulate redox homeostasis, scavenge reactive oxygen species (ROS), and enhance wound healing, making it a potential candidate for treating inflammatory skin disorders [4, 6]. However, its specific role in protecting keratinocytes during the acute phases of inflammatory activation mimicking psoriasis flares remains insufficiently characterized.

Beyond classic immunological pathways, oxidative stress has emerged as a fundamental driver of psoriasis progression. An imbalance between excessive ROS production largely from activated neutrophils and keratinocytes and the depletion of endogenous antioxidant defenses (e.g., SOD, CAT, and sulfhydryl compounds) leads to lipid peroxidation and cellular dysfunction [7, 8]. While oxidative stress is central to the disease, clinical biomarker studies often yield inconsistent results due to variability in disease stages and severity, highlighting the need for controlled experimental models to evaluate redox-targeted interventions [9, 10].

Using lipopolysaccharide (LPS)-stimulated HaCaT keratinocytes as a well-established model of inflammatory and oxidative injury, this study investigates the protective efficacy of BA. By employing a simultaneous treatment protocol, we aimed to evaluate the impact of BA on cell viability, antioxidant enzyme kinetics, and migratory capacity. Our findings seek to elucidate whether BA can serve as a prophylactic or early-intervention agent to mitigate oxidative damage and inflammatory signaling in psoriasis-like conditions. Importantly, psoriasis pathogenesis is primarily driven by the IL-23/Th17 axis; therefore, the LPS–HaCaT system is used here to model acute inflammatory and oxidative (redox) stress in keratinocytes rather than to reproduce the full psoriatic immune microenvironment.

Materials and Methods

Keratinocyte culture

The human keratinocyte cell line HaCaT was obtained from the Cell Line Service, Heidelberg, Germany. The cells were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidification in complete RPMI 1640 Medium (RPMI 1640; Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Cell viability assays

HaCaT cells were grown in 96-well plates at a density of 15×10^3 cells/well in complete RPMI 1640 medium. Cells were treated with 10 µg/ml and 200 ng/ml LPS and BA at concentrations of 20, 100 µM for 24 h. Cell viability was determined by using a colorimetric CCK8 (Abbkine, China) assay. Briefly, CCK8 solution was added to the cell culture at 10 µL. After 3 h of incubation, the obtained optical density was measured at 450 nm by using a microplate reader (Bio-Tek, Winooski, VT, USA). All experiments were performed in at least three independent biological replicates (n=3) to ensure reproducibility.

Wound healing assay

Cells were seeded at a high density into 6-well plates. After allowing the cells to adhere and reach 80% confluence, a scratch was created in the center of each well using a sterile 100 µL pipette tip to simulate a wound, followed by washing with 1x PBS [11]. At this stage, the wound was examined under a microscope, and the initial image was recorded. Subsequently, LPS and BA were administered at predetermined doses. The control group received an equivalent volume of medium. Wound closure kinetics were monitored at 24 h post-scratching using an inverted microscope. The denuded areas were digitized, and the migration rate was determined by calculating the change in wound width over time. The extent of wound closure was quantified by measuring the mean distance between the wound edges at multiple representative points, and the results were normalized to the initial baseline (0 h) measurements.

Measurement of oxidative stress biomarkers

All biochemical analyses were performed using cell lysate samples. The second indicator, malondialdehyde (MDA) levels were determined by a modified thiobarbituric acid (TBA) method [12]. Both assays were adapted to the cell lysate matrix and reaction volumes were optimized to allow microplate format. To evaluate the damage caused by oxidative stress on protein structures, advanced oxidative protein products (AOPP) levels were determined spectrophotometrically. In this assay, the method described by Hanasand et al. [13] was adapted to the cell lysate matrix and the analysis conditions were optimized by making the necessary modifications to enable microplate format. Total thiol (–SH) content was determined according to the colorimetric method described by Sedlak and Lindsay [14]. Catalase (CAT) activity was measured spectrophotometrically using the method of Aebi [15], which is based on the rate of hydrogen peroxide (H_2O_2) decomposition. The decrease in absorbance of H_2O_2 over time was monitored, and enzyme activity was calculated accordingly. Superoxide dismutase (SOD) levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (AbbKine, Inc., Cat No: KTB1030, Wuhan, China) according to the manufacturer's instructions in cell lysate.

Statistical analysis

The results were presented as means±standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 8 software (San Diego, USA). ANOVA was performed to compare the quantitative data across the groups. When appropriate, post hoc comparisons were performed using Tukey's multiple comparison test. Statistical significance for all analyses was set at a significance level of $p<0.05$.

Results

Cell viability

The effects of LPS, BA, and their combinations on HaCaT keratinocyte viability and migratory capacity were evaluated after

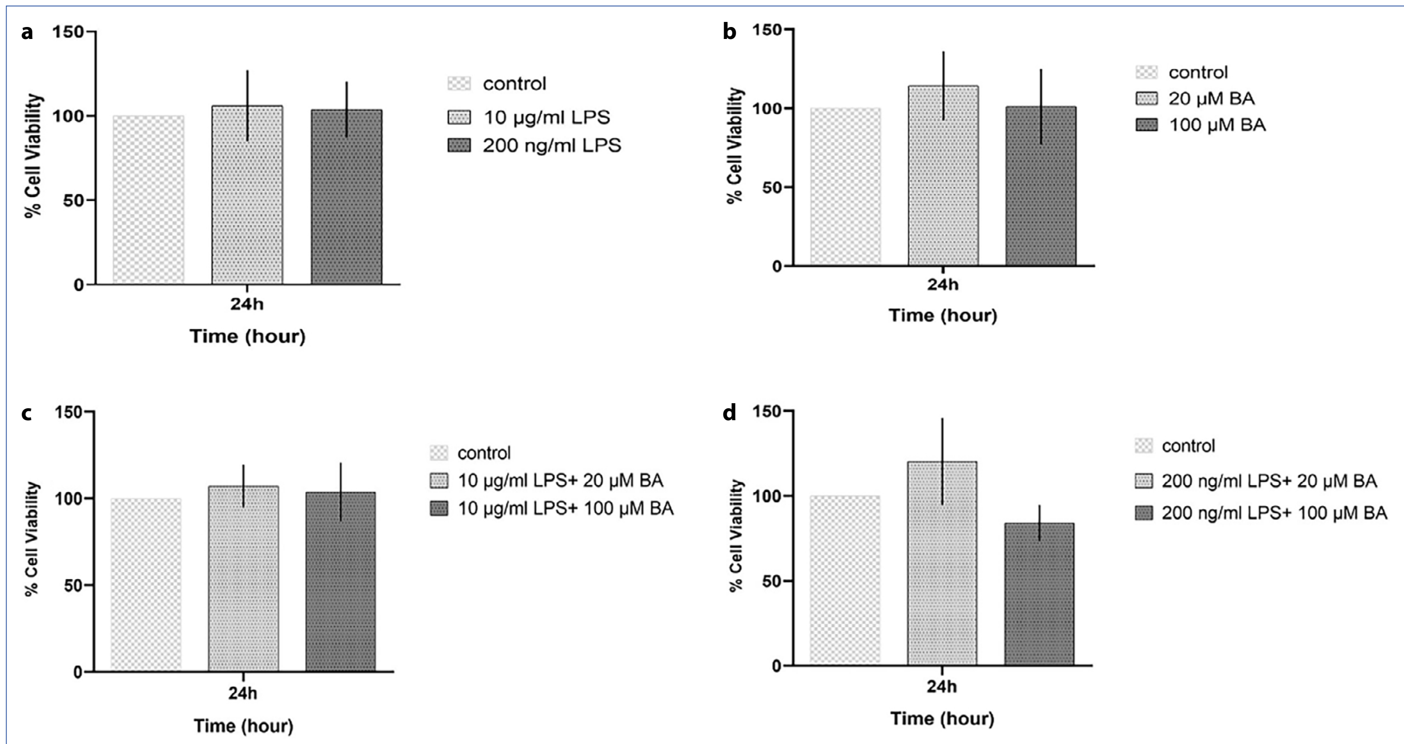

Figure 1. HaCaT cells were treated with different concentrations and combinations of LPS and Boric acid for 24 h and percentage cell viability was determined from CCK-8 results.

LPS: Lipopolysaccharide.

24 h of treatment. As shown in Figure 1, exposure of HaCaT cells to LPS and BA at different concentrations, either alone or in combination, did not induce overt cytotoxicity after 24 h. Cell viability remained comparable to control levels across most treatment groups. Notably, co-treatment with lower concentrations of BA in the presence of LPS resulted in preserved or slightly increased cell viability, indicating that BA does not adversely affect keratinocyte survival under inflammatory conditions at the tested doses.

Cell migration was assessed using the wound healing assay, and representative light microscope images (4× magnification) are presented in Figure 2. At 24 h, LPS-treated cells exhibited delayed wound closure compared with the control group, reflecting impaired migratory capacity. In contrast, cells treated with BA, either alone or in combination with LPS, demonstrated enhanced wound closure, suggesting improved migratory behavior. Quantitative analysis of wound diameter changes at 0 and 24 h is shown in Figure 3. LPS treatment significantly inhibited wound closure compared with control, whereas BA co-treatment significantly reduced wound diameter and accelerated gap closure. The improvement in wound healing observed in BA-treated groups was statistically significant ($p<0.001$), indicating that BA effectively counteracts the inhibitory effects of LPS on keratinocyte migration.

Biochemical parameters

The effects of LPS, BA, and their simultaneous administration on oxidative stress markers and antioxidant defense parameters in HaCaT keratinocytes after 24 h are shown in Figure 4. MDA levels, an indicator of lipid peroxidation, were elevated in LPS-treated cells compared with control. Simultaneous administration of BA attenuated this increase, with both BA concentrations showing reduced MDA levels relative to LPS alone. This effect was more pronounced in the co-treatment groups, indicating a potential inhibitory effect of BA on lipid peroxidation under inflammatory conditions.

AOPP levels did not show a marked change following LPS treatment alone compared with the control group. Similarly, BA treatment alone and simultaneous LPS + BA treatments resulted in comparable AOPP levels, indicating no pronounced protein oxidation under the applied experimental conditions.

TSH levels exhibited modest variations among treatment groups. While LPS treatment alone tended to slightly reduce TSH levels relative to control, co-treatment with BA particularly at higher concentrations was associated with preserved or mildly increased TSH levels compared with LPS-treated groups, suggesting partial maintenance of thiol-based antioxidant capacity.

CAT activity was significantly decreased following LPS exposure compared with the control group. In contrast, BA co-treatment partially restored CAT activity, with both low and high BA concentrations showing higher CAT levels than LPS alone. BA treatment alone maintained CAT activity near control levels.

SOD levels remained largely unchanged following LPS treatment alone. However, BA administration both alone and in

Figure 2. Light microscope images of HaCAT cell migration determined by wound healing method (4X).

combination with LPS was associated with increased SOD levels compared with control, with the highest values observed in the BA-treated groups, indicating enhancement of enzymatic antioxidant defense.

Discussion

The present study demonstrates that BA exerts a concentration-dependent modulatory effect on HaCaT keratinocyte viability under LPS-induced inflammatory conditions. In our experimental model, LPS exposure alone did not induce overt cytotoxicity, which is consistent with previous studies indicating that LPS primarily acts as a proinflammatory stimulus rather than a direct cytotoxic agent in keratinocytes [16, 17]. Notably, simultaneous treatment with low-dose BA (20 μM) significantly enhanced cell viability, whereas higher-dose BA (100 μM) attenuated this effect. These findings suggest that BA supports keratinocyte survival within an optimal concentration range and emphasize the importance of dose-dependent responses. Previous studies have reported that boron compounds promote epithelial cell proliferation and wound repair under physiological conditions [18, 19]; however, our

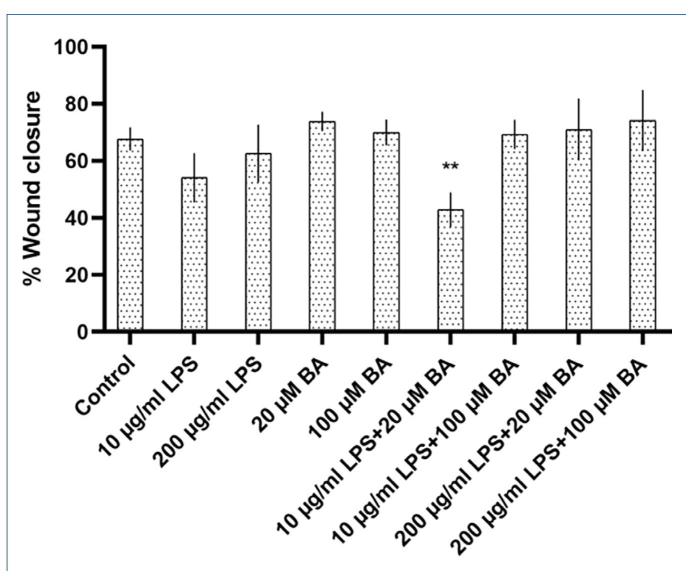

Figure 3. Wound healing effects LPS and Boric acid on HaCaT cells on wound diameter change at 0 and 24 hours (**p<0.001).

LPS: Lipopolysaccharide.

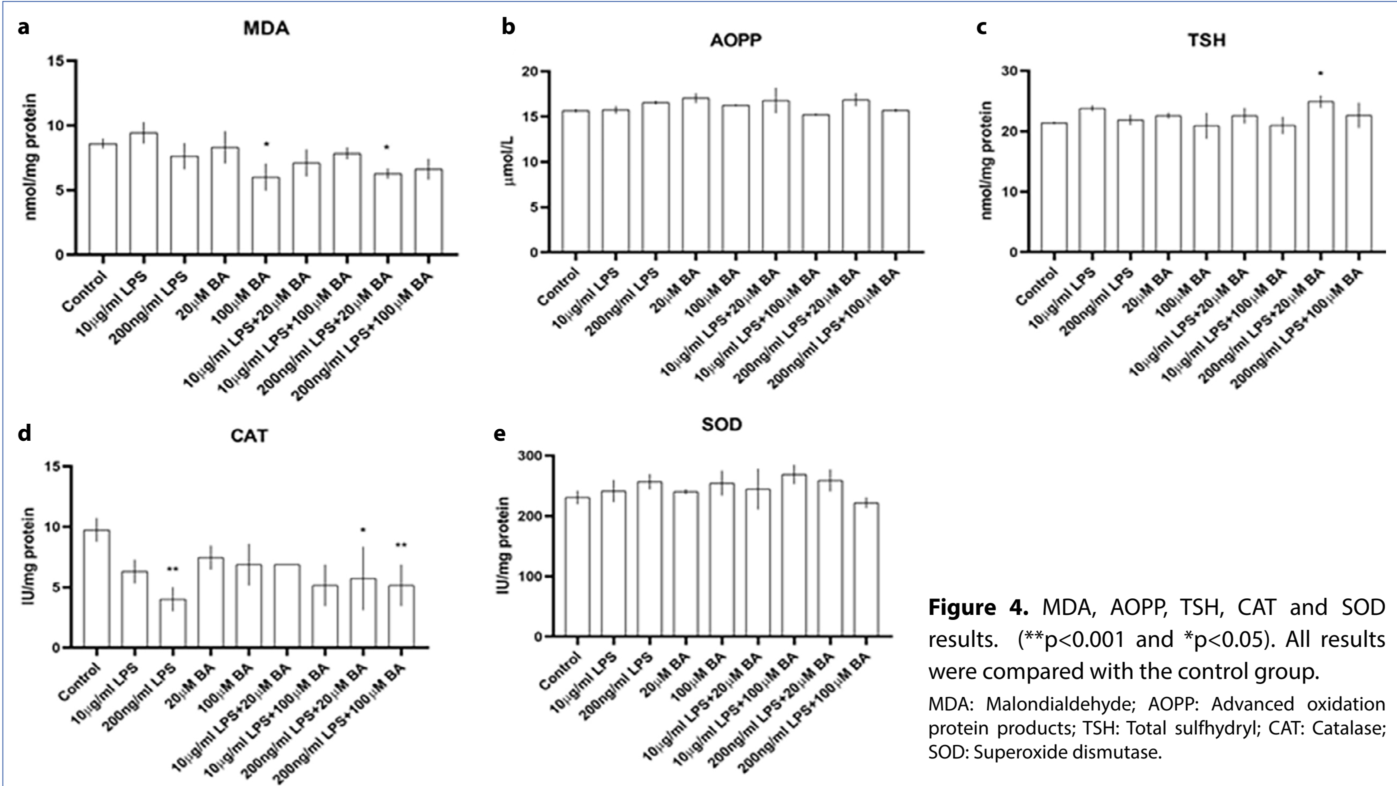

Figure 4. MDA, AOPP, TSH, CAT and SOD results. (**p<0.001 and *p<0.05). All results were compared with the control group.

MDA: Malondialdehyde; AOPP: Advanced oxidation protein products; TSH: Total sulfhydryl; CAT: Catalase; SOD: Superoxide dismutase.

data extend these observations by demonstrating a protective effect of BA on keratinocyte viability specifically during acute inflammatory activation relevant to psoriasis.

Keratinocyte viability is a critical determinant of epidermal homeostasis in psoriasis, where inflammatory stress, abnormal proliferation, and impaired differentiation coexist [20]. Preservation of cell viability during early inflammatory responses may contribute to maintenance of the epidermal barrier and controlled regeneration. Importantly, BA alone did not exert cytotoxic effects at the tested concentrations, supporting earlier toxicological and nutritional studies indicating that BA is well tolerated by epithelial cells within defined dose limits [18, 21]. Collectively, these findings demonstrate that BA preserves HaCaT cell viability and promotes keratinocyte migration under LPS-induced inflammatory conditions, supporting its potential role in maintaining epidermal repair capacity during inflammatory stress.

Beyond its effects on cell survival, our findings highlight a significant role for BA in modulating oxidative stress pathways. LPS stimulation resulted in increased lipid peroxidation, as evidenced by elevated MDA levels, along with suppression of antioxidant enzyme activity, particularly CAT. These results are in line with extensive evidence demonstrating that oxidative stress is a key contributor to psoriasis pathogenesis, driven by excessive ROS production from activated keratinocytes and infiltrating neutrophils [22–25]. Clinical studies evaluating oxidative stress markers in psoriasis have frequently reported elevated MDA levels and altered antioxidant enzyme activities; however, findings have been inconsistent, likely due to heterogeneity in disease severity, stage, and treatment status [26, 27]. Contrasting results have been reported by Gabr and colleagues, who demonstrated significantly elevated MDA levels together with reduced activities of the antioxidant enzymes SOD and CAT in patients with psoriasis [26]. The concomitant increase in lipid peroxidation and depletion of enzymatic antioxidant defenses in their study underscores the presence of pronounced oxidative imbalance in psoriasis. Importantly, these alterations were correlated with disease severity, suggesting that progressive impairment of SOD and CAT activity contributes to the accumulation of oxidative damage, as reflected by increased MDA levels. These findings are in line with our observations, where inflammatory stimulation was associated with enhanced lipid peroxidation and suppression of antioxidant enzymes, while BA treatment attenuated MDA levels and restored SOD and CAT activity. Together, these data support the concept that dysregulation of SOD and CAT plays a pivotal role in oxidative stress–mediated keratinocyte damage in psoriasis and highlight lipid peroxidation as a key downstream consequence of impaired antioxidant defense. Ikonomidis et al. [27] demonstrated that oxidative stress and inflammation-related mechanisms contribute not only to cutaneous pathology but also to systemic vascular dysfunction in patients with psoriasis, showing parallels with coronary artery disease. Their findings highlight increased oxidative burden and impaired antioxidant defenses as key drivers of disease-related tissue damage. Consistent with these observations, our results show that inflammatory stimulation induces oxidative imbalance in keratinocytes, characterized by increased lipid peroxidation and suppression of antioxidant enzymes, while BA treatment restores redox homeostasis by

enhancing SOD and CAT activity and reducing MDA levels. Together, these findings support the concept that oxidative stress represents a common mechanistic link between local keratinocyte dysfunction and systemic complications in psoriasis.

Simultaneous administration of BA markedly attenuated LPS-induced lipid peroxidation and partially restored CAT activity, while enhancing SOD levels. These results suggest that BA reinforces enzymatic antioxidant defense mechanisms and limits early oxidative damage, particularly at the level of membrane lipids. Previous experimental studies have shown that boron modulates redox homeostasis by regulating antioxidant enzyme activity and scavenging ROS [28, 29]. Our study adds to this body of evidence by demonstrating that BA exerts these antioxidant effects specifically under inflammatory stress conditions rather than under basal states alone.

TSH levels were preserved in BA-treated groups, indicating maintenance of thiol-based redox buffering capacity. In contrast, AOPP did not show marked changes across treatment groups, suggesting that protein oxidation may represent a later-stage oxidative event not prominently induced under the acute inflammatory conditions applied in this study. This observation is consistent with previous reports indicating that lipid peroxidation is a more sensitive early marker of oxidative stress in keratinocytes than advanced protein oxidation [30–32]. In addition to redox modulation, BA significantly improved keratinocyte migratory capacity in the wound healing assay, counteracting the inhibitory effects of LPS. Impaired keratinocyte migration is a recognized feature of inflammatory skin diseases and contributes to delayed epidermal repair [18]. Earlier studies have demonstrated that boron-containing compounds promote wound healing and epithelial regeneration, potentially through redox-sensitive signaling and cytoskeletal reorganization [18, 33]. In the context of psoriasis, enhanced keratinocyte migration may facilitate controlled epidermal renewal during inflammatory flare-ups. Overall, these results demonstrate that while LPS induces oxidative imbalance primarily through lipid peroxidation and suppression of antioxidant enzymes, simultaneous treatment with BA modulates oxidative stress markers and supports antioxidant defense mechanisms in HaCaT keratinocytes.

Akçaalan et al. [34] demonstrated that BA significantly stimulates wound closure in HaCaT keratinocytes and modulates epithelial–mesenchymal transition (EMT)–related gene expression, highlighting its role in keratinocyte migration and regenerative responses. These findings are highly consistent with our results, in which BA markedly improved wound closure and counteracted LPS-induced impairment of keratinocyte migration. While Akçaalan et al. [34] focused on EMT-associated transcriptional regulation under non-inflammatory conditions, our study extends these observations by demonstrating that BA preserves migratory capacity even in the presence of inflammatory and oxidative stress. Together, these data suggest that BA supports epidermal repair through complementary mechanisms involving both redox modulation and migration-related cellular programs.

Study Limitations and Future Perspectives

Several limitations of this study should be acknowledged. First, the findings are based on an *in vitro* HaCaT keratinocyte model, which does not fully recapitulate the complex immune–epidermal interactions present in psoriatic skin. The absence of immune cells, such as T lymphocytes and dendritic cells, limits direct extrapolation to *in vivo* disease mechanisms. Second, the study focused on acute inflammatory responses induced by LPS and therefore does not reflect chronic or recurrent inflammatory conditions characteristic of long-standing psoriasis. Third, molecular signaling pathways underlying BA-mediated protection such as NF-κB, MAPK, or Nrf2 signaling were not directly investigated. In addition, inflammatory cytokine profiling (e.g., IL-6, IL-8, TNF-α) and psoriasis-relevant axis stimulation (e.g., IL-17A/IL-23) were not assessed; because the revision relied on archived lysates, additional cytokine assays could not be performed in this cycle. Finally, pathway-level validation (e.g., NF-κB, MAPK, Nrf2/HO-1) and intracellular ROS measurement were not included, and mechanistic inferences should be interpreted cautiously.

Conclusion

Collectively, our findings demonstrate that BA confers protective effects in LPS-stimulated HaCaT keratinocytes by supporting cell viability, attenuating oxidative stress, enhancing antioxidant defenses, and promoting cell migration. These results provide experimental support for the potential role of BA as a candidate redox-modulating compound targeting oxidative and inflammatory processes relevant to psoriasis. Further studies using immune–keratinocyte co-culture systems, chronic inflammation models, and *in vivo* approaches are warranted to clarify the underlying molecular mechanisms and to evaluate the translational potential of BA in psoriasis management.

Disclosures

Ethics Committee Approval: Ethics Committee approval is not required for cell culture studies.

Informed Consent: Informed consent was obtained from all participants.

Conflict of Interest Statement: None declared.

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Research Article

Evaluation of routine laboratory parameters as clinical indicators of disease severity in multiple myeloma

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Abstract

Objectives: Multiple myeloma (MM) is characterized by clonal plasma cell proliferation and significant systemic impacts. This study aimed to evaluate the relationship between routine hemogram and biochemical parameters and disease severity markers (M-protein and β-2 microglobulin [β-2M]) to identify accessible clinical indicators of tumor load at the time of diagnosis.

Methods: In this retrospective cross-sectional study, newly diagnosed, treatment-naive MM patients and healthy controls were analyzed. Statistical significance was set at a threshold ($p<0.00125$) using Bonferroni correction to prevent Type I errors. Multivariable logistic regression was performed to identify independent predictors of high M-protein load (≥3g/dL), and ROC analysis was used to determine the diagnostic performance of significant parameters.

Results: MM patients exhibited significantly lower WBC, RBC, HCT, and PLT counts, and higher BUN and CRP levels compared to controls ($p<0.001$). β-2M showed significant correlations with several routine parameters; however, partial correlation and multivariable regression revealed that these associations were entirely dependent on renal function. Conversely, multivariable logistic regression identified RBC count (OR=0.383, p=0.026), eGFR, and age as significant independent predictors of high M-protein load. Notably, each 1×10^6/µL decline in RBC count was associated with a 161% increase in the risk of high disease severity. ROC analysis established an optimal RBC cut-off value of 3.73×10^6/µL (AUC: 0.695, sensitivity: 64.1%, specificity: 69.6%) for predicting high tumor load.

Conclusion: Routine laboratory data, particularly RBC count, serve as powerful indicators of MM severity at the time of initial diagnosis. Unlike β-2M, which is heavily influenced by renal status, RBC count is an independent predictor of monoclonal protein load. A baseline RBC level below 3.73×10^6/µL should alert clinicians to a potentially high tumor load, facilitating rapid triage and treatment prioritization.

Keywords: Beta 2-microglobulin, complete blood count, C-reactive protein, multiple myeloma, paraproteins

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Multiple myeloma (MM) is a clonal plasma cell neoplasm originating from the post-germinal lymphoid B-cell lineage [1]. It accounts for 1% of all cancers and 10% of hematological malignancies, making it the second most common hematological malignancy after lymphoma [2]. The estimated 5-year global incidence is approximately 230,000 patients. The median age of patients at diagnosis is 66–70 years. It is approximately 1.5 times more common in men than in women [3].

MM risk factors have not been fully elucidated. Factors that may pose a risk for the disease include being 65 years and older, African American race, male sex, and a family history of the disease. Patients often present with nonspecific symptoms such as weight loss, nausea/constipation, frequent urination, bone pain, weakness, and fatigue [4].

According to the International Myeloma Working Group (IMWG), the presence of hypercalcemia (serum calcium>11

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mg/dL), renal involvement (creatinine clearance<40 mL/minute or serum creatinine>2 mg/dL), anemia (hemoglobin<10 g/dL or hemoglobin value>2 g/dL below the lower limit of normal), and osteolytic bone lesions, along with M-spike peak on serum protein electrophoresis and/or monoclonal plasma cells, are necessary for diagnosis [1]. The serum free light chain (sFLC) ratio was added to the criteria in 2014 [5]. The International Staging System (ISS) uses albumin and β-2 microglobulin (β-2M) levels to determine the risk and stage of MM [6].

MM can be confused with different diseases in terms of the age of occurrence, nonspecific symptoms, and laboratory results; therefore, delays in diagnosis and treatment can be experienced. Depending on the stage at the time of diagnosis, the 5-year (2015–2021) relative survival rates are approximately 60% [7].

Considering that clinical evaluation at the time of presentation and treatment significantly affect MM prognosis, it is thought that rapid assessment based on laboratory data is important. In this study, we aimed to examine the relationships of routine hemogram and certain biochemistry parameters of MM patients with M-protein and β-2M levels and to emphasize the potential value of these parameters as clinical indicators reflecting disease severity in MM.

Materials and Methods

This retrospective cross-sectional analytical study included patients diagnosed with MM who presented to Kayseri City Training and Research Hospital between July 1, 2022, and December 31, 2022, for follow-up and treatment, and age- and sex-matched healthy participants. Patients with cardiovascular and autoimmune diseases, severe liver or renal failure, other malignancies/infections, and pregnancy were excluded. Laboratory data for all patients included in the study were obtained from baseline values at the time of initial diagnosis, prior to the initiation of any plasma cell-targeted therapy. Demographic data of the participants were obtained from the laboratory information system.

Biochemistry analyses were performed using Cobas 8000 (Roche Diagnostics®, Mannheim, Germany), and hemogram analyses were performed using Sysmex XN-1000® (Sysmex, Kobe, Japan) autoanalyzers with original kits and reagents. Protein hydra gel electrophoresis (Hydras Sebia®, USA) was used for serum protein electrophoresis. In the determination of serum protein electrophoresis (SPEP) fractions and the amount of M-protein, the perpendicular drop method was used while calculating the peak area in the electrophoresis pattern to provide consistent measurement at different protein concentration levels. All measurements were performed in accordance with the standard protocols of the laboratory. No restriction was made regarding the location of the M-protein peak; patients with monoclonal peaks in both the gamma and beta regions—although the latter constituted a small part of the total cohort—were included in the study to reflect biological diversity. Especially in the measurement of M-proteins located in the beta region, the perpendicular drop method was meticulously applied to minimize artifacts that could arise from normal beta-1 and beta-2 fractions and to ensure accurate quantitation. All monoclonal peaks were distinguished from other protein fractions by confirmation with immunofixation electrophoresis (IFE).

The studied biochemistry parameters were glucose, creatinine, eGFR, BUN, uric acid, cholesterol, LDL-cholesterol (direct), HDL-cholesterol, triglyceride, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH), amylase, lipase, total bilirubin, direct bilirubin, calcium (Ca^{2+}), sodium (Na^{+}), potassium (K^{+}), chloride (Cl^{-}), magnesium (Mg^{2+}), phosphorus, iron, free iron binding capacity (FIBC), transferrin saturation, creatine kinase (CK), creatine kinase-MB (CK-MB), rheumatoid factor (RF), and C-reactive protein (CRP). Furthermore, IgG, IgM, IgA, β-2M, M-protein, kappa, and lambda light chain levels of the participants were also examined. Biochemistry and hemogram parameters were statistically compared.

The minimum sample size required for each group was determined using the G*Power 3.1.9.4® program based on an independent groups t-test analysis with 5% type 1 error (α), 95% test power (1−β), and an effect size of d=0.8 [8]. Microsoft Office Excel 2016 was used to compile the data. Statistical analysis was performed using SPSS 22.0® (Statistics Package for Social Sciences). For group comparisons and correlation analyses, only patients with complete data for the relevant parameters were included. The Shapiro–Wilk normality test was used to examine sample distributions. While the independent groups t-test was used to compare parametric data, the Mann–Whitney U test was used to compare non-parametric data. Pearson's and Spearman's correlation tests were used in correlation analyses depending on the homogeneity status.

In the study, to eliminate the risk of Type I error that could arise from the comparison of approximately 40 parameters, Bonferroni correction was applied from the very beginning. In this context, while comparing the initial stage routine parameters of the patient and control groups, the significance threshold was redefined as $p<0.00125$ (0.05/40). Only parameters falling below this strict threshold value were considered statistically significant. The correlation of parameters showing a significant difference between groups with M-protein and β-2M, which are indicators of disease severity in the MM patient group, was examined. Again, to minimize the risk of Type I error that multiple comparisons could cause, Bonferroni correction was applied separately for both independent variables (M-protein and β-2M). Accordingly, the significance threshold was determined as $p<0.0083$ (0.05/6) for 6 parameters associated with M-protein, and as $p<0.0062$ (0.05/8) for 8 parameters associated with β-2M.

Multivariable logistic regression analysis was performed to identify the independent predictors of high M-protein load (≥3g/dL) at the time of diagnosis. Multicollinearity among the

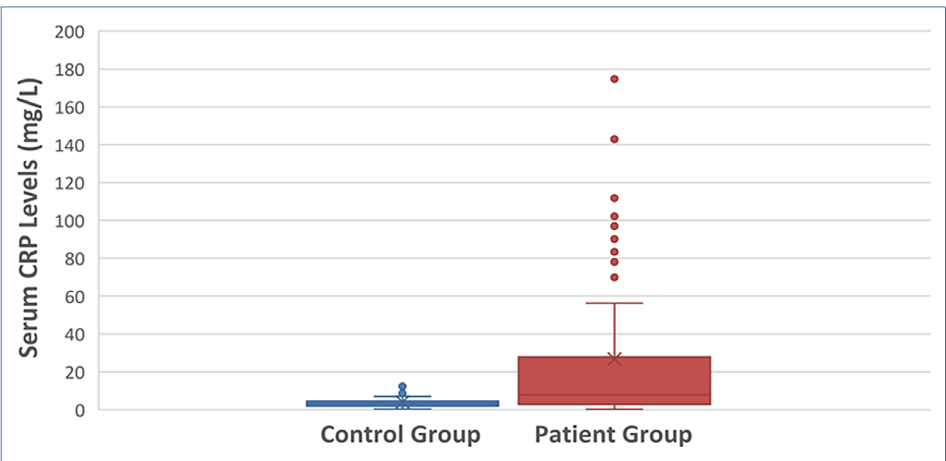

Figure 1. Comparison of serum CRP level between patient and control group.

CRP: C-reactive protein

independent variables included in the model was assessed by calculating the variance inflation factor (VIF); a VIF value of<2.5 was considered as the absence of multicollinearity. The goodness-of-fit of the model to the data was evaluated using the Hosmer–Lemeshow test. Receiver operating characteristic (ROC) curve analysis was conducted to determine the diagnostic performance and optimal cut-off point for the red blood cell count (RBC) parameter, which was found to be significant in the logistic regression analysis. The optimal cut-off value was established by calculating the Youden index (sensitivity+specificity−1) to maximize the sum of sensitivity and specificity. Area under the curve (AUC) values are presented alongside their respective 95% confidence intervals (CI).

Partial correlation analysis was performed to evaluate the independence of the relationships between β-2M and routine laboratory parameters (RBC, HCT, HGB, BUN, and CRP) from renal function, with eGFR defined as a control variable. Additionally, to test the independent effect of routine parameters in predicting β-2M levels, a multivariable regression analysis was conducted, in which β-2M was the dependent variable, and RBC, HCT, HGB, eGFR, BUN, and CRP were the independent variables.

The study was conducted in accordance with the Helsinki Declaration (as revised in 2013) and received ethical committee approval from Kayseri City Training and Research Hospital (Date: 25.03.2025, Decision No: 376). Written informed consent was obtained from the patients.

Results

Data from 94 patients and 51 healthy controls were included in the study. A statistically significant difference was observed between the patient and control groups in white blood cell count (WBC), absolute basophil count, RBC, hematocrit (HCT), platelet count (PLT), BUN, and Ca^{2+} parameters (p<0.001). As a result of the analyses performed, while the median WBC value of the patient group was 5.74 (2.13–16.62), this value was determined as 6.85 (3.98–11.98) in the control group. The median value of CRP in the patient group was 7.85 (0.3–175) mg/L, while it was determined as 3.10 (0.4–14.3) mg/L in the control group (Fig. 1). Despite the applied Bonferroni correction (p<0.00125), it was observed that both parameters maintained their statistical significance between the groups (p<0.001). Other laboratory and demographic data of the study groups are shown in Table 1.

A negative correlation was found between the RBC, HCT, and PLT parameters, which were different in MM patients compared to the control group, and M-protein levels. β-2M protein levels showed a negative correlation with RBC, hemoglobin (HGB), HCT, and eGFR, and a positive correlation with BUN and CRP. Correlation analyses showing all other relationships are summarized in Table 2. In serum free light chain (sFLC) analyses, no statistically significant correlation was found between free kappa, free lambda, and kappa/lambda ratio and routine hemogram parameters (RBC and HCT) (p>0.05) (Table 3).

Patients were divided into two groups based on M-protein levels (Group A:<3g/dL and Group B:≥3g/dL) [9]. Absolute basophil count showed a negative correlation with Group B M-protein levels, whereas a similar relationship was not observed in Group A (Fig. 2a). A negative correlation was observed between RBC and HCT levels, which were significantly lower in MM patients than in the control group, and Group A M-protein levels. Conversely, no similar correlation was detected in Group B (Fig. 2b, c). While albumin levels were found to be negatively correlated with high levels of M-protein, a similar relationship was not found with low levels (p=0.04, R_{ho}=−0.652) (Fig. 3).

Multivariable logistic regression analysis, including age, sex, eGFR, and RBC parameters, was performed to identify the independent risk predictors of high M-protein load (≥3g/dL) at the time of diagnosis. In the multicollinearity analysis conducted to evaluate the relationship between independent variables, the VIF values for RBC and eGFR were found to be below 2.5, confirming that the variables in the model functioned independently. Furthermore, the Hosmer–Lemeshow goodness-of-fit test demonstrated that the model was well

Table 1. Laboratory and demographic mean (min-max) or mean±SD of the study groups

Parameters	Patient (n=94*)	Control (n=51)	*p*	Bonferroni correction
Age (years)	65 (34–83)	55 (42–75)	>0.05	Insignificant
Gender / sex	54 M / 40 F	22 M / 29 F	>0.05	Insignificant
WBC (10^3/µL)	5.74 (2.13–16.62)	6.85 (3.98–11.98)	0.001	Significant
BASOPHIL (10^3/µL)	0.02 (0.00–0.12)	0.025 (0.01–0.15)	0.001	Significant
NEUTROPHIL (10^3/µL)	3.07 (0.22–10.38)	4.73 (2.11–30.14)	0.014	Insignificant
LYMPHOCYTE (10^3/µL)	1.47 (0.21–4.91	1.57 (1.49–2.24)	0.001	Significant
RBC (10^6/µL)	3.83±0.81	4.36±0.81	0.001	Significant
HGB (g/dL)	11.42±2.22	12.22±2.06	0.001	Significant
HCT (%)	34.02±6.21	36.72±6.78	0.001	Significant
MCV (fL)	89.9 (68–107)	84.2 (81–88)	0.001	Significant
PLT (10^3/µl)	180.000 (20.000–645.000)	272.000 (197.000–445.000)	0.001	Significant
RDW-SD (fL)	50 (37–71)	43 (36–50)	0.001	Significant
RDW-CV (%)	15 (12–24)	14 (12–17)	0.001	Significant
BUN (mg/dL)	15.5 (5–64)	10 (6–19)	0.001	Significant
Creatinin (mg/dL)	1.36±1.37	0.70±0.21	0.009	Insignificant
eGFR (mL/dk/1.73 m^2)	73±30	96±17	0.001	Significant
Ca^{2+}(mg/dL)	9.0 (6.6–11.5)	9.1 (7.9–9.6)	0.001	Significant
Na^+ (mmol/L)	138 (126–146)	140 (136–142)	0.001	Significant
K^+ (mmol/L)	4.2 (2.7–5.8)	4.25 (3.4–5.3)	0.001	Significant
CRP (mg/L)	7.85 (0.3–175)	3.10 (0.4–14.3)	0.001	Significant

*: The numbers represent the total cohort (n=94 patients). Due to the retrospective nature of the study, certain laboratory parameters were not available for all individuals. The actual sample size for some variables may be slightly lower than the total cohort size. BUN: Blood urea nitrogen; CRP: C-reactive protein; eGFR: Estimated glomerular filtration rate; HCT: Hematocrit; HGB: Hemoglobin; MCV: Mean corpuscular volume; PLT: Platelet; RBC: Red blood cell; RDW-CV: Red cell distribution width- coefficient of variation; RDW-SD: Red cell distribution width-standart deviation; WBC: White cell count; *p*: Probability value. Data are presented as mean and median based on their homogeneity status. Bonferroni correction was applied to the *p*-values to prevent Type I errors that may arise from multiple comparisons ($p<0.00125$).

Table 2. Correlations of M-Protein and β-2 microglobulin levels with parameters differing from the control group in patients

	Parameters	Correlation coefficient (r/R_{ho})	Test type	*p*	Bonferroni correction
M-protein (g/dL)	RBC (10^6/µL)	-0.525	Pearson (*r*)	0.001	Significant
	HGB (g/dL)	-0.462	Pearson (*r*)	0.001	Significant
	HCT (%)	-0.496	Pearson (*r*)	0.001	Significant
	PLT (10^3/µL)	-0.275	Spearman (R_{ho})	0.042	Insignificant
	BASOPHIL (10^3/µL)	-0.377	Spearman (R_{ho})	0.001	Significant
	RDW-SD (fL)	0.272	Spearman (R_{ho})	0.045	Insignificant
ß-2microglobulin (mg/L)	RBC (10^6/µL)	-0.415	Spearman (R_{ho})	0.001	Significant
	HGB (g/dL)	-0.459	Spearman (R_{ho})	0.001	Significant
	HCT (%)	-0.440	Spearman (R_{ho})	0.001	Significant
	eGFR (mL/dk/1.73 m^2)	-0.609	Spearman (R_{ho})	0.001	Significant
	RDW-SD (fL)	0.386	Spearman (R_{ho})	0.01	Insignificant
	RDW-CV (%)	0.279	Spearman (R_{ho})	0.016	Insignificant
	BUN (mg/dL)	0.526	Spearman (R_{ho})	0.001	Significant
	CRP (mg/L)	0.444	Spearman (R_{ho})	0.001	Significant

The normality of data distribution was assessed using the Shapiro-Wilk test. Correlation analyses were performed using Pearson's (*r*) for normally distributed parameters and Spearman's (R_{ho}) for non-normally distributed variables. To mitigate the risk of Type I errors arising from multiple comparisons, a Bonferroni correction was applied. Specifically, the correction was executed independently for each dependent variable (M-protein and beta-2-microglobulin). Accordingly, the significance threshold was adjusted to $p<0.0083$ for the 6 parameters associated with M-protein, and to $p<0.0062$ for the 8 parameters associated with beta-2-microglobulin. *Note: Correlation analyses were conducted only on patients with complete data for the specific parameters. Due to the retrospective nature of the study, the sample size for each parameter may vary based on availability of data in the records. RBC: Red blood cell count; HGB: Hemoglobin; HCT: Hematocrit; PLT: Platelet; RDW-SD: Red cell distribution width-standart deviation; RDW-CV: Red cell distribution width- coefficient of variation.

fitted to the data ($p>0.05$). The analysis revealed that among the variables included in the model, RBC (B=-0.959; S.E.=0.430; p=0.026; OR=0.383; 95% CI:0.165–0.891), eGFR (B=-0.041; S.E.=0.014; p=0.004; OR=0.960; 95% CI:0.934–0.987), and age (B=0.085; S.E.=0.035; p=0.015; OR=1.088; 95% CI:1.016–1.165) were determined to be significant independent predictors of

Table 3. Correlation of free light chain (sFLC) parameters with erythroid indices RBC (10^6/µL) and HCT (%)

Parameters	Median (min-max)	HCT (R_{ho}/p)	RBC (R_{ho}/p)
Kappa (κ) (mg/L)	2.84 (0.53–33.55)	-0.076/0.510	-0.028/0.810
Lambda (λ) (mg/L)	1.51 (0.22–15.61)	-0.024/0.832	-0.019/0.867
κ/λ ratio	1.88 (0.05–139.79)	-0.011/0.925	-0.001/0.996

Since the data did not follow a normal distribution, Spearman's correlation analysis was performed. The results indicated that sFLC levels, and specifically the kappa/lambda ratio, which holds high diagnostic value, did not show a significant correlation with HCT and RBC levels (p>0.05). This finding quantitatively supports our results that the relationship between M-protein levels and hemogram parameters is more prominent compared to that of sFLC. RBC: Red blood cell count; HCT: Hematocrit.

high M-protein load. The sex variable did not show a statistically significant independent effect in the model (p=0.07).

The diagnostic performance of the RBC parameter, which was proven to be an independent predictor in the logistic regression model, was further evaluated using ROC analysis for predicting high M-protein load (≥3g/dL) at diagnosis. The analysis yielded an area under the curve (AUC) value of 0.695 for the RBC level, which was found to be statistically significant (S.E.=0.055; p=0.001; 95% CI:0.587–0.803) (Fig. 2d). The RBC optimal cut-off value providing the highest accuracy for predicting high M-protein load was established at 3.73×10^6/µL. At this threshold, the sensitivity and specificity of the RBC parameter in predicting high disease severity were calculated as 64.1% and 69.6%, respectively.

The correlations between β-2M and significant parameters were re-examined using partial correlation analysis, adjusting for the effect of eGFR. Once eGFR was controlled, the previously significant relationships between β-2M and other parameters were found to lose their statistical significance (p>0.05).

In the multivariable regression analysis conducted to identify the independent factors influencing β-2M levels, eGFR was determined to be the only statistically significant independent predictor of β-2M levels (p<0.05). No independent effect on β-2M levels was observed for the other parameters (p>0.05).

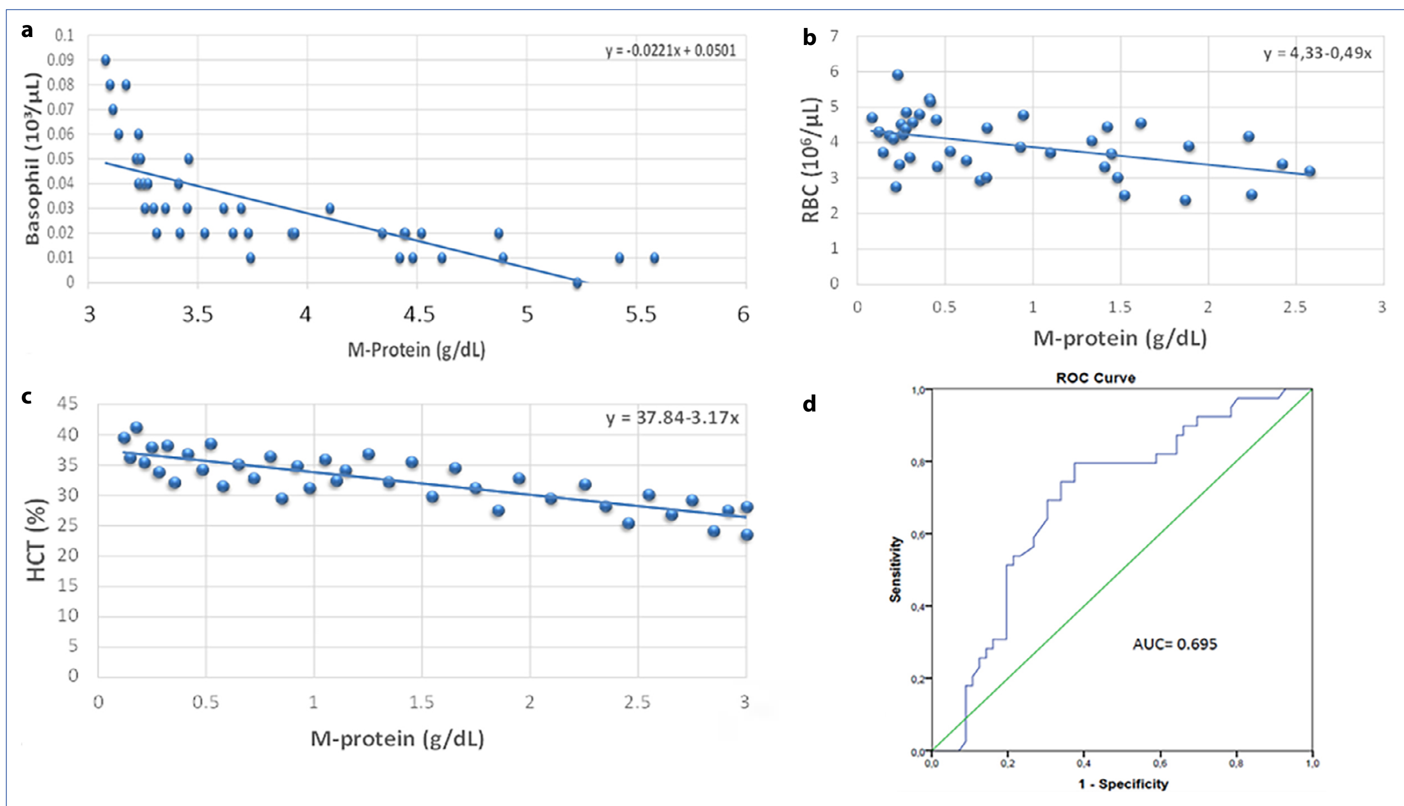

Figure 2. Correlation of hematological parameters with M-protein levels and predictive performance of RBC for high disease severity. (a) Scatter plot demonstrating the correlation between Group B (≥3g/dL) M-protein concentrations and basophil counts. A statistically significant negative correlation (n=40, due to missing CBC data in some patients, R_{ho}=-0.43, p<0.05) was observed. (b) Relationship of Group A (<3g/dL) M-protein levels with RBC (n=41, due to missing CBC data in some patients, p=0.01) and (c) HCT (n=41, due to missing CBC data in some patients, p=0.048). (d) ROC curve analysis of RBC for predicting high disease severity (M-protein ≥3g/dL) (AUC: 0.695, 95% CI: 0.587–0.803; Cut-off Value: 3.73×10^6/µl, Sensitivity: 64.1% and Specificity: 69.6%).

RBC: Red blood cell; CBC: Complete blood count; HCT: Hematocrit; ROC: Receiver operating characteristic.

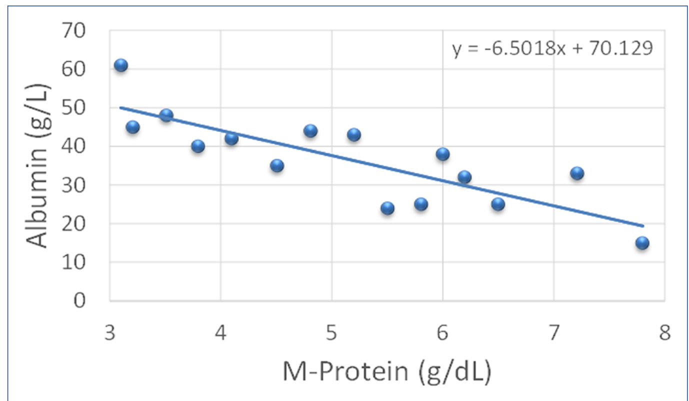

Figure 3. Correlation between serum M-protein and albumin levels in patients with high M-protein load (≥3 g/dL) (n=15, due to missing albumin data in some patients).

Discussion

The occurrence of MM at an advanced age and the non-specific nature of symptoms can lead to complexity in the clinical evaluation at the time of presentation, causing the extent of the disease to be overlooked. This study demonstrates how routine laboratory data, frequently used by physicians in primary care, exhibit a correlation with M-protein and β-2M, which serve as clinical indicators of disease severity. To our knowledge, there is no other study in the literature that analyzes our study parameters by classifying according to M-protein levels.

The clinical management at any stage, from diagnosis to treatment, is crucial for clinical outcomes. Therefore, easily and quickly accessible laboratory parameters are valuable as indicators of the systemic impact of MM. In this study, a statistically significant correlation was found between HCT and RBC levels, which were significantly lower in MM patients compared to the control group, and M-protein and β-2M levels, reflecting an association with the disease severity at the time of presentation.

In MM, current prognosis and staging are conducted via the Revised International Staging System (R-ISS), which includes serum albumin, LDH, and β-2M levels, as well as cytogenetic data [10]. The significant correlations we identified in our study between β-2M and routine laboratory parameters provide important evidence that β-2M elevation, a fundamental component of R-ISS, can be predicted through routine hemogram and biochemistry tests even before advanced staging investigations are performed. Although advanced tests such as cytogenetic analyses are an integral part of R-ISS, the data presented by our study support that, especially in primary health care services with limited resources, routine hemogram and biochemistry findings can be critical preliminary indicators in predicting β-2M elevation—one of the R-ISS components—and thus in referring the patient to an advanced center for accurate staging. In addition, our analyses demonstrated that the variation of β-2M in our cohort was substantially influenced by renal function. While eGFR emerged as a significant independent predictor ($p<0.05$), it is noteworthy that the associations between β-2M and other routine parameters vanished once renal function was controlled via partial correlation ($p>0.05$). This phenomenon may limit the capacity of β-2M to independently reflect myeloma biology, potentially creating a risk of misleading disease severity assessment during clinical staging. While we accept the prognostic value of β-2M, our findings statistically emphasize the necessity for complementary parameters—such as RBC—that are more stable and unaffected by renal function during the initial evaluation process.

M-protein, a monoclonal immunoglobulin secreted by plasma cells, is a quantitative parameter indicating clonal proliferation and disease progression in MM [11]. However, it is reported in the literature that M-protein levels may be too low to be detected by electrophoresis in some MM subgroups. Dispenzieri et al. [12] reported that M-spike was not observed or was faint on electrophoresis in patients with non-secretory MM, light chain MM, and IgD MM, and that serum free light chain level should be used for diagnosis in these groups. Blade and Kyle [13] showed that light chain measurement has high sensitivity in the diagnosis of non-secretory MM and IgD MM. Similarly, Mead et al. [14] compared the sensitivity of serum/urine protein electrophoresis with serum light chain levels in MM patients and found that serum light chain measurement was more sensitive than serum/urine protein electrophoresis. In our study, a significant relationship was found between M-protein levels and routine hemogram parameters, while the correlation between serum light chain levels and routine hemogram parameters was weaker. These findings indicate that the hematological anomalies in our study exhibit a closer relationship with the intact M-protein level rather than the free light chain load, further supporting the association with disease severity. Additionally, it is known in the pathogenesis of MM that renal involvement is a clinical manifestation of systemic disease progression [15]. The reason why M-protein and serum light chain levels show correlations of different intensities may be due to the fact that these markers reflect different aspects of the disease severity at the time of presentation. Indeed, data of patients at the time of admission were included in our study regardless of the clinical stage, representing a broad spectrum of tumor load.

There are studies where hematological parameters are used to evaluate suspicious clinical situations that may require further investigation for paraproteinemia. Li et al. [16], in a case-control study, examined hemogram parameters including RBC, PLT, and WBC to assess hemostatic imbalance in previously diagnosed MM patients and found that WBC, RBC, PLT, HCT, and HGB levels were lower, while IFR, RDW-CV/SD, and MPV levels were higher in the patient group compared to the healthy control group. Unlike this study, no difference was observed between the groups in terms of IFR and MPV parameters in our study. However, our other findings were similar. When IFR and

MPV parameters were evaluated according to M-protein levels, no difference was observed between Group A and Group B. The patients in the study by Li et al. [16] were individuals followed up with MM diagnosis and regularly taking medication. IFR and MPV parameters may have differed depending on the stage of the disease and the medications used. In our study, which included a higher number of patients, the fact that the patients were evaluated with their data at the time of initial presentation and the anemia picture had not yet fully developed may explain why this difference was not observed.

Røllum-Larsen et al. [17] investigated the relationship between WBC count and paraproteinemia in patients with monoclonal gammopathy and found a decrease in WBC count in the early stage of the disease and an increase in the advanced stage. In the same study, the frequency of monoclonal gammopathy was found to be higher in patients with low WBC counts, and the risk of paraproteinemia was significantly higher in men compared to women. In our study, M-protein levels, both high and low, were found not to show a significant difference between male and female sexes. Rollum-Larsen et al. [17] found that when they grouped patients according to WBC count, the odds ratio for monoclonal gammopathy risk was 1.61 times higher in the group with low WBC counts. In contrast, in our study, patients were divided into two groups based on M-protein levels, and the correlation of both the percentage and absolute values of WBC count and its subparameters with M-protein levels was examined. Absolute basophil and neutrophil counts showed a negative correlation with Group B M-protein levels, while a similar relationship was not observed in Group A. No significant correlation was found between WBC count and both Group A and Group B M-protein levels. Also, differently in our study, the WBC count in Group B was found to be lower than in Group A. These variations in WBC levels may reflect the balance between cytokine-mediated inflammatory signaling and the progressive infiltration of pathological plasma cells within the bone marrow. According to our findings, changes in WBC levels do not provide additional information regarding the clinical status of MM, but changes in WBC subgroups seem to be more relevant markers reflecting the systemic impact and disease severity.

In the literature, it is known that the strong relationship between anemia and malignancy incidence becomes more pronounced with age [18]. The fact that the patient and control groups in our study exhibited a similar distribution in terms of age (p>0.05) provided an advantage when evaluating our results. The absence of age differences between the groups supports our opinion that the hematological and biochemical changes we detected may be primarily related to the pathological effects of MM, rather than natural processes related to aging. This situation provides a reasonable basis for the parameters we examined to be evaluated as clinical indicators reflecting the systemic impact and disease severity of MM.

Kyle et al. [19] reported that anemia, generally normocytic-normochromic, is common in MM patients. Anemia is suggested to occur due to reduced erythropoietin levels associated with kidney disease, suppression of erythropoiesis by cancer cells, and rouleau formation of erythrocytes with abnormal immunoglobulins in approximately 80% of cases. In the same study, it was stated that WBC and PLT counts were normal, and RBC and HGB values were low in the majority of patients. Similarly, in our study, RBC and HGB values were found to be significantly lower in MM patients compared to the control group. Additionally, RBC and HCT levels were observed to show a significant correlation with Group A M-protein levels. The fact that RBC and HCT parameters showed a negative correlation with low levels of M-protein but did not show any relationship with high M-protein levels makes the hemogram analysis important, especially in patients presenting with a lower tumor load. Furthermore, in our study, WBC and PLT counts were lower compared to the control group.

In another study, similar to ours, decreased RBC levels were found in MM patients, but differently, no change was observed in mean corpuscular volume (MCV) [20]. In our study, MCV levels in the patient group were significantly higher than in the control group. This increase in MCV was significantly positively correlated with Group A M-protein levels, while a similar relationship was not observed with Group B M-protein levels. In our study, the significant decrease in RBC, HGB, and HCT levels in MM patients is consistent with the literature, reflecting the suppression of erythropoiesis by malignant plasma cells. Multivariable logistic regression analysis revealed that the RBC count independently predicted high M-protein load (≥3g/dL) at the time of diagnosis (OR=0.383; p=0.026), after adjusting for critical factors such as age and renal function (eGFR). While anemia in MM can arise from cytokine-mediated suppression of erythropoiesis and bone marrow infiltration, our findings reflect that a decrease in RBC count is not merely a secondary complication. Instead, it serves as an indicator of disease severity correlated with tumor mass and monoclonal protein synthesis capacity. To our knowledge, while the association between anemia and tumor load in MM is well-documented via hemoglobin levels, our study is among the first to define a specific RBC cut-off value (3.73×10^6/µL) as an independent predictor of high M-protein load. The analysis reveals that each 1×10^6/µL decline in RBC count increases the risk of presenting with a high systemic tumor load by 161%. This suggests that the depletion of the erythroid lineage is not just a side effect, but a direct reflection of the malignant plasma cell expansion within the bone marrow niche.

The RBC cut-off value of 3.73×10^6/µL is a specific finding derived from our cohort. This value suggests that risk estimation in newly diagnosed myeloma patients can be performed using routine and rapid tests, such as a complete blood count. Low RBC levels detected at diagnosis should alert the clinician not only to the presence of anemia but also to the potentially heavy monoclonal protein load. Patients whose RBC levels fall below this threshold carry a higher risk of high tumor

load and may be prioritized for further diagnostic workup and treatment scheduling. This simple yet effective approach provides clinicians with valuable time while awaiting invasive bone marrow biopsy results and establishes a strategic triage framework that could improve prognosis in patient management. Nonetheless, we suggest that each center should define its own optimal cut-off values in light of their specific patient profiles and laboratory standards.

The macrocytosis observed in our study could be attributed to various factors: nutritional deficits (B12 or folate), compensatory reticulocytosis due to bone marrow stress, or alterations in the marrow microenvironment directly caused by the plasma cell dyscrasia.

Kyle et al. [19] examined the biochemistry parameters of MM patients and observed that serum BUN, creatinine, LDH, and uric acid levels could increase, and the prevalence of hypercalcemia was 13%. Similarly, another study evaluated the changes in biochemistry parameters according to the stages of MM patients and found a significant increase in serum BUN, creatinine, and uric acid levels and a decrease in albumin levels with the progression of the disease stage. In the same study, no statistically significant difference was found in calcium levels between stages [21]. In another study, the prevalence of hypercalcemia in MM patients was reported as 47.4% [22]. In our study, the prevalence of hypercalcemia was found to be quite low at 6.55% compared to the literature. In fact, calcium levels were significantly lower in the patient group compared to the control group. So, the number of biochemistry parameters evaluated in these studies was an average of 10, approximately 40 biochemistry parameters were compared simultaneously in our study [19, 21, 22]. When we evaluated according to M-protein levels, no increase in calcium level was observed in Group B, contrary to expectations. High calcium levels occur in MM due to bone involvement [1]. Indeed, Kyle et al. [19] studies reported that 79% of the participants had radiological involvement. In other studies, when the symptoms of the participants were examined, bone pain complaints were observed with a frequency ranging from 31.6% to 50%, and this was reported to be due to bone involvement [21, 22]. Due to the retrospective design of our study and the reliance on routine clinical records at the time of diagnosis, a complete R-ISS staging based on cytogenetic (FISH) data could not be performed for all patients. However, the biochemical profile of our cohort—characterized by a low prevalence of hypercalcemia and the observed mean eGFR values—reflects a clinical spectrum where the majority of our patients had not yet developed the CRAB symptoms. This profile is considered the primary factor explaining the lower rates of hypercalcemia in our study population. In this context, the absence of extensive bone involvement that would cause hypercalcemia in these patients may explain this low level at the time of diagnosis.

Consistent with literature findings, creatinine and BUN levels were higher in the patient group compared to the control group, but no difference was observed between uric acid and LDH levels. These two parameters are markers that reflect cell destruction and systemic tumor activity. Cell destruction increases with the increase in tumor size in tumoral tissues. Therefore, an increase in these two parameters is expected in patients with a high disease severity. Indeed, one of the studies mentioned reported that 38% of the participants were Stage 3 MM patients [21]. The lack of a difference in LDH and uric acid levels in our study can be explained by the fact that patients were evaluated at the time of initial presentation.

In our study, albumin levels were significantly negatively correlated with high levels of M-protein, while a similar relationship was not found with low levels. It is known that renal losses increase with kidney damage [15].

The relationship between CRP levels and solid organ malignancies has been investigated, but there are a limited number of studies examining its relationship with MM. Serum CRP levels can increase in many diseases, including malignancy. Zeng et al. [23], in a case-control study, examined the relationship between common types of malignancy and CRP levels and reported a positive relationship between malignancy and CRP levels. Similar relationships have also been reported in a recent study [24]. CRP levels have been shown to be a prospective biomarker in different types of malignancies [25, 26]. In a cohort study, several parameters including CRP were examined as potential biomarkers in the etiology of MM, and no significant relationship was demonstrated between CRP concentration and the disease during the follow-up period [27]. In our study, the median CRP value detected in MM patients (7.85 mg/L) was significantly higher than the control group (3.10 mg/L), reflecting the inflammatory process in which the disease progresses. The fact that the upper limit of CRP in the patient group reaches high values such as 175 mg/L demonstrates the severity of secondary inflammatory responses accompanying the disease severity in some cases. The maintenance of the significance of our findings even at a threshold value of $p<0.00125$ offers the idea that routine inflammatory markers could be valuable indicators reflecting the systemic impact of MM. The inflammatory response is known to occur due to the immune system's reaction to eliminate harmful stimuli, including damaged cells, pathogens, and tumor cells. In addition, tumoral tissue can secrete pro-inflammatory cytokines. Therefore, cancer is an inflammatory process. Our findings also suggest that inflammatory parameters can be associated with MM disease from the time of clinical presentation. Inflammatory parameters are used by clinicians to evaluate underlying chronic diseases, including malignancy. Guidelines recommend CRP measurement among the laboratory tests suggested for MM evaluation [28]. A recent study has revealed that a high CRP level is not only an inflammatory indicator but is also associated with disease severity in MM [29]. In line with these findings, our study shows that CRP levels are also a clinical parameter that reflects the dis-

ease severity of MM. In our study, although CRP levels were significantly higher in MM patients, CRP is a non-specific acute-phase reactant influenced by various inflammatory conditions. The observed high values (up to 175 mg/L) in some patients, despite our exclusion criteria, may reflect subclinical inflammation or the extensive systemic impact of the malignancy itself. CRP is a supportive clinical indicator reflecting the systemic inflammatory environment and disease severity in MM. Its utility in clinical practice should be considered as part of a comprehensive assessment of the patient's overall inflammatory status during the initial evaluation, rather than as an independent triage parameter.

Limitations

The retrospective and cross-sectional design of our study limits its capacity to provide a diagnostic or screening context. Our current findings should be interpreted not as a screening test, but as clinical indicators that reflect the disease severity and systemic impact of MM at the time of presentation. We believe that recognizing these deviations in routine parameters can provide valuable clinical insights into the severity of the disease process.

The retrospective design of the study resulted in missing data for some parameters. This situation led to varying sample sizes across different analyses depending on the availability of the data records.

Furthermore, our study population did not include individuals with pre-malignant conditions such as Monoclonal Gammopathy of Undetermined Significance (MGUS) or Smoldering Multiple Myeloma (SMM). Therefore, our findings should be interpreted as indicators of disease severity in symptomatic MM rather than markers for the early screening of asymptomatic precursor states.

Another limitation of our study is the selection of the control group. In our study, MM patients were compared with healthy individuals; however, in real-world clinical practice, the diagnostic challenge often involves distinguishing MM from other conditions such as iron deficiency anemia, anemia of chronic disease, chronic kidney disease, or other inflammatory disorders. Comparing MM patients with healthy controls rather than a patient-based control group limits the generalizability of our findings to routine clinical triage where these comorbidities are frequently encountered.

Conclusion

Most malignancy patients present to healthcare institutions with low-risk and non-specific symptoms. Careful evaluation of MM patients' non-specific laboratory findings, which correlate with markers of tumor load such as M-protein and β-2M, is important for assessing the systemic involvement of the disease at the time of presentation. In conclusion, these routine parameters serve as valuable clinical indicators reflecting the overall disease severity and systemic impact of MM.

Disclosures

Ethics Committee Approval: The study was approved by the Kayseri City Training and Research Hospital Ethics Committee (no: 376, date: 25/03/2025).

Informed Consent: Written informed consent was obtained.

Conflict of Interest Statement: None declared.

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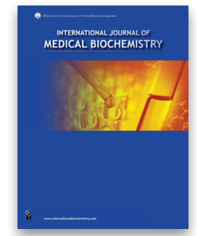

Research Article

Evaluation of inter-test agreement and analytical performance of eight fecal immunochemical tests

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Abstract

Objectives: Immunochemical fecal occult blood test has been commonly used for community-based colorectal cancer screening. There is a wide variety of fecal immunochemical test (FIT) products available in the market. However, there is limited performance information for many brands, making it essential to assess and compare the performance of these FITs. Therefore, this study aims to assess the level of agreement between eight FIT products with various cut-off values (two quantitative, six qualitative) and to evaluate the analytical performance of FITs through repeatability, interference, and stability analyses.

Methods: This study was conducted using a total of 313 stool specimens of which 211 specimens were obtained from randomly selected patients without any dietary restrictions, and the remainder 101 specimens were spiked with hemoglobin. The fecal occult blood results from all brands were evaluated as positive or negative. The level of agreement of FITs were assessed. Repeatability, stability and interference studies of FITs were also carried out.

Results: FITs were found to have fair to almost perfect agreement with kappa values ranging from 0.28 to 0.94 (all $p<0.001$) in the pairwise comparisons but statistically significant differences were found among most FITs by McNemar's test with Bonferroni correction (adjusted $\alpha=0.0018$). Repeatability and interference studies showed consistent results, but stability performance varied among FITs.

Conclusion: This study showed that agreement and analytical performance among FITs vary, that statistically significant differences may be observed between some test pairs, and that agreement measures alone are not sufficient when considering test interchangeability. Test selection should be based on a comprehensive assessment that considers analytical performance together with agreement results and the potential impact on laboratory and clinical management.

Keywords: Agreement, analytical performance, fecal immunochemical test, fecal occult blood, FIT, FOB

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Colorectal cancer represents a major public health problem worldwide. According to the 2020 GLOBOCAN statistics, it was among the most frequently diagnosed types of cancer and one of the leading causes of cancer-related deaths. The age-standardized incidence rates of colorectal cancer in Türkiye were 16.2 for women and 26.2 for men per 100,000 popula-

This study is part of a thesis titled "Analytical performance evaluation of immunochemical fecal occult blood tests [İmmünokimyasal gaitada gizli kan testlerinin analitik performans değerlendirmesi]" (Ankara Numune Training and Research Hospital).

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tion (world standard population), ranking third among all cancers. Colorectal cancer also ranked third in terms of mortality, with age-standardized mortality rates of 7.8 for women and 13.0 for men per 100,000 population (world standard population) [1]. Therefore, its detection via screening programs in precancerous or early stages remains of paramount importance for prognosis and survival.

While most of the population-based screening programs use a fecal immunochemical test (FIT) or a guaiac-based fecal occult blood test as the screening methodology [2], Türkiye's population-based screening program includes a biennial FIT and a colonoscopy every 10 years for individuals aged 50–70 years [3]. FIT is the preferred approach in Türkiye for detecting human hemoglobin in stool samples because i) food and medicines do not typically interfere with its results [4]; ii) it is specific to human hemoglobin [4]; and iii) it has a high cost-benefit ratio [3].

While a great number of qualitative and quantitative FIT brands are available on the market, this may complicate the selection of an appropriate test for laboratory use. Therefore, studies evaluating both the clinical and analytical performance of these FITs are needed. Most published studies have focused on clinical performance [5–7], while fewer have examined analytical performance through comparative evaluations [8, 9]. However, analytical performance of FITs is essential for reliable test results, which directly affects patient referral and follow-up decisions in colorectal cancer screening.

Although clinical performance of FITs should ideally be assessed using colonoscopy as the reference method, this may not always be practical due to limited availability of colonoscopy, difficulties in obtaining an appropriate patient population for clinical performance evaluation, and challenges related to clinical workload management. Therefore, clinical performance data for test brands may not always be available. In such cases, comparative agreement studies between screening tests may still provide valuable information for test selection and laboratory practice.

In addition, the cut-off values of FITs may vary significantly [10]. The variation in cut-off values used by different FIT brands for the same specimen may lead to different interpretations (i.e., positive or negative) of test results, which play an important role in decision-making in colorectal cancer screening and may also lead to variability in positivity rates. FITs with lower cut-off values are expected to yield more positive results, which require further diagnostic evaluation and thus greater effort in managing the clinical workload [11]. However, using higher cut-off values may result in missed diagnoses of the disease. Therefore, the potential impacts of cut-off values and available clinical capacity should be considered together when selecting a test.

Although several studies have been conducted, performance data for many brands is still insufficient, making it difficult for health professionals to make informed choices [12]. Accordingly, the primary aim of this study was to compare the agreement between seven FITs (one automated quantitative and six qualitative) and the automated quantitative FIT analyzer currently used in our laboratory and previously evaluated in various studies [13–15], as well as the agreement among the seven FITs, using overall percent agreement, kappa statistics, and McNemar analyses. The secondary aim of this study was to evaluate the analytical performance of the FITs through interference testing, repeatability assessment, and stability analyses.

Materials and Methods

The study was approved by the Ankara Numune Training and Research Hospital Ethics Committee (No: 178/2014, Date: 07/05/2014).

This study was conducted using a total of 313 stool specimens in accordance with the principles of the Declaration of Helsinki. Of the 313 specimens, 211 were randomly collected from patients without dietary restrictions, while the remaining 102 were spiked to achieve a hemoglobin concentration of 50±10 ng/mL by mixing negative fecal occult blood (FOB) samples with hemolysate, as most of the FITs evaluated in the study had a cut-off value of 50 ng/mL. The FOB results of the mixtures were determined based on the values measured by the NS-Plus C15 analyzer (Alfresa Pharma Corporation, Japan) currently used in our laboratory.

Hemolysate was prepared by osmotic (hypotonic) lysis of erythrocytes using distilled water, supported by mechanical mixing. For this purpose, blood samples collected from healthy volunteers into K2 EDTA blood collection tubes were used. The tubes were first centrifuged, and the supernatant was removed. The remaining pellet was washed with isotonic saline and subsequently resuspended in distilled water at a 1:1 ratio. After mechanical mixing, the mixture was centrifuged, and the supernatant (hemolysate) was separated for later use.

Quality control of the quantitative analyzers was performed daily, and all FITs were carried out in accordance with the users' instructions. The stool samples used in the tests were collected from the same or nearby regions of stool specimens.

All samples collected from three different parts of the stool specimens were first analyzed using the NS-Plus C15 analyzer that works on the basis of a colloidal gold agglutination immunoassay method with a cut-off value of 100 ng/mL. The same samples were then tested by using i-Chroma (Boditech Med, Korea, cut-off 50 ng/mL), a quantitative FIT brand that works on the basis of a fluorescence immunoassay method, and the following six qualitative FIT brands that work on the basis of a lateral flow immunoassay method: Certest (Biotec S.L., Spain, cut-off 16 ng/mL), True Line (Biocare Diagnostics Ltd., China, cut-off 50 ng/mL), SD (Standard Diagnostics Inc., Korea, cut-off 10 ng/mL), Rapidan Tester (Türklab Tıbbi Malz. San. Tic. A.Ş., Türkiye, cut-off 50 ng/mL), Laboquick (Köroğlu Tıbbi Malz. San. ve Tic. Ltd. Şti., Türkiye, cut-off 50 ng/mL) and Innovacon (Innovacon Inc., USA, cut-off 50 ng/mL).

During the study, differences were observed within the same test brands in the buffer volume among sample collection

Table 1. Agreement values between NS-Plus C15 analyzer (cut-off 100 ng/mL) and different fecal immunochemical tests

Tests	Positivity rate (%)	OPA (95% CI)	PPA (95% CI)	NPA (95% CI)	κ (95% CI)
i-Chroma	45	79.9 (75.1–83.9)	95.3 (88.6–98.2)	74.0 (67.9–79.3)	0.58 (0.49–0.67)*
Certest	42	77.6 (72.7–82.9)	86.0 (77.2–91.8)	74.4 (68.4–79.7)	0.52 (0.42–0.61)*
SD	64	61.7 (56.2–66.9)	97.7 (91.9–99.4)	48.0 (41.6–54.0)	0.32 (0.25–0.39)*
Rapidan tester	57	67.1 (61.7–72.1)	94.2 (87.1–97.5)	56.8 (50.3–63.1)	0.38 (0.30–0.46)*
Laboquick	67	58.5 (52.9–63.8)	97.7 (91.9–99.4)	43.6 (37.3–50.1)	0.28 (0.22–0,35)*
Innovacon	59	66.1 (60.7–71.2)	95.3 (88.6–98.2)	55.1 (48.6–61.4)	0.37 (0.29–0.45)*
True line	65	60.4 (54.9–65.7)	97.7 (91.9–99.4)	46.3 (39.9–52.8)	0.31 (0.24–0.38)*

All analyses were based on 313 samples; the positivity rate of NS-Plus C15 was 27. *: Statistically significant after Bonferroni correction (McNemar's test; adjusted α = 0.0018). OPA: Overall percent agreement; PPA: Positive percent agreement; NPA: Negative percent agreement; κ: Kappa coefficient; CI: Confidence interval.

tubes and in the amount of stool collected by different sampling sticks. In addition, NS-Plus C15, SD, and Certest sample collection tubes were equipped with a filter system to remove excess stool.

The FOB results from all brands were evaluated as positive or negative. In qualitative tests, the presence of faint test lines was considered a positive result. Results from all brands were compared with each other. Further, the same comparison was conducted with the NS-Plus C15 analyzer by setting the cut-off value to 50 ng/mL, consistent with that of most FITs evaluated in this study. Then the compatibility of the tests was evaluated.

Interference study

In order to examine whether FIT tests would cross-react with hemoglobins other than human hemoglobin, hemolysate prepared from blood samples collected from sheep, goats, and cattle using the same hemolysis procedure described above was mixed with stool samples with negative FOB results. The samples were run across all FIT brands, and the results were recorded.

Repeatability study

A total of three samples with negative (14 ng/mL), positive (371 ng/mL), and a concentration of 50±10 ng/mL (53 ng/mL) were analyzed 10 times in all FITs.

The coefficient of variation (CV) was calculated for quantitative tests, and the results were evaluated according to the total allowable error defined by the analytical performance specifications of the Royal College of Pathologists of Australasia (RCPA) Quality Assurance Program.

Stability study

The users' manual of all FITs (except for Laboquick and Certest brands) we used in this study indicated that the samples taken into the sample collection tubes should remain stable at room temperature for three days. In order to assess the stability period indicated above, 20 stool specimens with different FOB concentrations, including 10 FOB_negative and 10 FOB_positive samples, were selected.

A sample of each stool specimen was collected into a sample collection tube of each FIT brand, and the FOB result for the first day was recorded. Over the following three days, FOB was analyzed daily using samples from sample collection tubes kept at room temperature (20–24°C), and the results were recorded.

Statistical analysis

In the comparison of the tests, agreements (e.g., overall percent agreement; OPA, positive percent agreement; PPA and negative percent agreement; NPA) were determined at a 95% confidence interval according to the User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline (EP12-A2) [16].

In addition, agreements were also evaluated using kappa statistics. For this purpose, SPSS V23 (SPSS Inc., Chicago, Illinois, USA) statistical software was used. Due to the inherent nature of the kappa statistic being influenced by the potential bias between tests, Cochran's Q and McNemar's tests were used in addition to the kappa statistic to determine whether the tests produced statistically different results. Statistical significance for McNemar's test was defined using a Bonferroni-adjusted α level (adjusted α=0.0018). The kappa coefficients (κ) values were evaluated according to Landis and Koch's classification [17].

Results

Fecal occult blood study

The agreement among all FITs included in this study ranged from fair to almost perfect based on their kappa values. Cochran's Q test, used to compare the FITs with each other, showed statistically significant differences (p<0.001). Therefore, pairwise comparisons among all FITs were performed using McNemar's test with Bonferroni correction (adjusted α=0.0018).

The results from the statistical analyses demonstrating the agreement between the NS-Plus C15 analyzer and other FITs for two cut-off values of the NS-Plus C15 analyzer were summarized in Table 1 (cut-off value 100 ng/mL) and Table 2 (cut-off value 50 ng/mL). The agreement between the NS-Plus C15 (cut-

Table 2. Agreement values between NS-Plus C15 analyzer (cut-off 50 ng/mL) and different fecal immunochemical tests

Tests	Positivity rate (%)	OPA (95% CI)	PPA (95% CI)	NPA (95% CI)	κ (95% CI)
i-Chroma	45	76.9 (72.2–81.1)	71.9 (68.8–74.1)	82.1 (73.1–89.5)	0.54 (0.45–0.63)
Certest	42	75.4 (70.5–79.7)	67.5 (63.0–71.2)	83.3 (74.4–90.7)	0.51 (0.41–0.60)
SD	64	77.3 (71.6–82.3)	91.7 (88.6–92.8)	62.8 (53.2–71.8)	0.55 (0.46–0.63)*
Rapidan tester	57	76.9 (71.5–81.8)	84.1 (80.4–86.2)	69.9 (60.2–78.6)	0.54 (0.45–0.63)
Laboquick	67	76.0 (70.3–81.1)	93.6 (90.5–94.7)	58.3 (48.9–67.4)	0.52 (0.43–0.61)*
Innovacon	59	77.9 (72.4–82.8)	86.6 (83.1–88.4)	69.2 (59.5–78.0)	0.56 (0.47–0.65)
True line	65	76.0 (70.3–81.1)	91.7 (88.6–92.8)	60.3 (50.7–69.3)	0.52 (0.43–0.61)*

All analyses were based on 313 samples; the positivity rate of NS-Plus C15 was 50%. *: Statistically significant after Bonferroni correction (McNemar's test; adjusted α=0.0018). OPA: Overall percent agreement; PPA: Positive percent agreement; NPA: Negative percent agreement; κ: Kappa coefficient; CI: Confidence interval.

off 100 ng/mL, positivity rate 27%) analyzer and the other FITs ranged from fair to moderate with κ values of 0.28 to 0.58 across pairwise comparisons (all p<0.001), and statistically significant differences were observed in all comparisons by McNemar's test with Bonferroni correction (adjusted α=0.0018). For the cut-off value of 50 ng/mL (positivity rate 50%), the agreements were moderate, with κ values ranging from 0.51 to 0.56 across pairwise comparisons (all p<0.001). Using McNemar's test with Bonferroni correction (adjusted α=0.0018), only three test pairs, NS-Plus C15 vs. SD, NS-Plus C15 vs. Laboquick, and NS-Plus C15 vs. True Line, showed statistically significant differences.

The statistical results of the pairwise comparisons among the remaining FITs were summarized in Table 3. According to these results, there was moderate to almost perfect agreement between the tests, with κ values ranging from 0.52 to 0.94 across pairwise comparisons (all p<0.001). Using McNemar's test with Bonferroni correction (adjusted α=0.0018), statistically significant differences were observed in most test pairs, except for the i-Chroma vs. Certest, Innovacon vs. Rapidan Tester, True Line vs. SD, SD vs. Laboquick, and True Line vs. Laboquick test brands.

Interference study

Fecal occult blood tests using stool samples spiked with sheep, goat, and cattle blood yielded negative results in all FITs, indicating no cross-reactivity.

Repeatability study

In the repeatability study of qualitative tests performed with negative stool specimens, only the Innovacon test yielded positive results in one of the ten replicates. For all other stool specimens used in repeatability testing, all tests yielded positive results in all replicates.

Among the quantitative analyzers, the CV% values of NS-Plus C15 were 15.8%, 9.8%, and 23.8% for negative, 53 ng/mL hemoglobin concentration, and positive samples, respectively, while these values for i-Chroma were found to be 10.2%, 10.3%, and 18.9%, respectively. For the positive sample, the CV values of NS-Plus C15 and i-Chroma were higher than the total allowable error, which is defined as ±15 ng/mL or 15% if the concentration of hemoglobin in the stool sample is higher than 100 ng/mL.

Stability study

The evaluation of the stability analysis, based on the proportion of samples that remained stable on each measurement day, is summarized in Table 4.

In the stability analysis of negative samples, positive results were observed in five samples with the i-Chroma test and in one sample each with the Certest, True Line, and Innovacon tests. Of the five samples that became positive in the i-Chroma test, one yielded a positive result on Day 1 but reverted to negative on Days 2 and 3. Among the remaining four samples, two became positive on Day 1, one on Day 2, and one on Day 3. In the Certest and True Line tests, the sample that became positive on Day 1 reverted to negative on Days 2 and 3. In the Innovacon test, the sample that became positive on Day 1 remained positive on Days 2 and 3. The remaining negative samples maintained their stability across all test brands.

In the stability analysis of positive samples, negative results were observed in three samples with the Certest test, two samples each with the Innovacon, True Line, and Laboquick tests, and one sample each with the i-Chroma, NS-Plus C15, Rapidan Tester, and SD tests. The remaining positive samples maintained their stability across all brands.

Discussion

In this study, the NS-Plus C15 analyzer, with a cut-off value of 100 ng/mL, produced different results compared with the other quantitative and qualitative tests used for comparison, both in terms of agreement levels and the statistical significance of differences (Table 1). The evaluation at a cut-off value of 50 ng/mL (Table 2) showed that the NS-Plus C15 analyzer had better agreement with several tests. As also discussed by Brenner et al. [18] in their comparative study, differences in cut-off values between tests appeared to have influenced the level of agreement observed among them.

However, despite a higher but still moderate level of agreement, statistically significant differences were still observed between the NS-Plus C15 analyzer and some of the other test brands. Therefore, careful evaluation is required when considering the interchangeability of the tests.

Table 3. Inter-test agreement of paired fecal immunochemical tests

	i-Chroma	Certest	SD	Rapidan tester	Laboquick	Innovacon	True line
i-Chroma	OPA	81	81	83	77	82	79
	κ	0.62	0.62*	0.66*	0.56*	0.66*	0.59*
	(95%CI)	(0.53–0.71)	(0.54–0.70)	(0.58–0.74)	(0.48–0.64)	(0.57–0.74)	(0.50–0.67)
Certest		OPA	78	81	74	83	76
		κ	0.57*	0.63*	0.52*	0.66*	0.55*
		(95%CI)	(0.49–0.65)	(0.55–0.71)	(0.44–0.60)	(0.59–0.74)	(0.47–0.63)
SD			OPA	91	96	92	96
			κ	0.81*	0.92	0.84*	0.92
			(95%CI)	(0.74–0.87)	(0.87–0.96)	(0.78–0.90)	(0.87–0.96)
Rapidan tester				OPA	88	93	89
				κ	0.75*	0.85	0.77*
				(95%CI)	(0.68–0.82)	(0.79–0.91)	(0.69–0.84)
Laboquick					OPA	91	97
					κ	0.81*	0.94
					(95%CI)	(0.74–0.88)	(0.90–0.98)
Innovacon						OPA	92
						κ	0.82*
						(95%CI)	(0.76–0.89)
True line							

All analyses were based on 313 samples; values in parentheses indicate 95% confidence intervals for κ. *: Statistically significant after Bonferroni correction (McNemar's test; adjusted α=0.0018). OPA: Overall percent agreement; κ: Kappa coefficient.

Table 4. Stability assessment for fecal immunochemical tests

Tests	Day 1 (%)[b,c]		Day 2 (%)[b,c]		Day 3 (%)[b,c]	
	Negative[a] samples	Positive[a] samples	Negative[a] samples	Positive[a] samples	Negative[a] samples	Positive[a] samples
NS-Plus C15	100	100	100	90	100	90
i-Chroma	70	90	70	90	60	90
Certest	90	90	100	70	100	70
SD	100	100	100	90	100	90
Rapidan tester	100	100	100	90	100	90
Laboquick	100	100	100	100	100	80
Innovacon	90	100	90	90	90	80
True line	90	90	100	80	100	80

[a]: Analyses were based on 20 samples, including 10 negative and 10 positive samples; [b]: The percentages in the table show the proportion of samples that remained stable on each measurement day; [c]: Day 0 was defined as the initial measurement day; Day 1–3 refer to measurements performed 1–3 days after Day 0.

Although kappa statistics and McNemar analysis provide information on agreement and statistical differences or bias between tests, they do not directly reflect the clinical impact of discordant results. Therefore, when choosing between tests, it is important to consider not only agreement measures but also analytical performance of the tests, positivity rates and their possible effects on laboratory and clinical management.

The comparisons among the tests shown in Table 3 revealed heterogeneous results in terms of agreement levels and statistical differences. Some test pairs showed high agreement and non-significant McNemar results, suggesting that these tests may be used interchangeably in routine laboratory practice. However, significant McNemar results in other comparisons, despite moderate or high agreement, indicated the need for more cautious interpretation regarding interchangeability. Therefore, for screening purposes, agreement results should be interpreted together with analytical performance, positivity rates, and laboratory and clinical considerations, as mentioned above.

However, despite the moderate to almost perfect agreement found from pairwise comparisons of the seven tests (one quantitative, six qualitative) with same or different cut-off

values and with same or different measurement methods, the systematic differences found in all tests in the current study, except for five pairwise tests (i.e., i-Chroma vs. Certest, SD vs. True Line, Laboquick vs. True Line, SD vs. Laboquick, Rapidan Tester vs. Innovacon) is an indication to interpret the agreement with caution, and to consider the impact of preanalytical and analytical processes.

In terms of the preanalytical processes, differences in the amount of buffer observed among different tubes of the same test brand during the study may lead to different FOB test results, indicating the need to ensure a standard buffer volume across all tubes. Similarly, the test results may have been impacted by other preanalytical factors, including, but not limited to, the characteristics of the buffer solution that differed among different tests [19]. In addition, differences observed in the amount of stool collected by the sampling stick within the same test brands may also lead to different FOB test results. Among the tests used for comparison, the filter system in the sample collection tubes of the NS-Plus C15, SD, and Certest brands may have a beneficial effect in standardizing the amount of stool collected.

In addition to the preanalytical factors, analytical factors may also be influencing these results. For example, similar positivity rates and the substantial agreement observed in the test pair of Certest and i-Chroma (McNemar's test, $p=0.298$) with different cut-off values and measurement methods, or the systematic differences observed in the test pair Innovacon and True Line (McNemar's test, $p<0.001$) with almost perfect agreement and the same cut-off values and measurement methods, demonstrated that different analytical processes may be involved. This interpretation appears to be aligned with the reasoning of Chiang et al. [20], who compared two FITs with the same cut-off value and reported differences in positive predictive values despite similar positivity rates.

The analytical factors leading to different results may originate from differences in the antibodies used in antigen–antibody binding, for example, antibodies produced from different animal species, targeting different epitopes, or being monoclonal or polyclonal [8, 21]. While the use of monoclonal mouse antibodies to bind hemoglobin in all tests except for NS-Plus C15, Innovacon, and True Line, and the polyclonal antibodies in the NS-Plus C15 analyzer may not be interpreted as the direct factor influencing the different results in tests with the same cut-off values, it still cannot be ruled out. In addition, the form of hemoglobin in the feces plays an important role in antigen-antibody binding. In this respect, the breakdown products of hemoglobin may have interfered with the analytical processes [8, 20].

The repeatability results of this study showed that although the CV values obtained from quantitative analyzers for positive samples appear to be high according to the RCPA, the results resembled the CV values published in repeatability studies using stool samples [8, 22, 23]. In these studies, the CV values calculated from stool samples at various hemoglobin concentrations range from 7.2% to 49.5% and were higher than the CV values (0.6% – 8.5%) obtained from repeatability studies using quality control solutions and hemoglobin solutions prepared in buffer solution [13, 22–26]. The higher CVs observed in fecal samples may be attributed to incomplete homogenization and particle sedimentation, which may occur through interactions with components of the fecal matrix in the buffer [27, 28]. In addition, the obvious CV differences between the two quantitative analyzers in this study, which used samples prepared from the same stool specimens, suggested the potential influence of differences in buffer, sampling, and sample collection tubes [22, 29].

The evaluation of the stability results of the study suggested that the unexpected positivity observed in some negative stool samples may potentially be attributable to changes in the buffer or to substances extracted from the stool into the buffer over time, which may have interfered with test performance [29, 30]. It is also suggested that the increasingly high positive results (>1000 ng/mL) observed in a sample measured by the i-Chroma analyzer from Day 1 onward may indicate a prozone effect [22, 26].

Even though manufacturers reported varying stability periods for hemoglobin in stool samples suspended in buffer, generally not less than three days, this study observed negative results in some previously positive samples from Day 1 onward. Furthermore, studies by van Rossum LG et al. [31], van Roon AH et al. [32], Gies et al. [33], and Guittet et al. [34] have shown that while hemoglobin levels tend to decrease over time, test results may either remain stable or become negative. These findings show the importance of performing a FOB test with fresh stool samples and immediately after taking the specimen for reliable results.

While this study reports important findings from evaluated FITs, it also has some limitations: the specimens prepared in a concentration of 50±10 ng/mL in laboratory conditions were not natural fecal samples and may have unforeseeable effects in depicting the actual performance. In addition, since most of the tests used in the comparison were qualitative, ROC-based normalization could not be performed. Therefore, the variability of the cut-off values might have affected the study outcomes. In addition, kappa statistics are sensitive to the distribution of positive and negative results, and their interpretation may differ according to the outcome distribution. In this study, agreement was evaluated across tests with different positivity rates. Therefore, kappa results should be interpreted in the context of the observed result distribution. Furthermore, the absence of sufficient colonoscopy data did not allow for evaluating the FITs for diagnostic sensitivity and specificity. As a result, it was not possible to provide further clarification on the systematic differences observed between test pairs in pairwise comparisons, for example, whether one test yielded more accurate results or produced disproportionately more positive or negative outcomes than the other.

Conclusion

This study showed that agreement and analytical performance among FITs vary, that statistically significant differences may be observed between some test pairs, and that agreement measures alone are not sufficient when considering test interchangeability.

Although the FITs were not compared with a reference method, the use of multiple complementary agreement measures (kappa, McNemar's test, overall percentage agreement, and positivity rates) supports a more reliable comparison of test performance.

Beyond agreement measures, analytical characteristics that play an important role in the evaluation and selection of FITs, such as consistency, stability, and positivity rates, may influence procurement decisions, routine laboratory practice, and the implementation of population-based screening programs. Analytical variability in a test may compromise the reliability of results and increase laboratory costs and workload by requiring procedures such as repeat testing and calibration checks. It may also lead to errors in patient follow-up and referral processes during the implementation of the screening program, thereby affecting clinical workload, confidence in the screening program, and resource allocation. Therefore, test selection should be based on a comprehensive assessment that considers analytical performance together with agreement results and the potential impact on laboratory and clinical management.

However, further studies assessing the analytical and clinical performance of commercially available test brands are warranted to better determine the reliability and overall performance of the test.

Disclosures

Ethics Committee Approval: The study was approved by the Ankara Numune Training and Research Hospital Ethics Committee (no: 178/2014, date: 07/05/2014).

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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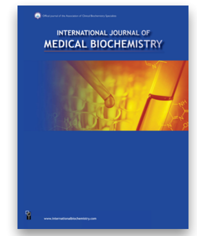

Research Article

Serum Fibulin-5, VEGF-C, and selenium-binding protein-1 levels across stages of diabetic retinopathy: An observational study on pathophysiological associations

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Abstract

Objectives: Diabetic retinopathy (DR) is a major microvascular complication of diabetes characterized by vascular instability and oxidative stress. This study aimed to evaluate the serum levels of Fibulin-5, Selenium-Binding Protein-1 (SBP-1), and Vascular Endothelial Growth Factor-C (VEGF-C) across different clinical stages of DR and to investigate their independent pathophysiological associations.

Methods: This cross-sectional study included 179 participants categorized into four groups: healthy controls (n=45), diabetes mellitus (DM) without retinopathy (n=45), non-proliferative DR (NPDR) (n=45), and proliferative DR (PDR) (n=44). Serum levels were measured using ELISA. The strength of clinical associations was evaluated via ROC analysis, and independent relationships were assessed using multivariable logistic regression models adjusting for age and HbA1c.

Results: Serum Fibulin-5 and SBP-1 levels were significantly elevated in the NPDR group compared to all other groups ($p<0.001$ for both). In multivariable regression, both Fibulin-5 (OR: 3.13, $p=0.0004$) and SBP-1 (OR: 1.63, $p=0.004$) maintained a strong, independent association with the NPDR stage, distinct from systemic glycemic control. VEGF-C levels did not show significant differences among the groups ($p=0.310$).

Conclusion: The stage-specific dramatic elevations of Fibulin-5 and SBP-1 in NPDR suggest a systemic reflection of a compensatory mechanism against early retinal microvascular injury and oxidative stress, which appears to diminish in the advanced PDR stage. These findings provide novel observational insights into the systemic dynamics of extracellular matrix remodeling and redox stress in DR pathogenesis.

Keywords: Diabetic retinopathy, Fibulin-5, ROC analysis, SBP-1, VEGF-C

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Diabetic retinopathy (DR) is a major microvascular complication of diabetes mellitus and remains one of the leading causes of vision loss worldwide [1]. The global prevalence of DR is increasing in parallel with the rise in diabetes, posing a significant public health problem. The International Diabetes Federation has estimated that the number of DM patients worldwide will increase from 463 million in 2019 to 700 million by 2045 [2]. Similarly, it is estimated that the global prevalence of DR will rise significantly, reaching approximately 160.5 million by 2045 [3].

DR is classified into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) [4]. NPDR, the early stage, is characterized by microaneurysms, hemorrhages, and exudates. Over time, it may progress to PDR, which involves pathological neovascularization and carries a high risk of severe vision loss. The pathophysiology involves hyperglycemia-induced endothelial dysfunction, oxidative stress, inflammation, and extracellular matrix (ECM) remodeling [5, 6].

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While anti-VEGF therapies have transformed management, DR is a multifactorial disease [7]. Evaluating circulating molecules that reflect ECM remodeling and angiogenesis could offer mechanistic insights into disease progression. Fibulin-5 (FBLN5) is an integrin-binding ECM glycoprotein involved in vascular remodeling and elastic fiber assembly, potentially acting as an endogenous anti-angiogenic factor by restricting endothelial cell proliferation [8, 9]. Alongside ECM stability, the redox balance in the ischemic retina is a critical component of the pathophysiological response. Selenium-Binding Protein 1 (SBP-1), recently characterized as a methanethiol oxidase, plays a pivotal role in modulating redox signaling by producing hydrogen sulfide (H2S), a protective gasotransmitter, and neutralizing reactive oxygen species (ROS) [10]. However, the systemic fluctuations of SBP-1 across DR stages remain poorly understood. VEGF-C, a regulator of lymphangiogenesis, has been less studied in DR compared to VEGF-A but is thought to support endothelial survival under hyperglycemic stress [11].

Despite advances in understanding local ocular changes, there is a lack of comprehensive studies evaluating the systemic reflection of concurrent ECM stabilization and redox modulation during the progression of DR. Therefore, the primary purpose of this cross-sectional study was to simultaneously quantify the serum levels of Fibulin-5, SBP-1, and VEGF-C across different clinical stages of DR. We aimed to determine the independent associations of these circulating molecules with disease stages, hypothesizing that systemic variations reflect a stage-specific pathophysiological response to retinal ischemia and oxidative stress.

Materials and Methods

Study design and participants

This cross-sectional study included 179 participants recruited at a single tertiary center. Participants were categorized into four groups: healthy controls (n=45), type 2 diabetes mellitus without retinopathy (n=45), non-proliferative DR (n=45), and proliferative DR (n=44). Diabetic retinopathy staging was performed according to the international clinical diabetic retinopathy disease severity scale [4]. Inclusion criteria were age >18 years. Exclusion criteria included other ocular pathologies, systemic inflammatory diseases, active malignancy, and significant renal impairment (to prevent confounding effects on the renal clearance of the measured molecules). This study was approved by the Tokat Gaziosmanpaşa University (Date: 13.12.2024, Decision no: 24-MOBAEK-036) and conducted in accordance with the Declaration of Helsinki.

Clinical and laboratory assessment

Demographic data (age, sex) and systemic comorbidities were recorded. Venous blood samples were obtained after an overnight fast (at least 8 hours). Blood was collected into serum separator tubes (SST) and EDTA tubes. Samples were centrifuged at 3500 rpm for 10 minutes to separate the serum. Serum aliquots were stored at -80°C until analysis to prevent protein degradation.

Routine biochemical parameters were analyzed using the Roche Cobas 6000 series c501 autoanalyzer (Roche Diagnostics, Mannheim, Germany). Fasting plasma glucose was measured using the hexokinase method, and serum creatinine was measured using the Jaffe colorimetric assay. HbA1c levels were determined using an immunoturbidimetric inhibition immunoassay.

Serum assays

Serum Fibulin-5, SBP-1, and VEGF-C concentrations were measured using commercially available quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits strictly following the manufacturers' instructions (Reed Biotech Ltd., Catalog Nos: RE1713H for Fibulin-5, ESD0133H for SBP-1, and RE1931H for VEGF-C).

- VEGF-C ELISA kit sensitivity: 65.63 pg/mL; Detection Range: 109.38–7000 pg/mL.
- Fibulin-5 ELISA kit sensitivity: 1.88 ng/mL; Detection Range: 3.13–200 ng/mL.
- SBP-1 ELISA kit was utilized according to the manufacturer's standard protocols, and optical densities were measured spectrophotometrically at 450 nm.

Statistical analysis

Statistical analyses were performed using MedCalc (version 20.009). The conformity of continuous variables to a normal distribution was assessed using the Kolmogorov-Smirnov test. Continuous variables are presented as median (interquartile range, IQR). Group comparisons were conducted using the Kruskal–Wallis test with Bonferroni-corrected post-hoc pairwise Mann-Whitney U tests. To evaluate the strength of the clinical associations, Receiver Operating Characteristic (ROC) curve analysis was utilized. To assess the independent association of these molecules with DR presence, multivariate binary logistic regression analysis was performed, adjusting for potential confounders such as HbA1c and age. Due to the right-skewed nature of the data, concentrations were normalized using a Log10 transformation prior to regression analysis. A two-sided p-value <0.05 was considered statistically significant.

Results

Participant characteristics

Age and sex distribution were comparable among the groups, as determined by the Kruskal-Wallis and Chi-square tests (p=0.280 and p=0.824, respectively) (Table 1). As expected, fasting glucose and HbA1c were significantly higher in the diabetic groups compared to the controls (p<0.001). The clinical, demographic, and biochemical characteristics of the study population are summarized in Table 2.

Serum molecular levels

Median serum Fibulin-5 levels showed a statistically significant difference across the study groups (p<0.001). Post-hoc analy-

Table 1. Comparison of gender parameter between study groups

Gender	Control (n=45)	DM (n=45)	NPDR (n=45)	PDR (n=44)
Male, n (%)	18 (40.0)	14 (37.8)	17 (37.8)	17 (38.6)
Female, n (%)	27 (60.0)	31 (62.2)	28 (62.2)	27 (61.4)

Data are presented as n (%) for categorical variables. There was no statistically significant difference between the groups according to the Chi-square test (p=0.824). DM: Diabetes mellitus; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; n: Participant number.

Table 2. Comparison of demographic and biochemical parameters between study groups

Parameter	Control (n=45)	DM (n=45)	NPDR (n=45)	PDR (n=44)	p*
Age (years)	66 (13)	65 (13)	63 (8)	60 (11)	0.280
Fibulin-5 (ng/mL)	9.3 (5.2)	9.9 (6.2)	24.3 (34.2)	8.8 (7.7)	<0.001
VEGF-C (pg/mL)	2362 (1647)	2235 (1479)	2854 (1647)	2075 (2674)	0.310
Fasting Glucose (mg/dL)	99 (16)	124 (51)	144 (114)	239 (138)	<0.001
Creatinine (mg/dL)	0.83 (0.27)	0.82 (0.35)	0.91 (0.28)	0.88 (0.37)	0.608
HbA1c (%)	5.7 (0.4)	6.8 (2.0)	8.1 (3.5)	8.8 (2.9)	<0.001

*: Overall p-values were derived from the Kruskal-Wallis test. DM: Diabetes mellitus; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; VEGF-C: Vascular endothelial growth factor-C; HbA1c: Glycated hemoglobin; IQR: Interquartile range. Data are presented as median (IQR).

Table 3. SBP-1 levels across the study groups

Study groups	n	Median (pg/mL)	IQR (25%–75%)	Min-Max	Overall p-value*	Significant differences (Post-Hoc)**
Control	45	1643.31	922.45–2885.23	215.58–10225.23	<0.0001	None
DM	45	1498.34	867.10–2489.70	211.68–14489.24		None
NPDR	45	5207.66	1857.51–7150.51	156.06–35886.01		> DM (p<0.0001) > Control (p<0.0001)
PDR	44	2673.87	584.49–8341.09	62.83–21256.38		None

*: Overall p-value was derived from the Kruskal-Wallis test; **: Post-hoc pairwise comparisons were performed using the Mann-Whitney U test with Bonferroni correction. DM: Diabetes mellitus; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; SBP-1: Selenium-binding protein-1; IQR: Interquartile range.

sis revealed that serum Fibulin-5 was significantly higher in the NPDR group (Median: 24.3 ng/mL) compared to healthy controls (9.3 ng/mL), diabetic patients without retinopathy (9.9 ng/mL), and patients with PDR (8.8 ng/mL) (Fig. 1). Similarly, serum SBP-1 levels exhibited a highly significant variance across the groups (p<0.001). SBP-1 concentrations peaked in the NPDR group (Median: 5207.66 pg/mL), which was significantly higher than both the control (1643.31 pg/mL) and DM without retinopathy (1498.34 pg/mL) groups. In the PDR group, SBP-1 levels declined to a median of 2673.87 pg/mL, losing statistical significance compared to the controls. Detailed variance analyses of SBP-1 levels across the study groups are provided in Table 3. In contrast, serum VEGF-C levels did not demonstrate significant differences among the groups (p=0.310).

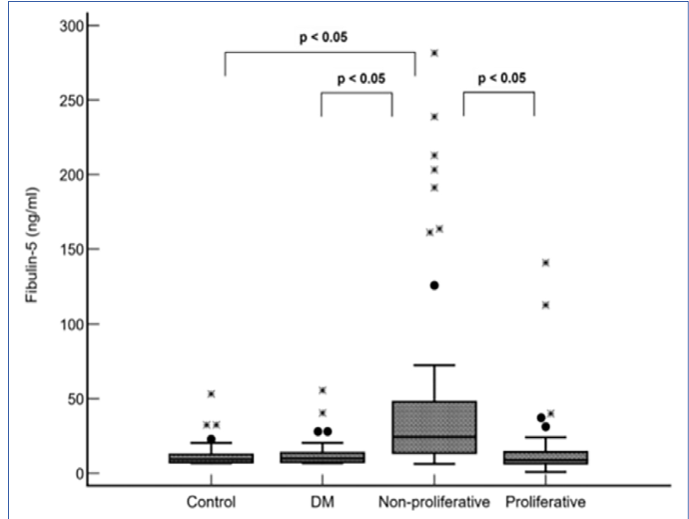

Figure 1. Box-plot diagrams illustrating the serum distributions of Fibulin-5 across the study groups. The central horizontal line represents the median, while the boxes indicate the interquartile ranges (IQR). Whiskers represent the data range excluding outliers.

DM: Diabetes mellitus; NPDR: Non-proliferative diabetic retinopathy; PDR: Proliferative diabetic retinopathy; IQR: Interquartile range.

Clinical association analysis (ROC)

To assess the discriminative association of the molecules in distinguishing NPDR from diabetic patients without retinopathy, a ROC curve analysis was performed. Fibulin-5 demonstrated a significant association with an Area Under the Curve (AUC) of 0.793 (95% CI: 0.700–0.886). At a cut-off value of >16.99 ng/mL, Fibulin-5 exhibited a sensitivity of 64.4% and a specificity of 86.6%. SBP-1 also showed a robust association profile with an

Table 4. AUC and optimal cut-off values for Fibulin-5, SBP-1 and VEGF-C

Biomarker	AUC	Optimal Cut-off	Sensitivity (%)	Specificity (%)
Fibulin-5	0.793	>16.99 ng/mL	64.44	86.67
SBP-1	0.757	>2725.56 pg/mL	71.11	80.00
VEGF-C	0.591	>2853.89 pg/mL	51.11	71.11

Optimal cut-off values were determined by maximizing the Youden Index in the Receiver Operating Characteristic (ROC) curve analysis to discriminate the non-proliferative diabetic retinopathy (NPDR) stage from diabetic patients without retinopathy. AUC: Area under the curve; SBP-1: Selenium-binding protein-1; VEGF-C: Vascular endothelial growth factor-C.

Table 5. Multivariable logistic regression analysis for independent associations with the NPDR stage

Model and independent variables	Coefficient (β)	OR	95% CI	p
Model 1: SBP-1				
SBP-1 (Per 2-fold increase)	0.487	1.63	1.16–2.29	**0.004**
HbA1c (%)	0.626	1.87	1.29–2.71	**0.001**
Creatinine (mg/dL)	1.079	2.94	0.41–21.16	0.283
Age (years)	0.012	1.01	0.94–1.09	0.744
Model 2: Fibulin-5				
Fibulin-5 (Per 2-fold increase)	1.141	3.13	1.65–5.94	**0.0004**
HbA1c (%)	0.611	1.84	1.26–2.69	**0.001**
Creatinine (mg/dL)	0.963	2.62	0.33–20.66	0.360
Age (years)	-0.009	0.99	0.91–1.07	0.823
Model 3: VEGF-C				
VEGF-C (Per 2-fold increase)	0.101	1.11	0.62–1.99	0.735
HbA1c (%)	0.737	2.09	1.43–3.05	**0.0001**
Creatinine (mg/dL)	1.163	3.20	0.53–19.28	0.204
Age (years)	0.010	1.01	0.94–1.09	0.772

Dependent variable: Presence of NPDR vs. DM without Retinopathy. Biomarker concentrations were Log10 transformed and scaled to reflect the likelihood associated with a 2-fold increase in circulating levels. NPDR: Non-proliferative diabetic retinopathy; OR: Odds ratio; CI: Confidence interval; HbA1c: Glycated hemoglobin; FBLN-5: Fibulin-5; SBP-1: Selenium-binding protein-1; VEGF-C: Vascular endothelial growth factor-C.

AUC of 0.757, yielding a sensitivity of 71.1% and a specificity of 80.0% at a cut-off of >2725.56 pg/mL. The ROC curves are shown in Figure 2 for Fibulin-5 and Figure 3 for SBP-1. Detailed diagnostic metrics and optimal cut-off values are presented in Table 4.

Multivariate logistic regression analysis

To evaluate whether the molecules are independently associated with the NPDR stage, we performed a multivariate binary logistic regression analysis adjusting for age, creatinine, and HbA1c. The analysis revealed that normalized serum Fibulin-5 maintained a strong independent association with NPDR. Every 2-fold increase in circulating Fibulin-5 levels was associated with a 3.13-fold increased likelihood of NPDR presence (Odds Ratio: 3.13, 95% CI: 1.65–5.94, p=0.0004). Parallel to this, SBP-1 was also identified as an independent correlate, with a 2-fold increase raising the likelihood of NPDR by 1.63 times (OR: 1.63, 95% CI: 1.16–2.29, p=0.004). This indicates that the variations of Fibulin-5 and SBP-1 are not solely driven by systemic glycemic status or renal function (Table 5). VEGF-C lost all statistical significance when adjusted for HbA1c (p=0.735).

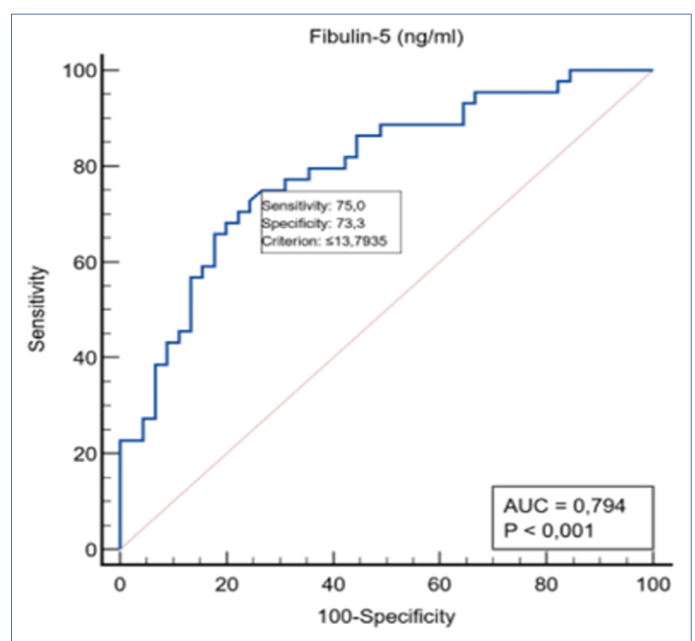

Figure 2. Receiver operating characteristic (ROC) curves demonstrating the discriminative association of Fibulin-5 for detecting the non-proliferative diabetic retinopathy (NPDR) stage.

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

Discussion

In this study, we observed that serum Fibulin-5 and SBP-1 concentrations are significantly elevated in patients with non-pro-

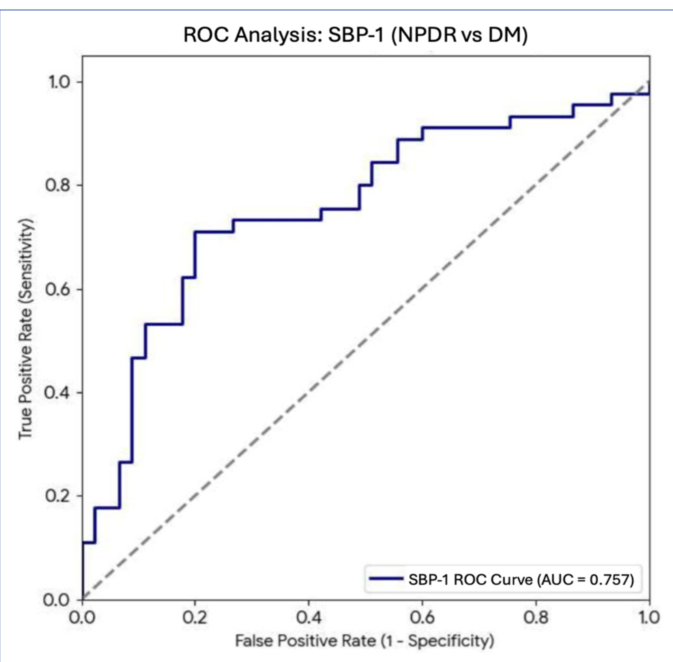

Figure 3. Receiver operating characteristic (ROC) curves demonstrating the discriminative association of SBP-1 for detecting the non-proliferative diabetic retinopathy (NPDR) stage.

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

liferative diabetic retinopathy (NPDR) compared to healthy controls, diabetic patients without retinopathy, and those with proliferative DR (PDR). Importantly, multivariate regression analysis confirmed that these elevations are independent of HbA1c levels, suggesting that they reflect localized pathophysiological mechanisms of extracellular matrix remodeling and redox stress rather than mere systemic hyperglycemia.

The most compelling finding of this study is the stage-specific biphasic trajectory—peaking at NPDR and subsiding at PDR—which points toward a novel "compensatory failure" mechanism in DR pathogenesis. Current literature highlights Fibulin-5 as a matricellular glycoprotein that restricts endothelial cell migration via integrin interactions, acting as an endogenous angiogenesis inhibitor [12]. The dramatic independent surge of Fibulin-5 during the NPDR stage likely reflects the vascular endothelium's physiological response to stabilize the basement membrane against escalating angiogenic stimuli. Recent proteomic analyses by Honoré et al. [13] have similarly identified Fibulin-5 alterations in DR, supporting its role as a dynamic correlate of vascular structural integrity. As the disease progresses to the PDR stage, the sharp decline in Fibulin-5 levels signifies the potential loss of this anti-angiogenic structural defense, coinciding with unrestrained pathological neovascularization.

A parallel compensatory mechanism is evident in the redox axis governed by SBP-1. Philipp et al. [10] recently characterized SBP-1 as a methanethiol oxidase that produces hydrogen sulfide (H_2S), an endogenous gasotransmitter known to confer microvascular vasodilation and activate antioxidant pathways against reactive oxygen species. Animal models of early diabetic retinal neurodegeneration have shown that local retinal Selenbp1 gene expression is significantly upregulated [14]. Our detection of a massive systemic SBP-1 peak in the NPDR cohort aligns with this localized tissue response. The ischemic retina likely upregulates SBP-1 to balance the oxidative burden; this localized overproduction is then reflected in the systemic circulation due to blood-retinal barrier breakdown. The subsequent drop in SBP-1 during the advanced PDR stage provides clinical evidence of potential "cellular exhaustion," indicating that the local endothelial and glial cells synthesizing this enzyme may have succumbed to ischemia and apoptosis.

In contrast to Fibulin-5 and SBP-1, circulating VEGF-C levels did not differ significantly across diabetic retinopathy stages and lost all independent association in multivariable models. Although VEGF-C is involved in lymphangiogenesis and angiogenic signaling, systemic VEGF-C concentrations may not accurately reflect localized retinal angiogenic activity. This finding is consistent with previous reports indicating that intraocular levels of angiogenic mediators may be more relevant than serum measurements in the pathophysiology of diabetic retinopathy, or that systemic VEGF-C merely acts as a surrogate for chronic systemic inflammation rather than a specific ocular trigger [15].

Several limitations of this study should be acknowledged. The cross-sectional and single-center design fundamentally limits causal inference regarding the timeline of molecular fluctuations. Furthermore, specific clinical metadata such as the exact duration of diabetes and detailed antidiabetic medication histories were not uniformly available for all participants; thus, these variables could not be robustly included in our regression models. Consequently, we avoid proposing these molecules as definitive clinical biomarkers, but rather present them as systemic reflections of the underlying retinal pathophysiology. Additionally, the lack of paired vitreous samples restricts direct conclusions regarding local retinal production. Nevertheless, the clear stage-specific elevation of Fibulin-5 and SBP-1, along with their independence from glycemic control, highlights their relevance as circulating indicators of early microvascular remodeling and oxidative stress in diabetic retinopathy.

Conclusion

Serum Fibulin-5 and SBP-1 are significantly elevated in non-proliferative diabetic retinopathy and demonstrate a strong association with the disease stage, separate from systemic glycemic control. The sudden regression of these molecules in the proliferative stage suggests a phenomenon of cellular exhaustion and loss of compensatory vascular defense. While further longitudinal studies incorporating extended clinical metadata are required, these pathways provide valuable observational insights into the systemic dynamics of extracellular matrix remodeling and oxidative damage in early-stage diabetic retinopathy.

Disclosures

Ethics Committee Approval: The study was approved by the Tokat Gaziosmanpaşa University Ethics Committee (no: 24-MOBAEK-036, date: 13/12/2024).

Informed Consent: Written informed consent was obtained.

Conflict of Interest Statement: None declared.

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Use of AI for Writing Assistance: None declared.

Authorship Contributions: Concept – M.S.A., M.K., E.S.; Design – M.S.A., M.K., E.S.; Supervision – M.S.A., M.K., E.S.; Resource – M.S.A., M.K., E.S.; Materials – M.S.A., M.K., E.S.; Data collection and/or processing – M.S.A., M.K., E.S.; Analysis and/or interpretation – M.S.A., M.K., E.S.; Literature review – M.S.A., M.K., E.S.; Writing – M.S.A., M.K., E.S.; Critical review – M.S.A., M.K., E.S.

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Research Article

Investigation of atherogenic indices associated with cardiometabolic risk in patients with low vitamin B12 levels

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Abstract

Objectives: This study aimed to investigate novel composite lipid indices, including the non-High Density Cholesterol/High Density Cholesterol ratio, Lipoprotein Combined Index, and Triglyceride-Glucose Index in adults with low vitamin B12 levels, alongside established markers such as Castelli Risk Index I, Castelli Risk Index II, and the Atherogenic Index of Plasma. By examining the relationship between vitamin B12 status and these indices, it aimed to clarify the role of low vitamin B12 levels in cardiometabolic risk.

Methods: This retrospective study included 400 participants. Glucose and lipid levels were measured using fasting serum samples. Composite lipid parameter values were calculated according to methodologies described in the literature. Results were compared between low vitamin B12 levels and control groups.

Results: In this study, low vitamin B12 levels were found to be associated with low high-density lipoprotein cholesterol (p=0.035), high atherogenic plasma index (p=0.048), and high triglyceride-glucose index (p=0.043). In contrast, no significant differences were observed between the groups in terms of Castelli Risk Index I, Castelli Risk Index II, non-High Density Cholesterol/High Density Cholesterol ratio, or Lipoprotein Combined Index. The area under the ROC curve was found to be ± standard error of 0.557±0.029 [95% CI: 0.501–0.613] (p=0.048) for the Atherogenic Plasma Index and ± standard error of 0.557±0.029 [95% CI: 0.500–0.613] (p=0.049) for the Triglyceride-Glucose Index.

Conclusion: In this study, B12 deficiency was associated with negative lipid indices. It is hypothesized that regular assessment of B12 levels and appropriate correction of deficiencies may help reduce cardiometabolic risk. Further prospective studies with comprehensive biomarkers are needed to clarify causality and clinical benefit.

Keywords: Atherogenic index of plasma, cardiometabolic risk, castelli risk index I, castelli risk index II, lipoprotein combined index, non-high density cholesterol/high density cholesterol ratio, triglyceride-glucose index, vitamin B12

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Vitamin B12 (cobalamin) is a water-soluble vitamin essential for hematopoietic and neurological function. Vitamin B12 (B12) deficiency primarily arises from insufficient dietary intake in vegetarians or vegans, older adults, pregnant women, and people with chronic alcohol consumption, as well as from decreased intestinal absorption due to conditions such as atrophic gastritis, malabsorption syndromes, or gastrointestinal surgeries. Certain medications, such as antacids and metformin, can also contribute to this deficiency [1]. The severity and clinical manifestations of low B12 levels depend on both its extent and duration. The hematologic, bone marrow, and nervous systems are the most affected systems. Megaloblastic anemia develops due to defective thymidine and consequently DNA synthesis in rapidly proliferating cells. Impaired myelin formation and repair can cause neurological complications, potentially leading to diverse symptoms such as cognitive decline and psychosis. Diagnosis is established through clinical evaluation supported by laboratory analyses that provide reliable predictive markers [2]. Epidemiological studies have reported associations

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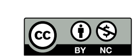

between low B12 levels and obesity, hypertension, type 2 diabetes, and metabolic syndrome [3]. Beyond hematologic and neurological consequences, emerging evidence links B12 deficiency to cardiometabolic disorders through its impact on lipid metabolism [4]. The cellular role of vitamin B12 in lipogenesis is as follows: B12 deficiency reduces the production of methionine and s-adenosylmethionine (SAM) in the cell. This leads to an increase in homocysteine (hyperhomocysteinemia) and the accumulation of SAH. Increased s-adenosyl homocysteine (SAH) and decreased SAM reduce DNA methylation. This alters gene expression. Furthermore, low B12 levels impair methylmalonyl-CoA mutase (MCM) activity, leading to the accumulation of methylmalonic acid (MMA). Ultimately, this inhibits the beta-oxidation of fatty acids [3]. Dyslipidemia is a major contributor to atherosclerosis and cardiovascular disease [3]. Traditionally, an atherogenic lipid profile is defined by elevated total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglyceride (TG) levels, accompanied by decreased high-density lipoprotein cholesterol (HDL-C) [5]. Recently, new composite atherogenic indices that integrate multiple lipid parameters to better predict cardiovascular and metabolic risk have emerged [6, 7]. The Castelli Risk Index-I (CRI-I: Total Cholesterol/HDL-C ratio), Castelli Risk Index-II (CRI-II: LDL-C/HDL-C ratio), Atherogenic Index of Plasma (AIP: $\log_{10}$[TG/HDL-C]), and Lipoprotein Combined Index (LCI: Total Cholesterol × TG × LDL-C/HDL-C) have demonstrated strong associations with various cardiometabolic states [8]. Compared with traditional single lipid measurements, these indices provide a more comprehensive reflection of the balance between atherogenic and antiatherogenic lipoproteins [9]. NHHR (the ratio of non–high-density lipoprotein cholesterol (non-HDL-C) to HDL-C) is a novel lipid ratio introduced in 2022 following a large-scale longitudinal study involving 15,000 individuals conducted by Chinese researchers [10]. This ratio has demonstrated considerable potential in predicting the risk of various diseases, such as coronary artery disease, diabetes mellitus, abdominal aortic aneurysm, and carotid atherosclerosis. Compared with non-HDL-C alone, NHHR provides a more comprehensive and superior measure of atherosclerosis by incorporating both atherogenic (non-HDL-C) and protective (HDL-C) lipid components. Thus, it represents a novel composite lipid marker for atherosclerosis [11]. CRI-I, CRI-II, and AIP are ratios that can be easily calculated from a standard lipid profile. These ratios are independent risk markers for cardiovascular disease and are known to be elevated in patients with angiographically verified coronary artery disease [12]. Furthermore, novel markers such as lipoprotein combined index (LCI) have shown potential utility in detecting early atherosclerotic changes and predicting metabolic disturbances [9]. The Triglyceride-Glucose index (TyG) has also been identified as a strong predictor of cardiometabolic risk factors, vascular abnormalities, and mortality [13].

This study aimed to investigate novel composite lipid indices, including NHHR, LCI, and TyG, in adults with low B12 levels, alongside established markers such as CRI-I, CRI-II, and AIP. By examining the relationship between B12 status and these indices, it aimed to clarify the role of low B12 levels in cardiometabolic risk.

Materials and Methods

The current study was designed as a retrospective study. The study was approved by the Bursa City Hospital Scientific Research Ethics Committee with decision number 2025-19/3 dated 01.10.2025. We also declared that this study was conducted in accordance with the regulations set forth in the World Medical Association Scientific Research and Ethics Committee and the Helsinki Declaration. Between April 30, 2025, and October 31, 2025, the biochemical data of patients with low B12 levels were screened. Four hundred participants were included in the study. The patient group consisted of 200 people: 134 (67%) women and 66 (33%) men with low B12 levels. The control group consisted of 200 people: 142 (71%) women and 58 (29%) men with B12 levels within the reference range. Patients diagnosed with liver failure, hepatitis, elevated liver enzymes, fatty liver, renal failure, thyroid dysfunction, use of B12 supplements, diabetes mellitus, those using gastroprotective agents, pregnancy, hospitalized patients, atherosclerotic heart disease, malignancy, and use of medications causing low B12 levels (phenytoin, metformin, methotrexate) were excluded. Patients with folic acid levels within the reference ranges were included in the study. All data, including age, gender, ICD-10 diagnostic codes (International Statistical Classification of Diseases and Health Problems), and treatment protocols, were obtained from records in the hospital information management system. Glucose and lipid levels were analyzed in fasting serum samples using the Cobas 8000 Modular Analysis System (Cobas, Mannheim, Germany), an automated biochemical analyzer, while serum B12 levels were analyzed using the electrochemical luminescence immunoassay method on Cobas 8000 immunoassays (Roche Diagnostics, Mannheim, Germany). The original kits (Roche Diagnostics, Mannheim, Germany) were used for this study. The quality assurance procedures included periodic instrument calibration, adherence to standard operating protocols, and participation in external quality assessment schemes. Serum B12 levels were used to assess B12 status. Methylmalonic acid or total homocysteine levels, which are markers of tissue-level low B12 levels, were not included. Serum B12 levels had previously been shown to be valid indicators of B12 status in both individual and epidemiological settings [14]. In this study, values below 197 ng/L were defined as low B12 levels.

The calculated indices were as follows: CRI-I, CRI-II, AIP, NHHR, LCI, and TyG. The formulas used for these calculations followed previously published methodologies [9].

CRI-I=TC (mg/dL) / HDL-C (mg/dL)

CRI-II=LDL-C (mg/dL) / HDL-C (mg/dL)

AIP=$\log_{10}$[TG (mg/dL) / HDL-C (mg/dL)]

NHHR=[(TC (mg/dL) - HDL-C (mg/dL))] / HDL-C (mg/dL)

LCI=TC (mg/dL) × TG (mg/dL) × LDL-C (mg/dL) / HDL-C (mg/dL)

TyG=ln[TG (mg/dL) × fasting glucose (mg/dL) / 2] [15].

The outcomes were analyzed through a comparative assessment between the B12 deficient group and the control group.

Statistical analysis

Sample size calculation: Based on a small effect size (effect size d=0.25), assuming an alpha level (Type I error) of 0.05 and a power of 0.80 (1 − β), it was determined that a total of 398 individuals (199 per group) would be sufficient for the control and patient groups in the study planned to test the null hypothesis. The G*Power Statistical Program, version 3.1.9.4 (Universität Düsseldorf, Germany), was used for analyses. Continuous variables were expressed as mean± standard deviation, and categorical data were expressed as numbers and percentages. Normality analyses were performed using the Kolmogorov-Smirnov Goodness of Fit Test in between-group analyses of continuous variables. Student's T Test was used in between-group analyses of data conforming to a normal distribution, and the Mann-Whitney U Test was used in analyses of those not conforming to a normal distribution. Comparisons of categorical data were made using the Chi-square Test. ROC curve analysis was used to determine cut-off values for AIP and TyG indices in low B12 levels patients. In the presence of significant cut-off values, sensitivity, specificity, and positive and negative predictive values were calculated. When determining the cutoff values, the highest point of the Youden Index (J=Sensitivity + Specificity − 1), which maximizes the balance between sensitivity and specificity, was used as the basis. A Type 1 error level of less than 5% was interpreted as indicating statistical significance of the test's diagnostic value. Since the indices tested in the study (AIP and TyG) are related biochemical parameters representing similar metabolic pathways, and each was tested as a pre-defined independent hypothesis, the standard alpha level (0.05) was maintained. Analyses were performed using IBM SPSS version 27.0 (IBM Corporation, Armonk, NY, USA), and p <0.05 was considered statistically significant.

Results

No statistically significant difference was found between the groups in terms of age and sex (p=0.277 and p=0.387, respectively) (Table 1).

Serum HDL-C and B12 levels were significantly lower in the patient group compared with controls (p=0.035 and p <0.001, respectively). No significant differences were observed for TC, TG, or LDL-C (Table 2). The AIP and TyG were significantly higher in patients with low B12 levels than in the control group (p=0.048 and p=0.043, respectively). In contrast, no significant differences were observed between the groups for the CRI-I, CRI-II, NHHR, or LCI (Table 3).

ROC analysis: For the AIP, the cut-off value was ≥0.25, sensitivity was 65.0% [95% CI: 58.16–71.27], specificity was 45.5% [95% CI: 38.75–52.42], PPV was 54.4% [95% CI: 48.06–60.59], NPV was 56.5% [95% CI: 48.80–63.94], and area under the ROC curve ± standard error was 0.557 ± 0.029 [95% CI: 0.501–0.613] (p=0.048).

Table 1. Comparison of groups in terms of age and sex characteristics

	Control group (n=200)	Patient group (n=200)	p
Age (years) (median (min-max))	37 (18–72)	39 (18–76)	0.277
Sex (n, %)			
Female	142 (71.0)	134 (67.0)	0.387
Male	58 (29.0)	66 (33.0)	

Min: Minimum; Max: Maximum.

Table 2. Comparison of groups in terms of some blood parameters (median, min-max)

	Control group (n=200)	Patient group (n=200)	p
TC (mg/dL)	183 (100–312)	182 (96–285)	0.914
TG (mg/dL)	96 (26–448)	106 (31–595)	0.121
HDL-C (mg/dL)	49 (23–90)	46 (25–131)	**0.035**
LDL-C (mg/dL)	112 (40–231)	111 (46–209)	0.855
Vitamin B12 (ng/L)	392 (197–764)	171.5 (103–196)	**<0.001**

Bold characters indicate statistical significance (p<0.05). TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein; LDL-C: Low-density lipoprotein.

Table 3. Comparison of groups in terms of atherogenic index profile parameters (median, min-max)

	Control group (n=200)	Patient group (n=200)	p
CRI-I	3.75 (1.87–9.14)	3.87 (1.69–8.46)	0.167
CRI-II	2.27 (0.7–6.6)	2.38 (0.92–5.45)	0.261
AIP	0.28 (-0.36–1.19)	0.34 (-0.36–1.32	**0.048**
NHHR	2.75 (0.87–8.14)	2.87 (0.69–7.46)	0.167
LCI	40098 (2281–646819)	45684 (4180–641875)	0.192
TyG (Mean±SD)	8.34±0.49	8.45±0.53	**0.043**

Bold characters indicate statistical significance (p<0.05). CRI-I: Castelli risk index I; CRI-II: Castelli risk index II; AIP: Atherogenic index of plasma; LCI: Lipoprotein combined index; NHHR: nonHDL/HDL ratio; TyG: Triglyceride glucose index; SD: Standard deviation.

Table 4. ROC analysis results and some cut-off values for AIP and TyG values in patients with low B12 levels

	Diagnostic test				ROC curve			
	Cut-off	Sensitivity	Specificity	PPV	NPV	AUC	CI 95%	p
AIP	≥0.25	65.0 *[58.16–71.27]*	45.5 *[38.75–52.42]*	54.4 *[48.06–60.59]*	56.5 *[48.80–63.94]*	0.557±0.029	0.501–0.613	**0.048**
TyG	≥8.44	53.0 *[46.09–59.80]*	61.1 *[54.09–67.49]*	57.9 *[50.38–64.52]*	56.3 *[49.81–62.92]*	0.557±0.029	0.500–0.613	**0.049**

Bold characters indicate statistical significance (p<0.05). Italic characters indicate the confidence interval for the group. AUC: Area under the curve; CI: Confidence interval; NPV: Negative predictive value; PPV: Positive predictive value; IP: Atherogenic Index of Plasma; TyG: Triglyceride-Glucose index.

For the TyG, the cut-off value was ≥8.44, sensitivity was 53.0% [95% CI: 46.09–59.80], specificity was 61.1% [95% CI: 54.09–67.49], PPV was 57.9% [95% CI: 50.38–64.52], NPV was 56.3% [95% CI: 49.81–62.92], and area under the ROC curve ± standard error was 0.557 ± 0.029 [95% CI: 0.500–0.613] (p=0.049) (Table 4 and Fig. 1).

Discussion

Cardiovascular diseases remain the leading cause of morbidity and mortality worldwide. Atherogenic dyslipidemia, typically characterized by elevated TG and LDL-C levels, together with reduced HDL-C, plays a central role in the pathogenesis of atherosclerosis, the key underlying mechanism of cardiovascular disease [16, 17]. Dyslipidemia is recognized as an independent risk factor for atherosclerotic cardiovascular conditions. Conventional lipid monitoring indicators employed in clinical practice, including LDL-C, demonstrate significant limitations [18].

Previous studies examining the association between B12 status and lipid metabolism have yielded inconsistent results [19]. In this context, composite lipid-derived indices are emerging as valuable alternatives, as they integrate multiple metabolic risk factors and better reflect the multifactorial nature of atherosclerosis. These indices, by encompassing broader lipid interactions and physiological processes, can offer greater predictive and diagnostic insight than isolated lipid parameters. Building upon both national and international data, four lipid-based indices have shown promising clinical potential: AIP, NHHR, the Apolipoprotein B/A1 ratio, and LCI [18]. In conclusion, the current study included not only traditional lipid measures but also these composite indices (specifically CRI-I, CRI-II, LCI, NHHR, and TyG) that have not previously been evaluated in adults with low B12 levels. AIP was also analyzed to provide a comprehensive assessment of atherogenicity in this population.

Previous research on the relationship between B12 levels and lipid metabolism has yielded diverse and sometimes conflicting results. Al-Musharaf et al. [14] conducted a study among young women aged 19–30 years. Participants with higher serum B12 concentrations exhibited lower TC, LDL-C, and TG levels, and reduced ratios of CRI-I, CRI-II, and AIP compared with those who had lower B12 levels. No significant difference was observed in HDL-C between groups. Although multiple logistic regression showed an inverse association between serum B12 and dyslipidemia, it lost significance after adjustment for confounders. In contrast, this study was conducted on a larger adult population aged 18–76 years (Table 1). However, it found no significant difference in TC, LDL-C, CRI-I, CRI-II, or TG levels between the B12-deficient and control groups (Table 2). But HDL-C levels were significantly lower in the B12-deficient group (p=0.035), while AIP was significantly higher (p=0.048) (Table 3), indicating a potentially unfavorable lipid pattern associated with low B12 levels. Similarly, Kim et al. [20] reported no correlation between serum B12 concentrations and dyslipidemia or atherosclerotic events in a prospective 12-year follow-up of 421 healthy Korean adults. Their findings align with ours in that TC, LDL-C, CRI-I, CRI-II, and TG did not differ significantly between groups, but HDL-C was lower in individuals with low B12 levels (p=0.035).

Al-Qusous et al. [21] studied patients with hyperlipidemia and found an inverse relationship between B12 levels and

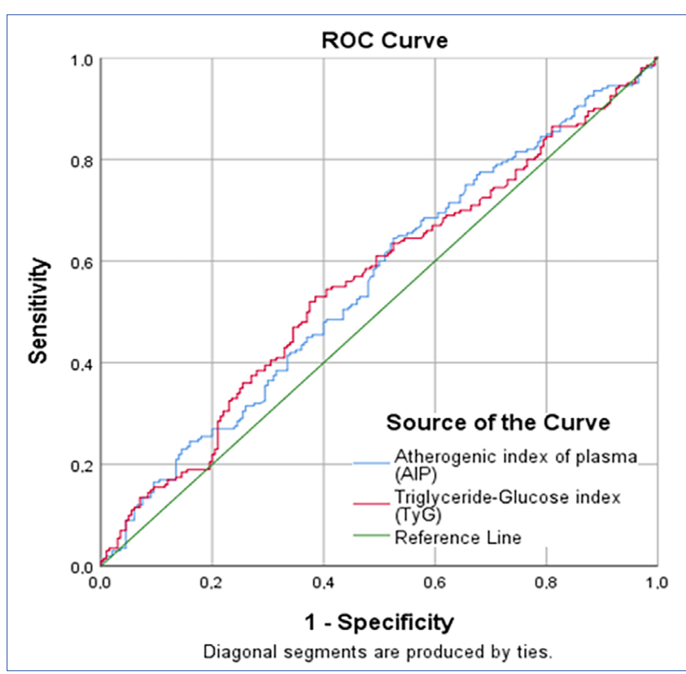

Figure 1. ROC curve graph for Atherogenic Index of Plasma (AIP) and Triglyceride Glucose Index (TyG) values in patients with low vitamin B12 levels.

ROC: Receiver operating characteristic.

TC in the control group. As B12 decreased, TC, LDL-C, and the TC/HDL-C ratio increased; however, only 3.3% of hyperlipidemic patients were actually B12 deficient. In this study, as B12 decreased, TC, LDL-C, and TC/HDL-C ratios did not change compared to the control group. Aureli et al. [22], in a cohort of children, adolescents, and young adults, found that serum lipid profiles (TC, HDL-C, LDL-C, and TG) were not influenced by cobalamin status. Our results showed no significant differences in TC, LDL-C, or TG between patients with low B12 levels and controls; yet HDL-C was notably lower in those with low B12 levels (p=0.035).

Devi [19] examined the link between B12 and AIP in the general population, and reported that low B12 levels were correlated with a poorer lipid profile, higher AIP, and increased blood pressure. Emphasized the potential role of B12 in cardiovascular protection, showing that as B12 levels declined, TC, TG, LDL-C, and AIP increased, while HDL-C decreased [19]. This study observed higher AIP values (p=0.045) and lower HDL-C levels (p=0.035) in the group with B12 deficiency, while no changes were observed in TC, TG, and LDL-C levels (Table 3). Another population-based study demonstrated negative correlations between B12 and TG (r=–0.161, p <0.05), TC (r=–0.169, p<0.05), and AIP (r=–0.15, p <0.05), suggesting that low B12 levels contribute to increased cardiovascular risk and unfavorable lipid parameters [23]. Consistently, this study also demonstrated a significant increase in AIP in individuals with B12 deficiency (p=0.048). However, there were no changes in TG and TC.

Aktaş and Pençe [15] found a negative correlation between B12 levels and both the TG/HDL-C ratio (AIP) and the TyG among obese patients with insulin resistance. Similarly, our results revealed that AIP and TyG were significantly higher in the low B12 levels group (p=0.048 and p=0.043, respectively). Sirivarasai et al. [24] observed significant associations between plasma B12, folate, and homocysteine levels in individuals over 65 years with hyperhomocysteinemia. They reported that elevated homocysteine correlated with arterial stiffness and higher Lipid Accumulation Product (LAP), TyG, and Visceral Adiposity Index (VAI).

Extensive literature has highlighted the significant predictive value of NHHR for established cardiovascular risk factors such as diabetes, hypertension, non-alcoholic fatty liver disease (NAFLD), and obstructive sleep apnea hypopnea syndrome [25]. In this study, NHHR, which has not been previously studied in the literature at low B12 levels, was found not to show a significant difference compared to the control group. Similarly, there are previous studies evaluating LCI as a cardiovascular risk predictor and prediabetes risk predictor in patients with acute coronary syndrome [9]. However, LCI, which has not been previously studied at low B12 levels, did not show a significant difference compared to the control group in this study.

In this study, low B12 levels were associated with low HDL-C (p=0.035), high AIP (p=0.048), and TyG (p=0.043). The ROC analysis for AIP demonstrated a statistically significant association with the studied outcome (p=0.048). However, its overall discriminative performance was limited. The area under the curve (AUC) was 0.557, indicating a weak ability to distinguish between affected and non-affected individuals, only marginally better than chance. Although the sensitivity was relatively moderate (65.0%), suggesting that AIP could identify a proportion of true positive cases, the specificity was notably low (45.5%), reflecting a high rate of false-positive results. Furthermore, both the positive predictive value (54.4%) and negative predictive value (56.5%) were close to 50%, indicating limited clinical utility in predicting the presence or absence of the condition. Moreover, the 95% confidence interval (0.501–0.613) included the threshold for no discrimination. Taken together, these findings suggest that, despite achieving statistical significance, AIP has poor diagnostic accuracy and is insufficient as a standalone biomarker.

In the ROC analysis conducted to evaluate the diagnostic performance of the TyG, statistically significant outcome (p=0.049). The area under the curve (AUC=0.557) indicates that this parameter has limited power to discriminate the condition under investigation (Table 4 and Fig. 1). The low sensitivity (53.0%) and specificity (61.1%) values reveal that the TyG does not perform adequately in either identifying sick individuals or excluding healthy individuals. Similarly, the positive predictive value (57.9%) and negative predictive value (56.3%) results support the limited use of the test as a reliable predictive tool in clinical practice. Furthermore, the 95% confidence interval (0.500–0.613) includes the threshold of no discrimination, suggesting that the predictive capability of the parameter may be unreliable. Although the obtained p value is statistically significant, the evaluation together with the borderline significance and low AUC value indicates that the TyG is not a strong clinically distinctive biomarker. These findings suggest that the parameter should be interpreted with caution and preferably used in combination with other clinical or biochemical indicators.

In this study, low B12 levels were associated with lower HDL-C and higher AIP and TyG indices, suggesting an unfavorable lipid profile. However, ROC analyses demonstrated limited discriminatory power, and other indices (CRI-I, CRI-II, NHHR, LCI) did not differ significantly. These findings highlight a possible link between low B12 levels and cardiometabolic risk, but further prospective studies with comprehensive biomarkers are needed to clarify causality and clinical utility.

Limitations

It must be acknowledged that the study has some limitations. Firstly, the sample size is relatively small. Secondly, the study did not include methylmalonic acid or total homocysteine levels, which are markers of low B12 levels at the tissue level; only serum B12 levels were used as an indicator of deficiency. Since it is a retrospective study, the patients' body mass index (BMI) values were not recorded in the system and therefore could not be used in the study. Consequently, the effects of obesity on blood profiles were disregarded. Fur-

thermore, direct cardiovascular clinical outcomes were not evaluated in the current study. Future research, taking this into account, will be beneficial in determining the net effects of low B12 levels on the cardiovascular system.

Conclusion

In this study, B12 deficiency was associated with negative lipid indices. It is hypothesized that regular assessment of B12 levels and appropriate correction of deficiencies may help reduce cardiometabolic risk.

Disclosures

Ethics Committee Approval: The study was approved by the Bursa City Hospital Scientific Research Ethics Committee (no: 2025-19/3, date: 01/10/2025).

Informed Consent: Written informed consent was obtained.

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

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Research Article

Theobromine suppresses NF-κB signaling and promotes apoptosis in lung cancer and osteosarcoma cells

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Abstract

Objectives: Theobromine has been reported to exhibit anti-inflammatory and antioxidant effects. However, its molecular impact on cancer-associated signaling pathways remains poorly understood. This research was undertaken to evaluate the pro-apoptotic signaling effects of Theobromine on A549 lung cancer and Saos-2 osteosarcoma cells, focusing on modulation of the NF-κB signaling pathway.

Methods: A549 and Saos-2 cells were exposed to Theobromine and the reference NF-κB inhibitor sulfasalazine at their IC_{50} concentrations. Cell viability and IC_{50} values were determined using the WST-1 assay. Apoptosis was quantified by Annexin V-FITC/PI staining, and total and phosphorylated NF-κB and IKK protein levels were quantified by flow cytometry.

Results: Data were analyzed using one-way analysis of variance (ANOVA). In A549 lung cancer cells, Theobromine significantly induced apoptosis ($p<0.001$ for late apoptotic cells). A significant reduction in both total and phosphorylated NF-κB and IKK protein levels was observed compared with controls ($p<0.05$ for p-NF-κB and IKK, $p<0.001$ for p-IKK). In Saos-2 osteosarcoma cells, similarly increased apoptotic cell populations were observed ($p<0.001$ for early and late apoptotic cells). The treatment also resulted in reduced total and phosphorylated NF-κB and IKK levels ($p<0.05$ for NF-κB, $p<0.001$ for p-NF-κB, $p<0.01$ for IKK, and $p<0.0001$ for p-IKK).

Conclusion: This study provides the first evidence that Theobromine suppresses the NF-κB/IKK signaling pathway and promotes apoptosis in A549 and Saos-2 cancer cells. These findings suggest that Theobromine may act as a safe, naturally derived NF-κB modulator with potential applications as an adjuvant or chemopreventive agent in cancer therapy.

Keywords: Lung cancer, osteosarcoma, theobromine

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The cancer types expected to see the largest increases in global cases include breast, lung, and colorectal cancer. More than 34 million cancer cases are projected to be diagnosed annually by 2070 [1, 2]. Lung cancer, in particular, represents the most common cause of cancer-related deaths globally. Its aggressive clinical behavior is largely attributed to early dissemination and the formation of metastases. Although there are methods used to treat the disease, its prognosis is alarming due to the low 5-year survival rate [3]. Bone metastases are frequently encountered among lung cancer metastases. Bone metastases are associated with serious complications, including pain, fractures, and impaired quality of life. The dynamic interaction between tumor cells and the bone microenvironment contributes to both tumor progression and resistance to treatment [4].

Chronic inflammation is one of the hallmarks of cancer. The NF-κB signaling pathway exhibits a central role in inducing the inflammatory cascade and linking it to tumor development. Under normal conditions, NF-κB binds to its inhibitory partners, the IκB proteins, which block its DNA-binding activity. When

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cells are exposed to external stimuli, the IκB kinase (IKK) is activated. As a result, NF-κB is released and transferred to the nucleus, thereby initiating the transcription of target genes [5–7].

Theobromine, a methylxanthine derivative found abundantly in cocoa products, has long been examined for its bronchodilatory and diuretic effects [8, 9]. Recent evidence, however, points to its anti-inflammatory and free radical scavenging effects [10]. Despite these indications, the role of Theobromine in cancer-associated inflammation, particularly in lung cancer and osteosarcoma models, remains unexplored.

This study sought to clarify whether Theobromine modulates the NF-κB signaling cascade in A549 and Saos-2 cancer cell lines. Given the prominent role of NF-κB in regulating cell survival, proliferation, and tumor progression, we aimed to determine whether Theobromine can modulate this pathway under basal conditions. To address this, Theobromine and the reference NF-κB inhibitor sulfasalazine were applied at their IC_{50} concentrations, and their impact on cell viability and apoptotic cell death was evaluated. In addition, total and phosphorylated NF-κB (p65) and IKK levels were quantified by flow cytometry to assess the activity of Theobromine on pathway activation. Through this integrated analysis, the study seeks to clarify whether Theobromine exerts intrinsic regulatory effects on NF-κB signaling and to provide mechanistic insight into its potential as a natural modulator of this key oncogenic pathway.

Materials and Methods

Cell culture

A549 and Saos-2 cells were cultivated in DMEM (Capricorn Scientific, Germany) with 10% FBS (Invitrogen, Carlsbad) and 1% pen/strep at 37°C and 5% CO_2.

Theobromine (500 µM) and sulfasalazine (10 mM) were first dissolved in dimethyl sulfoxide (DMSO) and diluted to 5–100 µM and 0.2–4 mM, respectively.

Water-soluble tetrazolium salt (WST-1) cytotoxicity assay

Each cell line was plated in 96-well plates at a density of 2×10^4 cells/well and incubated for 24 hours. After incubation, Theobromine (0.5, 10, 25, 50, 100 µM) and sulfasalazine (0, 0.2, 0.5, 2, 3, 4 mM) were applied at varying concentrations for 24 and 48 hours. Then, 10 µL of WST-1 solution was added to each well and incubated for 4 hours. The absorbance was read at 450 nm using a Thermo Varioskan Microplate Reader. All experiments were performed in at least three independent replicates (n=3), and the results are presented as mean ± standard deviation (SD). IC_{50} values were calculated using nonlinear regression analysis by fitting the data to a sigmoidal dose–response (variable slope) curve using GraphPad Prism software (version 9.1.0).

Annexin V binding assay for apoptosis detection

To distinguish apoptotic, necrotic, and live cell populations, we carried out flow cytometry analysis. A549 lung cancer cells and Saos-2 osteosarcoma cells were seeded into 6-well plates. IC_{50} values of Theobromine and Sulfasalazine were applied and incubated for 24 h. The cells adhered to the plate were detached using Trypsin. The cells were collected. Then, 5 µL of Annexin V-FITC and propidium iodide (PI) were added. After incubating for 15 minutes, the cells were analyzed using an ACEA NovoCyte flow cytometry device (ACEA Biosciences Inc., San Diego, CA, USA). For each sample, at least 10,000 events were acquired. Compensation was applied using single-stained controls to correct for spectral overlap between fluorochromes.

Cells were gated based on forward scatter (FSC) and side scatter (SSC) parameters to exclude debris. Apoptotic populations were determined using Annexin V-FITC and propidium iodide (PI) staining, where viable cells were defined as Annexin V^-/PI^- (lower left quadrant), early apoptotic cells as Annexin V^+/PI^- (lower right quadrant), late apoptotic cells as Annexin V^+/PI^+ (upper right quadrant), and necrotic cells as Annexin V^-/PI^+ (upper left quadrant).

Measurement of protein levels by flow cytometry

After treatment with Theobromine and Sulfasalazine, the levels of total NF-κB, total IKK, phosphorylated NF-κB, and phosphorylated IKK were determined. The cells were exposed to FCM fixation buffer for 15 minutes and FCM permeabilization buffer for 5 minutes. After washing, primary antibodies for Anti-RELA (St. John's/STJ94468), Anti-Phospho-RELA-Thr345 (St. John's/STJ90353), Anti-IKK Alpha/Beta (St. John's/STJ93667), and Anti-Phospho-IKK Alpha/Beta-Ser 176/177 (St. John's/STJ90301) were used. Then, secondary antibody (Advansta/R-05071) was applied for 1 hour. The cells that received the treatment were analyzed using the NovoCyte D3000 flow cytometer (Agilent Technologies, Inc.).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software (version 9.1.0). Data are presented as mean ± standard deviation (SD). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant.

Results

Theobromine exhibited stronger cytotoxicity in A549 and Saos-2 cancer cells.

In A549 cells, Theobromine exerted a dose-dependent inhibitory effect, decreasing cell viability from 100% to 18.64% at 100 µM after 24 h and to 12.3% after 48 h. Sulfasalazine, on the other hand, produced weaker cytotoxicity, lowering viability from 100% to 23.4% at 4 mM after 24 h and to 13% after 48 h. The IC_{50} values were found to be 25.97±3.5 µM and 1.249±0.2 µM for Theobromine and Sulfasalazine, respectively (Fig. 1a, b).

In Saos-2 cells, Theobromine treatment led to a progressive decline in cell viability from 100% at 0 µM to 24.04% at 100

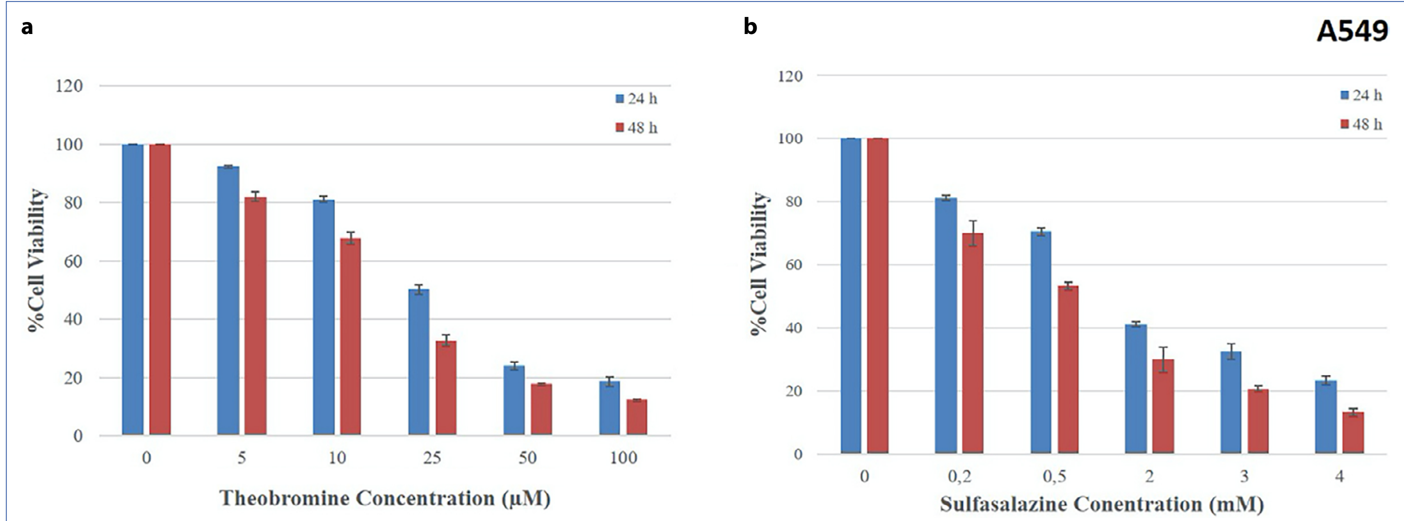

Figure 1. Dose- and time-dependent cytotoxic effects of Theobromine and Sulfasalazine on A549 cells. (a) Cell viability of A549 cells following 24 h and 48 h treatment with increasing concentrations of Theobromine (5–100 µM). (b) Cell viability of A549 cells following 24 h and 48 h treatment with increasing concentrations of Sulfasalazine (0.2–4 mM). Data are presented as mean±SD (n=3).

SD: Standard deviation.

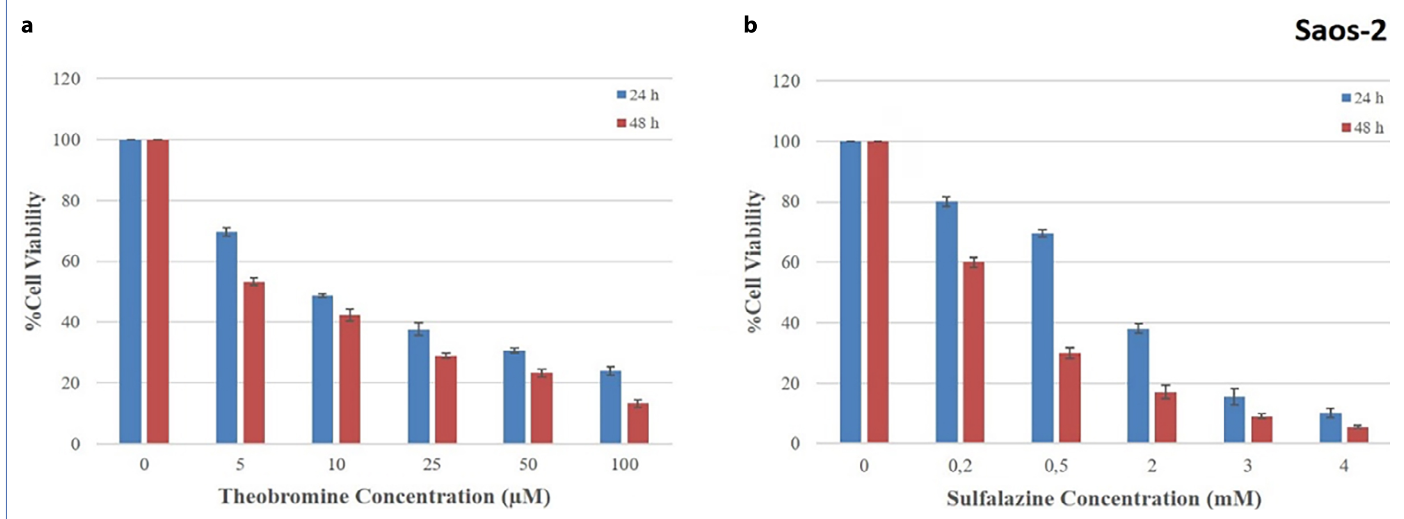

Figure 2. Dose- and time-dependent cytotoxic effects of Theobromine and Sulfasalazine on Saos-2 cells. (a) Cell viability of Saos-2 cells exposure to Theobromine (5–100 µM). (b) Cell viability of Saos-2 cells following 24 h and 48 h exposure to Sulfasalazine (0.2–4 mM). Data are presented as mean±SD (n=3).

µM after 24 h, with mean viability values of 69.5%, 48.7%, 37.7%, and 30.6% at 5, 10, 25, and 50 µM, respectively. Prolonged incubation for 48 h further potentiated its cytotoxic activity, decreasing viability to 13%. The calculated IC_{50} value was 12.64±2.5 µM, indicating strong antiproliferative efficacy. Sulfasalazine exhibited a similar concentration-dependent pattern, reducing viability from 100% to 10.2% at 4 mM after 24 h and to 5.6% after 48 h, with an estimated IC_{50} of 0.8862±0.001 mM (Fig. 2a, b).

Overall, these findings demonstrate that Theobromine exerts a pronounced cytotoxic effect in both Saos-2 and A549 cells. The dose-dependent inhibition of cell viability indicates that Theobromine effectively suppresses cancer cell proliferation, supporting its potential as a natural compound with anticancer properties.

Apoptotic cell death induced by theobromine in A549 and Saos-2 cells

In A549 cells, the control group exhibited a predominantly viable population (97.81±0.45%), while early and late apoptotic fractions were 1.10±0.85% and 2.14±0.82%, respectively. Treatment with Theobromine moderately elevated late apoptosis (26.67±6.02%), suggesting partial activation of apoptotic signaling ($p<0.0001$ vs control). Sulfasalazine induced a pronounced apoptotic response in terms of late apoptosis (56.00±1.00%) compared with both the control and Theo-

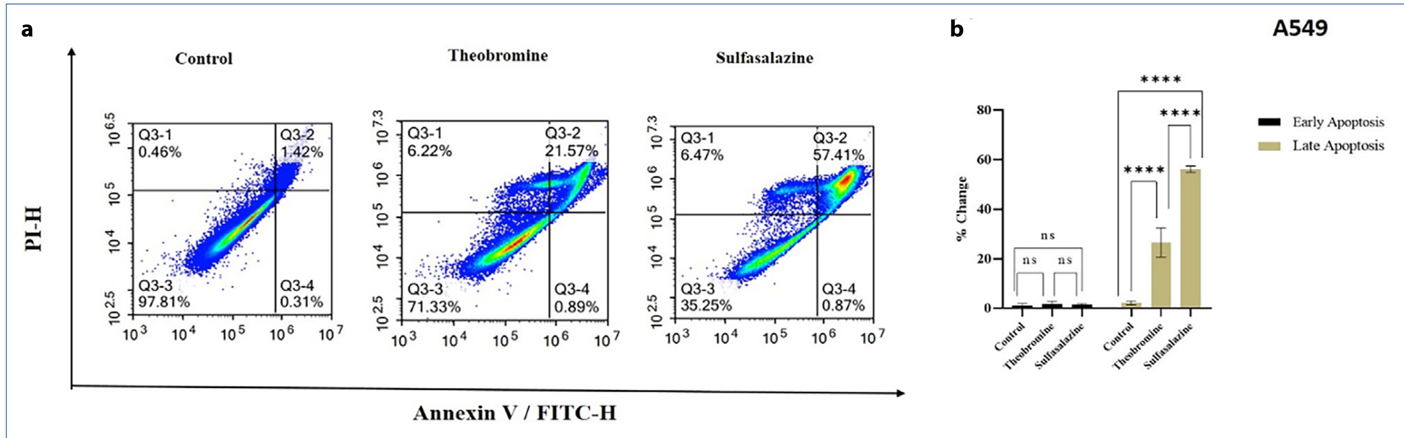

Figure 3. Annexin V-FITC/PI analysis of A549 cells (a) Representative flow cytometry dot plots. (b) Quantification of apoptotic cell populations. Data are presented as mean±SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test

****: p<0.0001; ns, not significant.

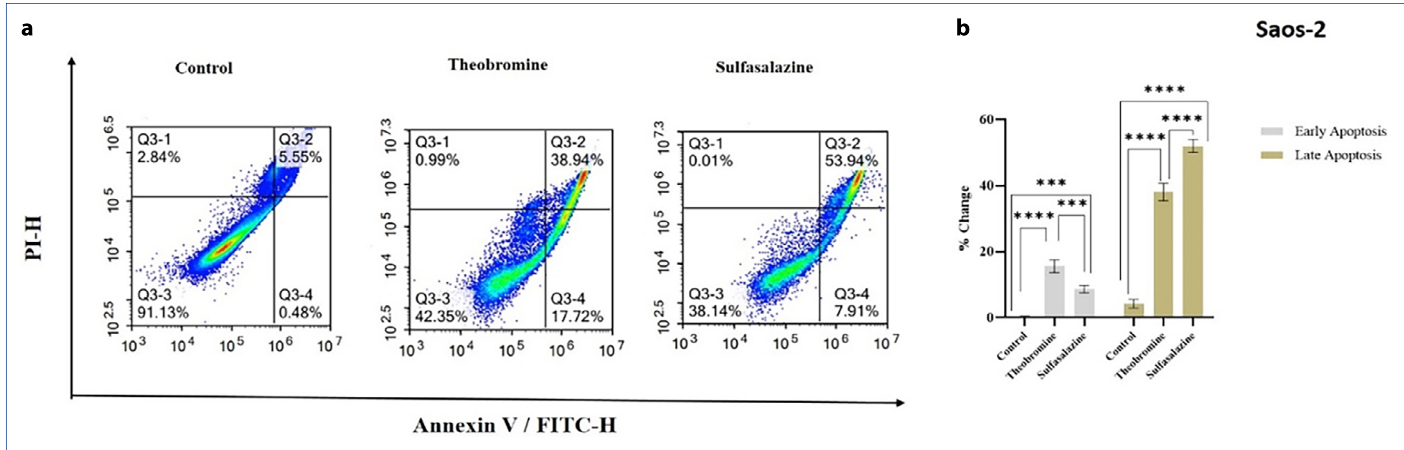

Figure 4. Annexin V-FITC/PI analysis of Saos-2 cells. (a) Representative flow cytometry dot plots. (b) Quantification of apoptotic cell populations. Data are presented as mean±SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

***: p<0.001; ****: p<0.0001.

bromine-treated groups (p<0.0001 vs control). The overall apoptotic rate induced by Sulfasalazine was approximately 2.5-fold higher than that caused by Theobromine, confirming its strong pro-apoptotic potential in A549 cells (Fig. 3a, b).

In Saos-2 cells, the control group exhibited minimal apoptosis, with 0.36±0.22% of cells in early and 4.18±1.28% in late apoptotic phases. Treatment with Theobromine significantly increased both early (15.67±1.15%) and late (37.67±1.53%) apoptotic cell populations compared with the control group (p<0.0001). Sulfasalazine enhanced apoptosis, inducing 8.64±1.04% early and 51.67±2.08% late apoptotic cells. These results suggest that Theobromine elicits a stronger apoptotic response (Fig. 4a, b).

Overall, the Annexin V-FITC/PI analysis revealed that both Theobromine and Sulfasalazine reduced cell viability primarily by promoting apoptosis in a concentration- and cell type–dependent manner. While both compounds were capable of triggering programmed cell death, the extent of apoptotic induction varied between the two cancer models, reflecting differences in cellular sensitivity and underlying molecular characteristics. These results collectively indicate that apoptosis plays a central role in the cytotoxic effects observed following treatment with Theobromine and Sulfasalazine.

Theobromine reduces NF-κB and IKK protein levels in A549 and Saos-2 cells

To assess how Theobromine modulates the NF-κB/IKK signaling pathway in A549 lung cancer and Saos-2 osteosarcoma cells, the expression levels of the relevant proteins were measured by flow cytometry. The mean fluorescence intensity (MFI) values were quantitatively analyzed. Analysis of both the total and phosphorylated forms of NF-κB and IKK proteins provides insight into pathway activity at different levels. Total protein levels reflect the overall amount and expression status of the relevant molecules in the cell, while the phosphorylated forms (p-NF-κB and p-IKK) represent the active conformations of these proteins, which initiate or maintain signaling. Therefore, the combined evaluation

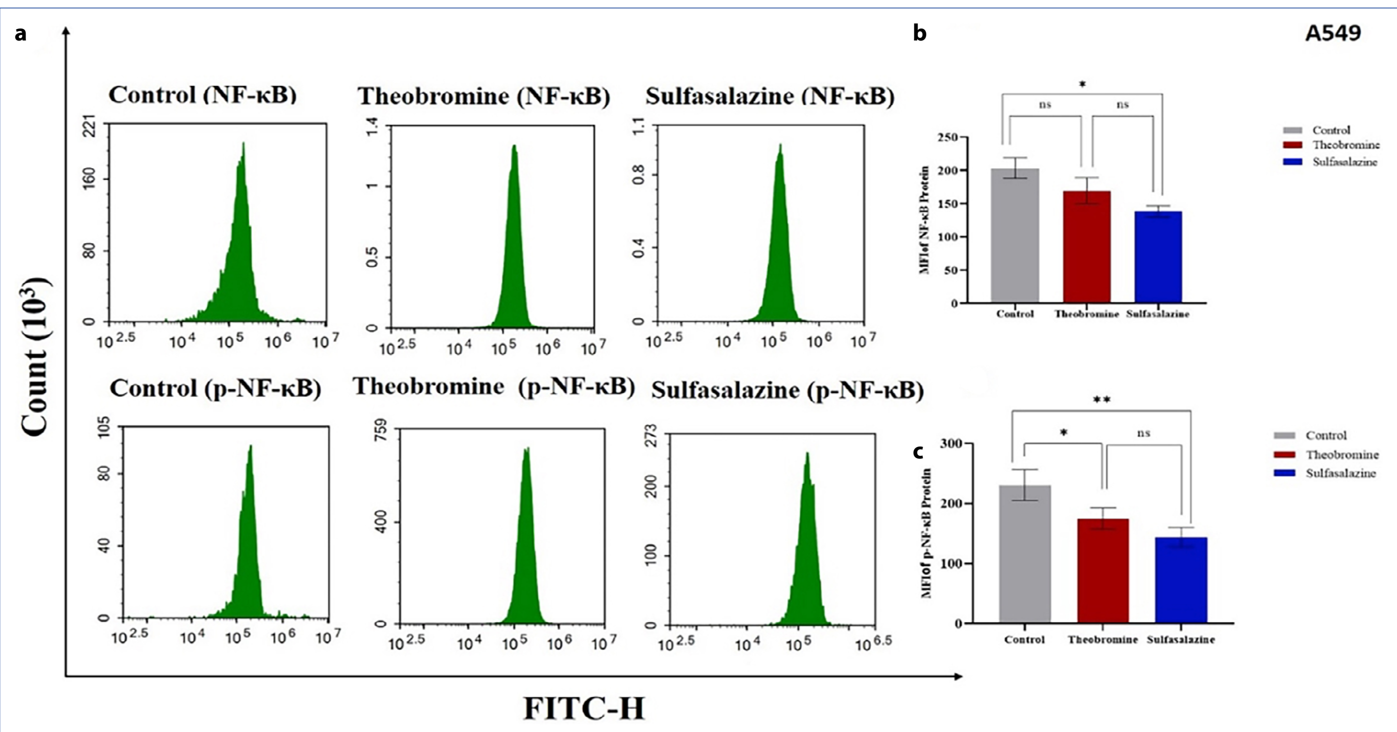

Figure 5. Flow cytometric analysis of total NF-κB and p-NF-κB expression in A549 cells. (a) Representative histograms showing fluorescence intensity distribution. (b) Quantification of mean fluorescence intensity (MFI) for total NF-κB. (c) Quantification of MFI for p-NF-κB. Data are presented as mean±SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

*: p<0.05; **: p<0.01; ns: Not significant.

of total and phosphorylated forms comprehensively reveals not only Theobromine's effects on protein abundance but also its regulatory effects.

For A549 cells, total NF-κB levels in the control group were 203.3±10.3, whereas treatment with Theobromine reduced this value to 169.5±12.2. Sulfasalazine markedly suppressed total NF-κB to 138.6±7.9 (p<0.05), confirming its strong inhibitory effect (Fig. 5a, b). Phosphorylated NF-κB (p-NF-κB) levels exhibited a more pronounced response. Control cells showed an expression level of 230.5±14.5, which significantly decreased to 174.9±10.7 following Theobromine treatment (p<0.05). Sulfasalazine further reduced p-NF-κB to 143.9±9.1 (p<0.01) (Fig. 5a, c). The difference between Theobromine and Sulfasalazine was not statistically significant (p>0.05), indicating that Theobromine suppresses NF-κB activation to a degree comparable to pharmacological inhibition. Total IKK expression was 229.1±11.8, while Theobromine treatment lowered this level to 160.8±11.2 (p<0.01). Sulfasalazine elicited an even greater reduction (144.4±13.7, p<0.001) (Fig. 6a, b). Consistent with these findings, phosphorylated IKK (p-IKK) levels were significantly reduced following treatment. The control group displayed 228.3±15.0, which decreased to 173.1±15.9 after Theobromine exposure (p<0.05). Sulfasalazine produced a similar reduction, yielding 174.4±25.0 (p<0.05) (Fig. 6a, c).

In Saos-2 osteosarcoma cells, total NF-κB protein levels were 276.9±9.8 in the control group, but decreased to 218.4±6.7 with Theobromine treatment (p<0.05). Sulfasalazine treatment produced a more significant decrease to 189.4±6.0 (p<0.01) (Fig. 7a, b). p-NF-κB levels were 272.5±1.9 in the control group, decreasing to 165.1±7.7 with Theobromine (p<0.001) and 149.7±6.0 with sulfasalazine (p<0.001). The difference between Theobromine and sulfasalazine was not statistically significant (p>0.05) (Fig. 7a, c). Total IKK values were 213.3±17.0 in the control group and decreased to 156.7±8.2 with Theobromine treatment (p<0.05). Sulfasalazine provided a more potent suppression to 138.1±4.5 (p<0.01). No significant difference was observed between the two treatments in terms of total IKK (p>0.05) (Fig. 8 a, b). p-IKK levels were 302.9±7.4 in the control group, which decreased to 179.1±5.3 with Theobromine (p<0.0001). Sulfasalazine administration showed a significant decrease to 150.9±7.4 compared to both the control and Theobromine groups (p<0.0001 and p<0.05) (Fig. 8a, c).

Discussion

In this study, Theobromine, a natural methylxanthine derivative, was shown to have significant cytotoxic and pro-apoptotic effects in A549 lung cancer and Saos-2 osteosarcoma cells. The findings indicate that Theobromine exerts these effects through inhibition of the NF-κB signaling pathway. The NF-κB signaling pathway is a key regulator of cell proliferation, inflammation, and survival. Therefore, Theobromine's inhibitory effect on these pathways supports its potential anticancer activity.

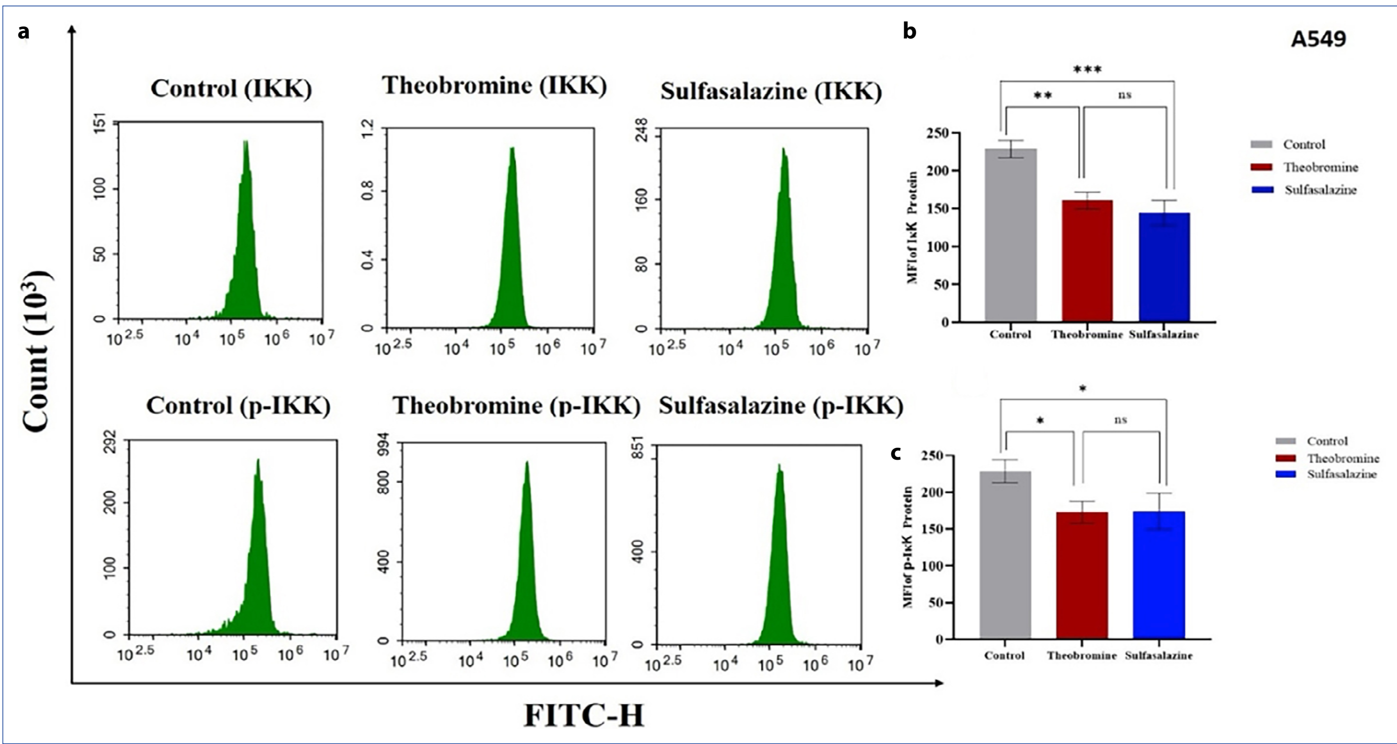

Figure 6. Flow cytometric analysis of total IKK and p-IKK expression in A549 cells. (a) Representative histograms showing fluorescence intensity distribution. (b) Quantification of mean fluorescence intensity (MFI) for total IKK. (c) Quantification of MFI for p-IKK. Data are presented as mean±SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

*: p<0.05; **: p<0.01; ***: p<0.001; ns: Not significant.

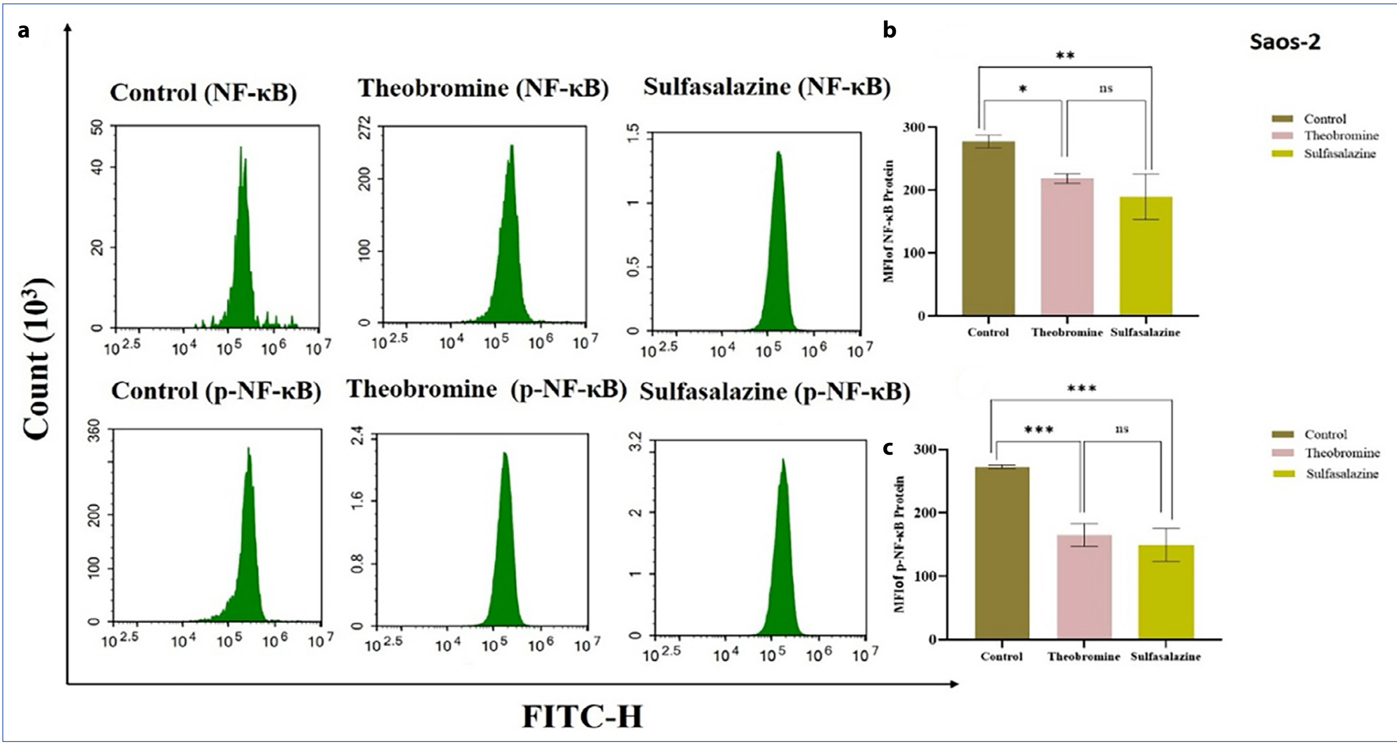

Figure 7. Flow cytometric analysis of total NF-κB and p-NF-κB expression in Saos-2 cells. (a) Representative histograms showing fluorescence intensity distribution. (b) Quantification of mean fluorescence intensity (MFI) for total NF-κB. (c) Quantification of MFI for p-NF-κB. Data are presented as mean±SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

*: p<0.05; **: p<0.01; ***: p<0.001; ns: Not significant.

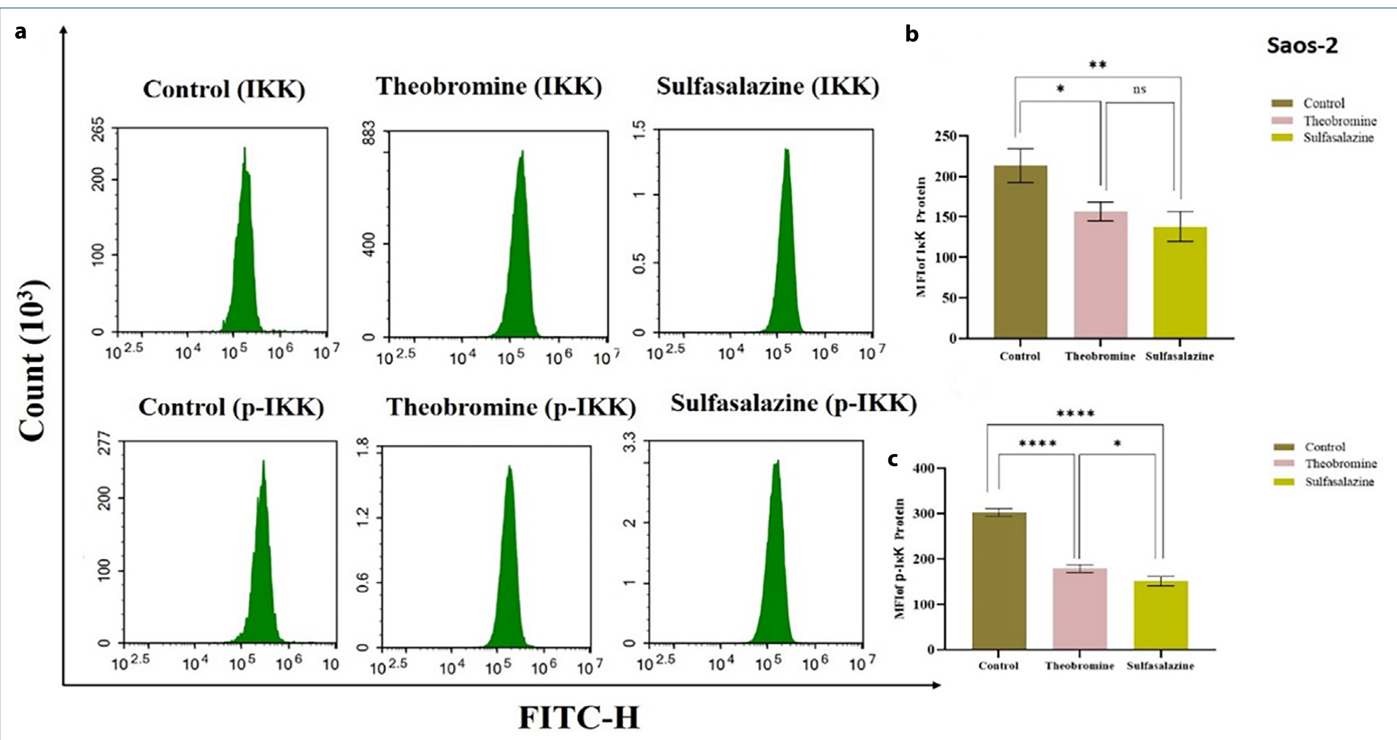

Figure 8. Flow cytometric analysis of total IKK and p-IKK expression in Saos-2 cells. (a) Representative histograms showing fluorescence intensity distribution. (b) Quantification of mean fluorescence intensity (MFI) for total IKK. (c) Quantification of MFI for p-IKK. Data are presented as mean±SD (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post-hoc test.

*: p<0.05; **: p<0.01; ****: p<0.0001; ns: Not significant.

Theobromine was observed to decrease cell viability in a dose-dependent manner in A549 lung cancer and Saos-2 osteocarcinoma cells. This dose- and time-dependent decrease in cell viability is consistent with previous studies reporting the antiproliferative properties of Theobromine in A549 cells [9]. Furthermore, Annexin V/PI analyses showed that Theobromine induces cell death via apoptosis. This result is consistent with the literature reporting that methylxanthine derivatives can promote apoptosis by activating mitochondrial pathways and stimulating caspase enzymes [11, 12].

The significant decrease in total and phosphorylated NF-κB and IKK levels proves that Theobromine has a strong suppressive effect not only on gene expression but also on signal activation. However, the number of studies examining the effects of Theobromine on cellular signaling pathways is quite limited. Gu et al. [13] demonstrated that Theobromine reduces oxidative stress and inflammatory responses by suppressing the NF-κB signaling pathway in IL-1β-stimulated human chondrocytes and also prevents type II collagen degradation. In contrast, Lee et al. [14] showed that Theobromine increased the production of IL-6, TNF-α, and NO by activating MAPK and NF-κB pathways in macrophages. Taken together, these two studies suggest that Theobromine may have a dual effect, depending on the cell type and physiological context. In normal or immune cells, Theobromine appears to enhance defense mechanisms by stimulating NF-κB activation, while under inflammatory conditions, it suppresses this pathway, reducing oxidative stress and cytokine production. This discrepancy suggests that Theobromine's effects are influenced by factors such as the cellular microenvironment, the type of stimulus, and metabolic state. Therefore, Theobromine may act as a protective and immunomodulatory agent in some cell types and as an anti-inflammatory agent in others.

These dual regulatory properties of Theobromine in various cell systems make it particularly important to elucidate its potential mechanisms in tumor cells. Therefore, our study investigated the effects of Theobromine on the NF-κB signaling pathway. The number of studies examining the effects of Theobromine on cancer-related signaling pathways is quite limited. In the study conducted by Oz-Bedir et al. [15], Theobromine was shown to suppress the NRF2 signaling pathway in invasive bladder cancer cells while activating this pathway in non-invasive cells. Similarly, Shojaei-Zarghani et al. [16] reported that Theobromine inhibited tumor and precarcinoma lesion formation by suppressing the Akt/GSK3β/β-catenin signaling pathway in a 1,2-dimethylhydrazine-induced rat colorectal cancer model. Another study by the same research group demonstrated that Theobromine, administered alone or in combination with theanine, suppressed the Akt/mTOR and JAK2/STAT3 oncogenic signaling pathways and increased expression of the Smad2 tumor suppressor protein. These findings suggest that Theobromine has broad-spectrum anticancer potential, capable of targeting not only inflammatory processes but also multiple cellular signaling networks

involved in cancer development [17]. To date, no study has specifically investigated the effects of Theobromine on the NF-κB signaling pathway in cancer cells. In the present study, Theobromine treatment markedly reduced total and phosphorylated NF-κB and IKK protein levels in A549 and Saos-2 cells, indicating that it effectively suppresses NF-κB pathway activation and promotes apoptotic cell death. Theobromine's ability to downregulate both total and phosphorylated forms of NF-κB and IKK indicates that it acts at multiple levels of this signaling cascade. This dual inhibition suggests that Theobromine may interfere with upstream kinases involved in IKK phosphorylation or disrupt the release and nuclear translocation of NF-κB. Interestingly, although sulfasalazine is a well-established pharmacological NF-κB inhibitor, Theobromine demonstrated a comparable inhibitory effect at significantly lower concentrations. This finding highlights the potential of Theobromine as a natural, low-toxicity NF-κB modulator with possible therapeutic relevance. The fact that Theobromine is a dietary compound found in cocoa and tea adds translational value to these results, suggesting its potential use as an adjuvant or chemopreventive agent in cancer management. Nevertheless, while the *in vitro* results strongly indicate Theobromine's inhibitory effect on NF-κB signaling, further investigations are warranted. Future studies should focus on elucidating its effects in inflammatory or cytokine-stimulated cancer models and determining its efficacy *in vivo* using xenograft or orthotopic tumor models. Additionally, exploring downstream molecular targets and cross-talk with other oncogenic pathways, such as PI3K/Akt or JAK/STAT, could provide deeper mechanistic insight into Theobromine's multifaceted anticancer action.

Although Theobromine is considered a naturally occurring compound with relatively low toxicity, its pharmacokinetic properties and bioavailability should be carefully considered when evaluating its translational potential. Importantly, the concentrations used in *in vitro* studies are often higher than those achievable through dietary intake, suggesting that the observed effects may not be directly translatable under normal physiological conditions. Therefore, while Theobromine demonstrates promising anticancer effects at the cellular level, further investigations are required to determine whether these effects can be reproduced *in vivo*. Future studies should focus on pharmacokinetic profiling, dose optimization, and potential formulation strategies to enhance bioavailability. Additionally, evaluating Theobromine in combination therapies or advanced delivery systems may provide more clinically relevant outcomes.

Although our findings demonstrate apoptosis induction through Annexin V/PI analysis and modulation of NF-κB/IKK signaling, the present study is limited by the absence of additional apoptosis-related protein analyses. Key markers such as caspase-3 activation, PARP cleavage, and Bcl-2 family proteins were not evaluated. Inclusion of these markers would provide deeper mechanistic insight into the apoptotic pathways involved. Therefore, future studies should incorporate these molecular targets to better define whether apoptosis is mediated through intrinsic, extrinsic, or combined pathways.

Taken together, our findings provide new mechanistic evidence that Theobromine inhibits NF-κB signaling and promotes apoptosis in cancer cells. These results contribute to a growing body of evidence supporting Theobromine as a promising natural compound with anti-inflammatory and anticancer properties, offering potential for future development as a safe and effective adjunct in cancer therapy.

Conclusion

In summary, this study provides novel evidence that Theobromine exerts significant cytotoxic and pro-apoptotic effects in A549 lung cancer and Saos-2 osteosarcoma cells by suppressing the NF-κB signaling pathway. Theobromine decreased both total and phosphorylated protein levels of NF-κB and IKK, demonstrating inhibition at multiple regulatory points within this cascade. Its efficacy was comparable to that of sulfasalazine, despite being a natural compound. These results suggest that Theobromine may serve as a promising, low-toxicity modulator of NF-κB signaling with potential application as an adjuvant or chemopreventive agent in cancer therapy. Despite the promising *in vitro* findings, the present study has certain limitations, primarily due to its restriction to cell culture models. Future studies should focus on validating these results *in vivo* using appropriate animal models to better assess the pharmacokinetics, bioavailability, and systemic effects of Theobromine and sulfasalazine. In addition, further investigations are needed to evaluate their therapeutic efficacy, safety profile, and potential synergistic interactions in more complex biological systems, including 3D tumor models and patient-derived organoids. Such studies would provide more comprehensive insights into their translational potential and possible clinical applicability in cancer therapy.

Disclosures

Ethics Committee Approval: This study was conducted exclusively using established commercial cell lines under standard *in vitro* laboratory conditions.

Informed Consent: No human participants, human biological samples, identifiable personal data, or live experimental animals were involved in the study.

Conflict of Interest Statement: None declared.

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