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## PUBLICATION FEE

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- Abstract: Hortin GL, King C, Kopp J. Quantification of rhesus monkey albumin with assays for human microalbumin [Abstract]. Clin Chem 2000;46:A140-
- Editorial: Demers LM. New biochemical marker for bone disease: is it a breakthrough? [Editorial]. Clin Chem 1992;38:2169–70.
- Letter to the Editor: Davey L, Naidoo L. Urinary screen for acetaminophen (paracetamol) in the presence of N-acetylcysteine [Letter]. Clin Chem 1993;39:2348–9.
- Book Chapter: Rifai N, Warnick GR. Lipids, lipoproteins, apolipoproteins, and other cardiovascular risk factors. In: Burtis CA, Ashwood ER, Bruns DE, editors. Tietz textbook of clinical chemistry and molecular diagnostics. 4th Ed. St. Louis (MO): Elsevier Saunders; 2006. p. 903-81.
- Book: Personal Author Harrell FE Jr. Regression modeling strategies. New York (NY): Springer; 2001. Bailar JC III, Mosteller F, editors. Medical uses of statistics. 2nd Ed. Boston (MA): NEJM Books; 1992:449 p.
- Book with Single Author Cohn PF. Silent myocardial ischemia and infarction. 3rd ed. New York: Marcel Dekker; 1993.
- Editor(s) as author: Norman IJ, Redfern SJ, editors. Mental health care for elderly people. New York: Churchill Livingstone; 1996
- Technical Reports Tschantz BA, Moran B. Modeling of the hydrologic transport of mercury in the Upper East Fork Poplar Creek (UEFPC) watershed. Technical Report for Lockheed Martin Energy Systems: Bethesda, MD, September 2004.
- Supplement Castelli WP. Lipids, risk factors and ischaemic heart disease. Atherosclerosis 1996;124 Suppl:S1-9.
- Epub ahead of print Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. [Epub ahead of print] Clin Chem February 6, 2009 as doi:10.1373/clinchem.2008.113035.
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Research Article

# Contribution of kynurenine/tryptophan ratio to early prediction of COVID-19 severity in the emergency department

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## Abstract

**Objectives:** Kynurenine is the breakdown product of tryptophan. The tryptophan metabolic pathway increases in COVID-19 infection. This study was designed to reveal the relationship between tryptophan and kynurenine levels and disease severity. Our study also aimed to explore the relationship between tryptophan-kynurenine levels and patient survival, need for mechanical ventilation, and length of hospital stay.

**Methods:** All 82 COVID-19 patients were grouped as severe and mild cases. Serum tryptophan and kynurenine levels were measured by the ELISA method. Receiver operating characteristic curves were generated to plot the KYN/TRP ratio and other variables. Multivariate logistic regression analyses were used to assess the strength of associations between risk factors and patient status. Categorical variables were compared.

**Results:** The kynurenine/tryptophan level was significantly higher ($p<0.001$), and the tryptophan level was significantly lower ($p=0.008$) in the severe group. With a cutoff point of 14.2, the kynurenine/tryptophan ratio had 56.1% sensitivity and 80.49% specificity in predicting COVID-19 severity. In the multivariate logistic regression analysis using age, troponin, platelet count, ferritin, and kynurenine/tryptophan levels, with a cut-off value of 0.34, the sensitivity and specificity were 92.6% and 87.8%, respectively. There was no significant difference in tryptophan and kynurenine levels in terms of patient survival, need for mechanical ventilation, and length of hospital stay.

**Conclusion:** The kynurenine/tryptophan ratio is valuable in evaluating clinical outcomes of COVID-19 patients, especially when used in conjunction with age, troponin, platelet count, and ferritin. It is useful in predicting the clinical course at the time of admission to the emergency department. To our knowledge, the kynurenine/tryptophan ratio, together with age, troponin, platelet count, and ferritin parameters, is the best model with the highest AUC that can be used to show early prediction of clinical outcomes in COVID-19.

**Keywords:** COVID-19, disease severity, indoleamine 2, 3 dioxygenase, kynurenine, tryptophan

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The COVID-19 virus has caused a global pandemic [1]. Proinflammatory cytokines cause pulmonary fibrinolysis and damage alveolar epithelial and endothelial cells. As a result, oxygen ($O_2$) and carbon dioxide ($CO_2$) exchange is prevented, and hypoxia occurs. Cytokine storms, hypoxia, and fibrosis are associated with a poor clinical course and increased mortality [2–4]. Controlling immune reactions is the most vital and challenging management process for COVID-19 patients [5].

Approximately 95% of tryptophan (TRP), an essential amino acid, is converted to kynurenine (KYN) and its metabolites in the liver and kidney. Two rate-limiting enzymes that metabolize TRP are tryptophan-2,3-dioxygenase (TDO) and indoleamine-2,3-dioxygenase 1/2 (IDO1/2) [6, 7]. Under physiological conditions, TDO, which is expressed mainly in the liver, is regulated by hormones such as cortisol, insulin, glucagon, and epinephrine, while IDO1 is expressed in monocytes and dendritic

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cells and is expressed as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) [8–10]. The recently identified IDO2 gene, which has 45% homology with IDO1, is expressed in the brain, liver, kidney, and epididymis. However, the metabolic activity of IDO2 is thought to be much lower than that of IDO1 [11]. In this study, the term 'IDO' refers to IDO1 unless stated otherwise. Local TRP depletion (via the stress-responsive kinase GCN2 pathway) and an increase in kynurenine metabolites (via the aryl hydrocarbon receptor (AhR)) inhibit the proliferation of T cells, convert naïve CD4+ T cells into Treg cells, and direct dendritic cells (DCs) and macrophages towards an immunosuppressive phenotype [12, 13]. By IDO activation, APCs produce inhibitory cytokines such as IL-10 and transforming growth factor β (TGFβ) rather than inflammatory cytokines such as IL-12. Therefore, IDO activation can change the nature of APCs and change the entire local environment from immunogenic to tolerogenic [14, 15].

The KYN/TRP ratio, which reflects IDO activity, is reportedly higher in COVID-19 patients than in controls [16, 17]. Paradoxically, in COVID-19, a cytokine storm occurs despite IDO activation [3]. There are some hypotheses on this topic. Firstly, the TRP metabolic pathway can occur in response to inflammation and represents an attempt to limit excessive immune reactivity. However, if it is not enough, a cytokine storm begins [16]. Secondly, immune tolerance resulting from the activation of the TRP metabolic pathway weakens the immune response to the virus and delays its clearance, thus leading to the development of acute respiratory distress syndrome (ARDS) and multiple organ failure [18].

The IDO enzyme is probably induced by increased levels of IFN-γ, IL-1, IL-6, TNF-α, and ROS in COVID-19 patients [19]. KYN, which increases with IDO activation, stimulates AhR and may cause "systemic aryl hydrocarbon receptor activation syndrome," which is associated with hyperinflammation, hypercoagulation, and multiple organ damage [20]. For this reason, it has been hypothesized that the TRP metabolic pathway may worsen the clinical course of COVID-19 and possibly reduce the patient's recovery potential [21].

Determining the severity of the disease before the onset of the cytokine storm in COVID-19 and starting appropriate treatment accordingly is life-saving. There is a need for parameters that shed more light on clinicians working under intense conditions. The results of our study will provide further information on the use of KYN and TRP levels at the time of admission to emergency departments and will help better predict the severity of COVID-19.

## Materials and Methods

### Participant acquisition and sample collection

The study included 82 patients aged >18 years who presented to the hospital emergency department between 01.01.2022 and 01.08.2022 and had a positive SARS-CoV-2 PCR test. The exclusion criteria included cancer, inflammatory diseases, rheumatological diseases, and allergic diseases that could affect IDO enzyme activity. Patients were grouped into severe and mild cases according to the criteria established by the Ministry of Health [22]. By evaluating the following criteria, patients with at least one criterion were included in the severe group. Patients who did not meet these criteria were considered mildly ill.

- Shortness of breath,
- Respiratory rate ≥28/minute,
- Oxygen saturation <93% or $PAO_2$ <60 mmHg despite oxygen support of 5 liters/minute or more
- $PAO_2/FIO_2$ <300,
- More than 50% involvement with clinical worsening on chest X-ray or tomography,
- Hypotension (systolic blood pressure <90 mmHg, >40 mmHg decrease from normal systolic blood pressure, mean arterial pressure <65 mmHg) or vasopressor requirement,
- Skin perfusion disorders, lactate >4 mmol/L, sequential organ failure assessment score (≥2 unit increase in SOFA score),
- Troponin elevation or arrhythmia.

Patients were followed for 4 months to determine their survival. Follow-up lasted 4 months, as surviving patients fully recovered during this time. All participants gave informed consent to participate in this study, which was approved by the local ethics committee. Yellow cap gel blood tubes (SARSTED) were used to collect blood samples during admission to the emergency department. Samples were allowed to clot for 20–30 min. Samples were centrifuged at 4000xg for 10 minutes. Serum obtained from blood samples was divided into two equal parts and placed in Eppendorf tubes. Samples were stored at -80°C until the day of analysis. Samples were kept at room temperature until completely thawed before analysis. All laboratory records, radiological imaging, and clinical examination findings were recorded.

### Sample analysis

Serum KYN levels were measured using a Human Kynurenine ELISA Kit (Sunred Biological Technology, China). Different standard concentrations were obtained by diluting the lyophilized standard included in the kit. Following the kit procedure, the absorbance of the examined samples was measured at 450 nm with an EBLX-800 microplate reader (BioTek Instruments, Inc., USA). Concentrations corresponding to sample absorbance were calculated by the formula obtained from the standard curve plot (sensitivity: 13.672 nmol/mL, assay range: 15 nmol/mL →4000 nmol/mL, intra-assay CV<10%). Serum TRP levels were measured using a Human Tryptophan ELISA Kit (Cloud Clone USCN, USA). Different standard concentrations were obtained by diluting the lyophilized standard included in the kit. Following the kit procedure, the absorbance of the examined samples was measured at 450 nm with an EBLX-800 microplate reader (BioTek Instruments, Inc., USA). Concentrations corresponding to sample absorbance were calculated by the formula obtained from the standard curve (sensitivity: 0.55 µg/mL, detection range: 1.23–100 µg/mL, intra-assay: CV<10%).

**Table 1. Demographic data and laboratory findings of patients infected with COVID-19 upon admission to the hospital**

| Parameters | Mild (n=41) | Severe (n=41) | p |
|---|---|---|---|
| Gender (male), n (%) | 16 (39) | 27 (66) | 0.013 |
| Age (years) | 56 (40–65) | 67 (56–82) | <0.001 |
| Oxygen saturation | 96 (94–97) | 89 (86–91) | <0.001 |
| Glucose (mmol/L) | 6.82 (5.62–8.41) | 8.15 (7.00–13.48) | <0.001 |
| Urea (mmol/L) | 5.16 (4.16–5.99) | 8.15 (5.16–14.15) | <0.001 |
| Creatinine (µmol/L) | 77.8 (62.8–94.6) | 89.3 (81.4–121.1) | 0.002 |
| AST* (U/L) | 26 (20–40) | 36 (29–47) | 0.020 |
| ALT† (U/L) | 24 (18–34) | 26 (18–33) | 0.714 |
| CRP‡ (mg/L) | 37.6 (13.9–87.2) | 114.78 (66.97–175.7) | <0.001 |
| Ferritin (µg/L) | 233 (108–650) | 625 (234–1105) | 0.006 |
| Procalcitonin (µg/L) | 0.07 (0.05–0.11) | 0.17 (0.12–0.43) | <0.001 |
| Laktate (mmol/L) | 1.51 (1.23–2.03) | 2.09 (1.57–3.25) | <0.001 |
| $PaO_2/FiO_2$ (mmHg) | 377.9 (310.2–402.1) | 300.2 (280.8–315.6) | <0.001 |
| Hemoglobin (g/L) | 13.2 (12.2–14.2) | 13.6 (11.6–14.9) | 0.752 |
| Hematocrit (%) | 38.2 (36–42.5) | 40.6 (35.4–45.4) | 0.207 |
| Platelet ($\times10^9$/L) | 206 (155–268) | 195 (166–262) | 0.802 |
| WBC§ ($\times10^9$/L) | 7.15 (5.21–9.74) | 7.24 (5.76–11.29) | 0.481 |
| Lymphocyte (%) | 20 (14.1–26.6) | 11.5 (8.7–20) | <0.001 |
| NLR‖ | 3.62 (2.49–5.51) | 7.04 (3.7–10.06) | <0.001 |
| D-dimer (mg/L) | 420 (260–889.45) | 710 (520–1830) | 0.004 |
| aPTT¶ (sec) | 30 (27.6–31.6) | 30.6 (27.7–32.7) | 0.484 |
| INR** | 0.99 (0.93–1.08) | 1.1 (1–1.21) | 0.002 |
| Troponin (ng/L) | 0.007 (0.004–0.015) | 0.014 (0.009–0.064) | <0.001 |
| Kynurenine (nmol/mL) | 268 (151–507) | 272 (187–1014) | 0.068 |
| Tyrptophan (ug/mL) | 23.2 (7.9–48.5) | 18.6 (7.4–36.7) | 0.008 |
| Kynurenine/Tyrptophan | 11.1 (4.7–28.1) | 15.0 (6.0–53.5) | <0.001 |
| Exitus, n | 3 | 23 | <0.001 |
| Need of mechanic ventilation, n | 0 | 9 | <0.001 |

Descriptive statistics are presented as medians (IQR) or frequencies with percentages. *: Aspartate aminotransferase; †: Alanine aminotransferase; ‡: C reactive protein; §: White Blood Cell; ‖: Neutrophil-to-lymphocyte ratio; ¶: Activated Partial Thromboplastin Time; **: International Normalized Ratio. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; CRP: C-reactive protein; $PaO_2$: Arterial partial pressure of oxygen; $FiO_2$: Fraction of inspired oxygen; WBC: White blood cell; NLR: Neutrophil-lymphocyte ratio; aPTT: Activated partial thromboplastin time; INR: International normalized ratio; n: Number.

### Statistical analysis

The Shapiro–Wilk test was used to determine whether the data were normally distributed. The results are presented as the mean±standard deviation, median (minimum-maximum), or frequency and percentage. Normally distributed data were compared with independent sample t-tests or one-way ANOVA. The Kruskal–Wallis and Mann–Whitney U tests were used for nonnormally distributed data. The Bonferroni correction was used as a multiple comparison test. Categorical variables were compared between groups using Pearson's chi-square and Fisher's exact tests. $p<0.05$ was considered to indicate statistical significance. All the statistical analyses were performed with IBM SPSS ver. 23.0.

Receiver operating characteristic (ROC) curves were generated to plot the KYN/TRP ratio and other variables. The sensitivity, specificity, positive likelihood ratio (+LR), and negative (-) LR were also determined for the optimal cutoff value. The performance of the KYN/TRP ratio and other variables in predicting patient status was determined using receiver operating characteristic (ROC) curves, with the area under the curve (AUC) being the primary interest. Multivariate logistic regression analyses were used to assess the strength of associations between risk factors and patient status, and curves were generated to determine optimal cutoff values. MedCalc Statistical Software version 20.015 was used for ROC analysis.

## Results

In the severe group, the KYN/TRP ratio reflecting IDO activity was significantly higher than that in the mild group ($p<0.001$), while the TRP level was significantly lower in the severe group ($p=0.008$). The KYN level was higher in severely ill patients than in healthy patients ($p>0.05$) (Table 1).

There was no significant difference between genders in KYN/TRP, TRP, or KYN levels. After the patients were grouped as se-

**Table 2. Diagnostic test results for the prediction of severe COVID-19**

| | AUC | Cutoff | Sensitivity % (95% CI) | Specificity % (95% CI) | Positive Likelihood ratio | Negative Likelihood ratio |
|---|---|---|---|---|---|---|
| Age | 0.736 | 52 | 87.80 (73.8–95.9) | 48.78 (32.9–64.9) | 1.71 | 0.25 |
| Kynurenine | 0.617 | 225.07 | 90.24 (76.9–97.3) | 34.15 (20.1–50.6) | 1.37 | 0.29 |
| Tyrptophan | 0.67 | 18.1 | 48.8 (32.9–64.9) | 80.5 (65.1–91.2) | 2.50 | 0.64 |
| Kynurenine/Tyrptophan | 0.71 | 14.2 | 56.1 (39.7–71.5) | 80.5 (65.1–91.2) | 2.88 | 0.55 |
| Ferritin | 0.675 | 226.8 | 80.49 (65.1–91.2) | 48.78 (32.9–64.9) | 1.57 | 0.40 |
| Platelet | 0.516 | 102 | 12.20 (4.1–26.2) | 100.00 (91.4–100.0) | – | 0.88 |
| Troponin | 0.747 | 0.026 | 41.46 (26.3–57.9) | 97.56 (87.1–99.9) | 17.00 | 0.60 |
| Kynurenine/Tyrptophan, Ferritin, Platelet, Troponin, Age | 0.94 | 0.39 | 92.6 (80.1–98.5) | 87.8 (73.8–95.9) | 7.60 | 0.08 |

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

vere or mild, it was observed that KYN levels were significantly higher in men (299 (151–404) nmol/mL) than in women (227 (168–507) nmol/mL) only in mild patients (p=0.010).

There was no correlation between the laboratory parameters in Table 1 and TRP-KYN levels. After patients were grouped into severe and mild groups, correlations were observed between KYN/TRP levels and Hb (p=0.009), Hct (p=0.003), INR (p=0.031), and aPTT (p=0.002) levels only in mild patients.

Only 9 patients died during treatment. There were no significant differences in TRP, KYN, or KYN/TRP levels between deceased and surviving patients. In addition, no significant differences were detected in TRP or KYN levels in terms of mechanical ventilation need or hospital stay duration.

ROC analysis was performed to determine the effect of TRP, KYN, and KYN/TRP levels on disease severity (Table 2).

According to the univariate logistic regression analysis, the AUC of TRP was 0.67, its sensitivity was 48.8%, its specificity was 80.5%, the AUC of KYN/TRP was 0.71, its sensitivity was 56.1%, and its specificity was 80.5%. Multiple logistic regression analysis was performed with various variations using the patients' demographic data and laboratory parameters. The combination of age, troponin level, platelet count, ferritin level, and KYN/TRP ratio had the highest AUC. According to the multivariate logistic regression analysis, the AUC was 0.94, the sensitivity was 92.6%, and the specificity was 87.8% in determining disease severity (Fig. 1).

## Discussion

Lower TRP and higher KYN levels have been reported in COVID-19 patients compared to healthy individuals [17, 23]. A meta-analysis of 1269 individuals, consisting of 794 COVID-19 patients and 475 controls, revealed a significant increase in the KYN/TRP ratio (p<0.0001) and KYN level, and a significant decrease in the TRP level [17]. In a study including 239 healthy individuals and 89 COVID-19 patients, it was observed that the KYN/TRP ratio in patients was 3.7 times higher than in healthy controls. The AUC for KYN/TRP levels at the time of COVID-19 diagnosis was 0.97 (95% CI=0.9463–0.9937, p<0.0001). Plasma TRP concentrations of the patients at admission were higher than the last sample before death (30.1±10.0 vs. 25.5±9.4 µmol/L), KYN (p=0.013) and KYN/TRP levels (p=0.080) lower [23]. McPadden et al. [24] reported that TRP levels in the cerebrospinal fluid of patients with and without COVID-19 were 19.4±16.4 and 24.8±18.9 µmol/L, respectively. In a meta-analysis study including 773 patients, KYN/TRP levels were found to be significantly higher in seriously ill patients (p<0.001) [17]. Similarly, we detected significantly lower TRP levels (p=0.008), higher KYN levels, and higher KYN/TRP ratios reflecting IDO activity (p=0.001) in severe patients compared to mild patients. Our results support the hypothesis that the TRP metabolic pathway is a potential marker of COVID-19 severity and can be targeted by therapeutic intervention.

Studies show that COVID-19 progresses worse in men than in women. Lionetto et al. [16] reported that KYN levels were higher in male patients than in female patients (p=0.004), but there was no gender-related difference in TRP levels. McPadden et al. [24] found higher hospitalization (OR 1.68, 95% CI=1.45–1.90) and in-hospital mortality (OR 1.87, 95% CI=1.33–2.63) rates in male patients. In our study, no significant difference was detected in TRP or KYN levels between genders in the severe group, while in the mild group, KYN

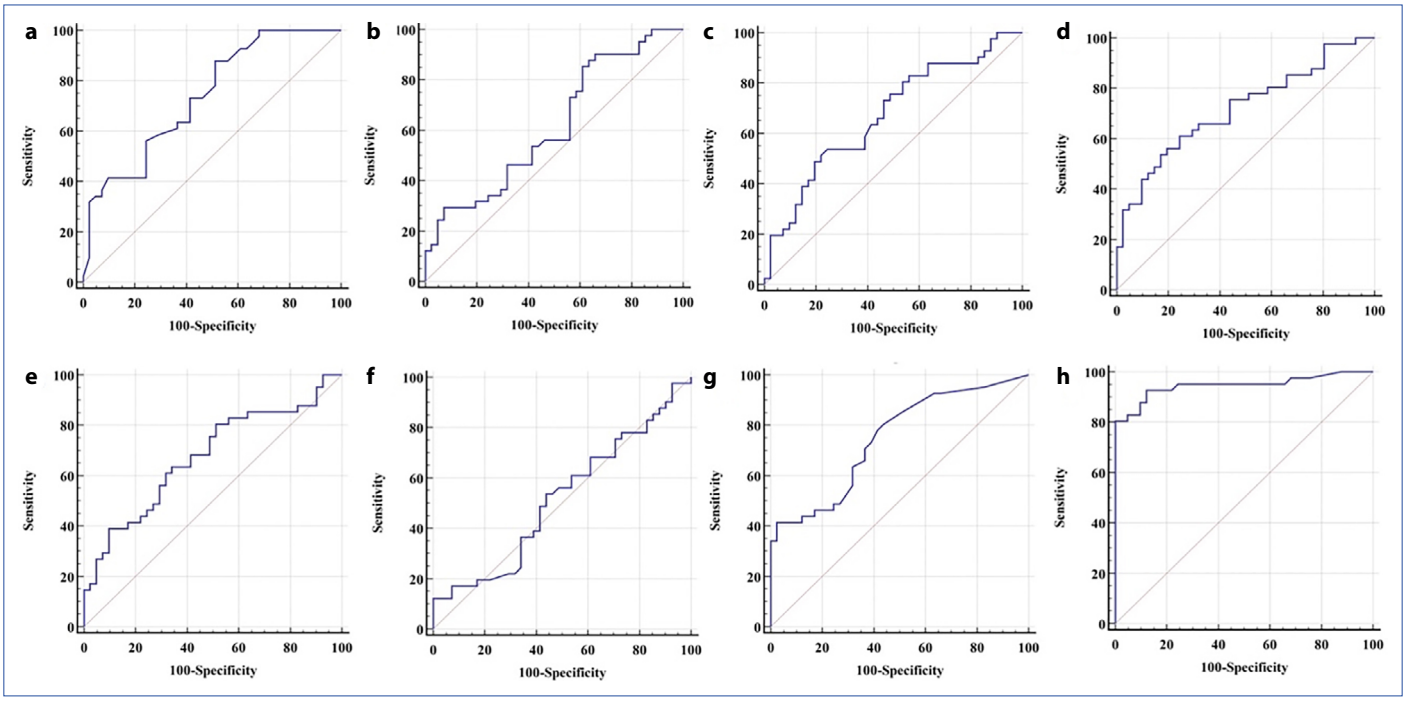


**Figure 1.** ROC analysis of univariate and multivariable logistic regression models for predicting severity of COVID-19. ROC analysis of parameters are shown in (a) age, (b) kynurenine, (c) tryptophan, (d) kynurenine/tryptophan, (e) ferritin, (f) platelet, (g) troponin, (h) kynurenine/tyrptophan, ferritin, platelet, troponin, age graphs.

ROC: Receiver operating characteristic.

levels were significantly higher in men (299 (151–404) nmol/mL) than in women (227 (168–507) nmol/mL) (p=0.010). This difference may be due to the rapid turnover rate of KYN in female patients.

ARDS is one of the causes of death in COVID-19 patients. Data obtained from a multicenter study showed that $PaO_2/FiO_2$, which reflects tissue oxygenation, was lower and lactate levels were higher in deceased patients [19]. Similarly, the $PaO_2/FiO_2$ ratio was significantly lower, and the lactate level was significantly higher in the severe group (p<0.001) in this study. However, we did not find a significant relationship between $PaO_2/FiO_2$ ratio, lactate level, mechanical ventilation need, and TRP-KYN levels.

In the study conducted by Michaelis et al. [25], no significant difference was found in the KYN/TRP ratio at the time of admission between deceased (n=24) and surviving (n=81) patients. However, after one week, a significant decrease in KYN levels was observed in the surviving group, while a significant increase in KYN levels was observed in the deceased group. Researchers stated that the decrease in KYN is associated with the improvement in the clinical course, while the increase in KYN may be a sign of worsening of the disease and poor outcomes. Similarly, in this study, there was no significant difference in TRP, KYN, or KYN/TRP levels at admission between deceased and surviving patients. However, since we did not measure TRP and KYN levels again in the following period, we could not reach such an interpretation. Additionally, there was no correlation between length of hospital stay and TRP, KYN, or KYN/TRP levels.

According to a meta-analysis, ferritin, D-dimer, procalcitonin, C-reactive protein, and troponin levels were associated with COVID-19 severity and mortality [26]. These parameters were significantly higher in the severe group but were not correlated with KYN or TRP levels in this study.

The TRP metabolic pathway likely contributes to the pathophysiology, severity, and progression of COVID-19 [17]. In this study, the KYN/TRP ratio had a sensitivity of 56.1% and specificity of 80.5% in predicting the severity of COVID-19, with an AUC of 0.71. Multivariate logistic regression analysis was performed with different parameter combinations to determine the role of these parameters in predicting disease severity. The highest AUC was reached by regression analysis of age, troponin level, platelet count, ferritin level, and KYN/TRP ratio. According to multivariate logistic regression analysis, sensitivity was 92.6%, specificity was 87.8%, and AUC was 0.94. Hu et al. [27] reported that the sensitivity and specificity of a model consisting of age, lymphocyte count, serum albumin level, and NLR in predicting severe COVID-19 were 90.5% and 84.2%, respectively. Liang et al. [28] reported the AUC as 0.77 in the COVID-19 Gram model they developed to predict critical diseases. According to the NEWS2 model developed by De Socio et al. [29], the AUC was 0.87. According to multivariate regression analysis in our study, AUC, sensitivity, and specificity were higher than other studies. When evaluated together with parameters such as age, troponin, and platelet count, ferritin, this result showed that the KYN/TRP ratio is more valuable than other prediction models in predicting disease severity in the early period.

### Limitations

Several limitations should be taken into account when interpreting these results. Due to the retrospective design, only routinely measured parameters were available for statistical analysis. Since all studies showed that there was a significant increase in KYN/TRP levels in the patient group compared to the control group and there was no study showing the opposite effect, we did not include the control group in our study to increase the patient population. We also had no information about how long the patients had been symptomatic. Therefore, it may be possible that these patients are at slightly different stages of COVID-19 disease. Measuring TRP and KYN levels on different days could explain their effect on the course of the disease more meaningfully. Therefore, a larger, long-term follow-up cohort with repeated measurements is required.

## Conclusion

The TRP metabolic pathway is activated in severe COVID-19 patients. To our knowledge, the KYN/TRP ratio, together with age, troponin, platelet count, and ferritin parameters, is the model with the best AUC for early prediction of clinical outcomes in COVID-19. Our study provided emergency physicians with more information and offered an alternative to better guide them early in predicting the severity of COVID-19.

**Ethics Committee Approval:** The study was approved by The University of Health Sciences Sisli Hamidiye Etfal Training and Research Hospital Health Application and Research Center Clinical Research Ethics Committee (No: 2933, Date: 25/08/2020).

**Authorship Contributions:** Concept – M.S.O., A.O., G.O.; Design – M.S.O., G.O., Z.M.Y.K.; Supervision – M.S.O.; Materials – A.O., E.A., M.S.O., Z.M.Y.K., E.S., G.O.; Data collection &/or processing – M.S.O., A.O., E.A.; Analysis and/or interpretation – M.S.O., G.O., E.S.; Literature search – M.S.O., Z.M.Y.K., E.S., E.A.; Writing – M.S.O.; Critical review – Z.M.Y.K., E.S., E.A., G.O., A.O.

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## References

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al; China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med 2020;382(8):727–33. [CrossRef]
2. Cao Y, Liu X, Xiong L, Cai K. Imaging and clinical features of patients with 2019 novel coronavirus SARS-CoV-2: A systematic review and meta-analysis. J Med Virol 2020;92(9):1449–59.
3. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LFP. The trinity of COVID-19: Immunity, inflammation and intervention. Nat Rev Immunol 2020;20(6):363–74. [CrossRef]
4. Leisman DE, Deutschman CS, Legrand M. Facing COVID-19 in the ICU: Vascular dysfunction, thrombosis, and dysregulated inflammation. Intensive Care Med 2020;46(6):1105–8.
5. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: A retrospective cohort study. Lancet 2020;395(10229):1054–62. [CrossRef]
6. Salter M, Pogson CI. The role of tryptophan 2,3-dioxygenase in the hormonal control of tryptophan metabolism in isolated rat liver cells. Effects of glucocorticoids and experimental diabetes. Biochem J 1985;229(2):499–504. [CrossRef]
7. Lee A, Kanuri N, Zhang Y, Sayuk GS, Li E, Ciorba MA. IDO1 and IDO2 non-synonymous gene variants: Correlation with crohn's disease risk and clinical phenotype. PLoS One 2014;9(12):e115848.
8. Isenović ER, Zakula Z, Koricanac G, Ribarac-Stepić N. Comparative analysis of tryptophan oxygenase activity and glucocorticoid receptor under the influence of insulin. Physiol Res 2008;57(1):101–7. [CrossRef]
9. Ball HJ, Sanchez-Perez A, Weiser S, Austin CJ, Astelbauer F, Miu J, et al. Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. Gene 2007;396(1):203–13.
10. Curti A, Trabanelli S, Salvestrini V, Baccarani M, Lemoli RM. The role of indoleamine 2,3-dioxygenase in the induction of immune tolerance: Focus on hematology. Blood 2009;113(11):2394–401. [CrossRef]
11. Fukunaga M, Yamamoto Y, Kawasoe M, Arioka Y, Murakami Y, Hoshi M, et al. Studies on tissue and cellular distribution of indoleamine 2,3-dioxygenase 2: The absence of IDO1 upregulates IDO2 expression in the epididymis. J Histochem Cytochem 2012;60(11):854–60. [CrossRef]
12. Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D, et al. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity 2005;22(5):633–42. [CrossRef]
13. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. J Immunol 2010;185(6):3190–8. [CrossRef]
14. Manlapat AK, Kahler DJ, Chandler PR, Munn DH, Mellor AL. Cell-autonomous control of interferon type I expression by indoleamine 2,3-dioxygenase in regulatory CD19+ dendritic cells. Eur J Immunol 2007;37(4):1064–71. [CrossRef]
15. Ravishankar B, Liu H, Shinde R, Chaudhary K, Xiao W, Bradley J, et al. The amino acid sensor GCN2 inhibits inflammatory responses to apoptotic cells promoting tolerance and suppressing systemic autoimmunity. Proc Natl Acad Sci U S A 2015;112(34):10774–9. [CrossRef]
16. Lionetto L, Ulivieri M, Capi M, De Bernardini D, Fazio F, Petrucca A, et al. Increased kynurenine-to-tryptophan ratio in the serum of patients infected with SARS-CoV2: An observational cohort study. Biochim Biophys Acta Mol Basis Dis 2021;1867(3):166042. [CrossRef]
17. Almulla AF, Supasitthumrong T, Tunvirachaisakul C, Algon AAA, Al-Hakeim HK, Maes M. The tryptophan catabolite or kynurenine pathway in COVID-19 and critical COVID-19: A systematic review and meta-analysis. BMC Infect Dis

2022;22(1):615.
18. Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: Immunosuppression by starvation? Immunol Today 1999;20(10):469–73. [CrossRef]
19. Gündoğan K, Akbudak İH, Hancı P, Halaçlı B, Temel Ş, Güllü Z, et al. Clinical outcomes and independent risk factors for 90-day mortality in critically ill patients with respiratory failure infected with SARS-CoV-2: A multicenter study in Turkish intensive care units. Balkan Med J 2021;38(5):296–303.
20. Turski WA, Wnorowski A, Turski GN, Turski CA, Turski L. AhR and IDO1 in pathogenesis of Covid-19 and the "systemic AhR activation syndrome:" A translational review and therapeutic perspectives. Restor Neurol Neurosci 2020;38(4):343–54.
21. Robertson J, Gostner JM, Nilsson S, Andersson LM, Fuchs D, Gisslen M. Serum neopterin levels in relation to mild and severe COVID-19. BMC Infect Dis 2020;20(1):942. [CrossRef]
22. Türkiye Cumhuriyeti Sağlık Bakanlığı. Covid-19 erişkin hasta tedavisi. Available at: https://covid19.saglik.gov.tr/Eklenti/43095/0/covid-19rehberieriskinhastayonetimivetedavi-12042022pdf.pdf. Accessed Jul 1, 2024.
23. Guo L, Schurink B, Roos E, Nossent EJ, Duitman JW, Vlaar AP, et al; also on behalf of the Amsterdam UMC COVID-19 Biobank Study Group. Indoleamine 2,3-dioxygenase (IDO)-1 and IDO-2 activity and severe course of COVID-19. J Pathol 2022;256(3):256–61. [CrossRef]
24. McPadden J, Warner F, Young HP, Hurley NC, Pulk RA, Singh A, et al. Clinical characteristics and outcomes for 7,995 patients with SARS-CoV-2 infection. PLoS One 2021;16(3):e0243291.
25. Michaelis S, Zelzer S, Schnedl WJ, Baranyi A, Meinitzer A, Enko D. Assessment of tryptophan and kynurenine as prognostic markers in patients with SARS-CoV-2. Clin Chim Acta 2022;525:29–33.
26. Huang I, Pranata R, Lim MA, Oehadian A, Alisjahbana B. C-reactive protein, procalcitonin, D-dimer, and ferritin in severe coronavirus disease-2019: A meta-analysis. Ther Adv Respir Dis 2020;14:1753466620937175. [CrossRef]
27. Hu H, Du H, Li J, Wang Y, Wu X, Wang C, et al. Early prediction and identification for severe patients during the pandemic of COVID-19: A severe COVID-19 risk model constructed by multivariate logistic regression analysis. J Glob Health 2020;10(2):020510. [CrossRef]
28. Liang W, Liang H, Ou L, Chen B, Chen A, Li C, et al; China Medical Treatment Expert Group for COVID-19. Development and validation of a clinical risk score to predict the occurrence of critical illness in hospitalized patients with COVID-19. JAMA Intern Med 2020;180(8):1081–9. [CrossRef]
29. De Socio GV, Gidari A, Sicari F, Palumbo M, Francisci D. National Early Warning Score 2 (NEWS2) better predicts critical Coronavirus Disease 2019 (COVID-19) illness than COVID-GRAM, a multi-centre study. Infection 2021;49(5):1033–8. [CrossRef]

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Research Article

# Evaluation of the analytical performance of 80 parameters analyzed in routine biochemistry laboratory by process sigma methodology

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## Abstract

**Objectives:** The Six Sigma methodology is also frequently used by clinical laboratories as an objective and quantitative way to measure quality. In our study, we aimed to evaluate the analytical performance of 80 tests using the Six Sigma methodology according to the CLIA (Clinical Laboratory Improvement Amendments) 2019, RICOS BV (Dr. Carmen RICOS Biological Variation) Desirable, and EFLM BV (European Federation of Clinical Chemistry and Laboratory Medicine Biological Variation) Desirable criteria.

**Methods:** The sigma values of 80 tests were calculated according to the TEa (Total Allowable Error) limits allowed by all three references using internal quality control and external quality control data. They were calculated monthly for 12 months, and the annual average was taken. Sigma values were calculated with the Six Sigma formula.

**Results:** Considering the total number of goals reached, the highest success rate of 60% was achieved according to the CLIA goals, while the lowest success rate of 36% was obtained according to the EFLM BV Desirable criteria. Although exactly the same laboratory data are used, this gap between the sigma values obtained according to the selected reference is especially noticeable in tests such as Na (Sodium), K (Potassium), Cl (Chloride), Calcium, HbA1c (Hemoglobin A1c), and Troponin T.

**Conclusion:** The Six Sigma protocol is one of the effective and universal tools for evaluating the performance of clinical laboratories. However, one of its biggest limitations is the lack of standardization in tolerance limits. The obtained performance varies according to the preferred reference. Therefore, we think that in the Six Sigma methodology, it is more feasible to select Total Allowable Error criteria from different references according to their suitability for the test.

**Keywords:** Biological variation, CLIA 2019, quality control, RICOS BV, six sigma, total allowable error

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Laboratory tests are performed on approximately 85% of individuals who present to healthcare facilities [1]. Since medical laboratory service, which is an important part of health services, is directly related to patient health, the accuracy of the results obtained from the medical laboratory and the correct functioning of the processes are crucial. The mortality rate due to laboratory errors varies from 0.05% to 0.61% [2]. Laboratory errors can lead to delayed diagnoses, misdiagnosis, incorrect treatment, increased risk to patient safety, increased costs, and lost time [3]. Medical laboratory processes are generally divided into three phases: preanalytical, analytical, and postanalytical. When evaluating laboratory errors by their phase, it has been shown that most errors occur in the preanalytical or postanalytical phase and few occur in the analytical phase [4]. Nevertheless, analytical quality is of critical importance to laboratories.

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Analytical error is the difference between the observed value and the true value and is divided into random error and systematic error. The random error can be negative or positive, its direction and magnitude are unpredictable, which is indicated by imprecision (CV) [5]. The systematic error, on the other hand, is an error with definite and measurable values that change the analysis result at a fixed and definite level. It always occurs in one direction [5]. It affects the accuracy of the analysis result and is indicated by bias. The sum of the random error and the systematic error is expressed as the total error (TE) and calculated with the formula 'Total Error = Bias+1.65xCV%' (equation-1) [5]. The total allowable error (TEa) for each test and its magnitude have been established as performance criteria by some organizations [6–8].

The Six Sigma methodology is used as an objective and quantitative method for measuring quality. Sigma expresses the frequency of defects as "defects per million possibilities (DPM)." A 6-sigma performance corresponds to 3.4 errors per million measurements, which is considered a world-class performance. Bias, CV, and TEa can be used to evaluate the quality of the analytical phase (Sigma value = (%TEa-%Bias) / CV%) (equation-2) [5]. The minimum acceptable performance for medical laboratories is a 3-sigma performance [5]. Different TEa limits are being used [6–8]. In this study, we aimed to evaluate the analytical performance of 80 tests studied in a clinical biochemistry laboratory by the Six Sigma method calculated with different TEa limits. Although there are many studies in the literature that apply the Six Sigma methodology to clinical chemistry testing in particular, it is very difficult to come across a study that holistically addresses such a broad test profile, almost all of the laboratory's tests, using up-to-date TEa resources.

## Materials and Methods

This study was conducted in the Clinical Biochemistry Laboratory of the Faculty of Medicine, Karadeniz Technical University, Farabi Hospital, with the approval of the Ethics Committee number 2014-168. Internal quality control and external quality control data for the period January 2014 to December 2014 were used in our study. Clinical chemistry parameters, hemogram, Hemoglobin A1c (HbA1c), immunoassay parameters, specific proteins, prenatal screening tests, coagulation parameters, and cardiac markers were included in the study. Thus, the annual average six sigma values of 80 parameters analyzed in the mentioned laboratory were calculated separately for each internal quality control level.

Analysis of routine clinical chemistry parameters was performed with a Beckman Coulter AU 5800 autoanalyzer, and that of the HbA1c test with a Biorad D10 autoanalyzer. The analysis of hemogram parameters was performed with a Beckman Coulter LH 780 autoanalyzer, and the analysis of prenatal screening parameters and the tests of DHEA-S (Dehydroepiandrosterone sulfate), TG (Thyroglobulin), ATG (Anti-thyroglobulin), ATA (Anti-thyroperoxidase), C-peptide, IGF-1 (Insulin-like growth factor 1), insulin, and PTH (Parathyroid hormone) were performed with a Siemens Immulite2000XPi autoanalyzer. Analysis of other immunoassay tests was performed with the Beckman Coulter DXI 800, other specific protein parameters were analyzed with the Siemens BN-II, coagulation parameters were analyzed with the Stago STAR Evaluation, and cardiac parameters were analyzed with the Roche Cobas Integra e 411.

In order to evaluate Albumin, ALP (Alkaline phosphatase), ALT (Alanine aminotransferase), amylase (AMY), AST (Aspartate aminotransferase), d.bil (Direct (Conjugated) bilirubin), t.bil (Total bilirubin), t.Ca (Total calcium), Cl (Chloride), HDL-K (High-density lipoprotein cholesterol), LDL-C (Low-density lipoprotein cholesterol), t.cholesterol (Total cholesterol), CK (Creatine kinase), creatinine, GGT (γ-glutamyltransferase), glucose, iron (Fe), LDH (Lactate dehydrogenase), Mg (Magnesium), PO4 (Phosphate), K (Potassium), t.protein (Total protein), Na (Sodium), TG (Triglycerides), urea, uric acid (u.acid) parameters (lot numbers: 51872, 51873) and Hemogram tests (lot numbers: 878000, 866400, 887900) Beckman Coulter internal quality control materials were used as internal quality control (IQC) material. IQC of the HbA1c parameter was performed with BIO-RAD Lyphocheck R Diabetes Control solution (lot numbers: 33871, 33872). IQC of cortisol, estradiol, ferritin, folate, FSH (Follicle-stimulating hormone), LH (Luteinizing hormone), HCG (Human chorionic gonadotropin), prolactin, total PSA (Total prostate-specific antigen), free T3, free T4, testosterone, TSH (Thyroid stimulating hormone), IGE (Immunoglobulin E), DHEA-S parameters were performed with BIO-RAD Lyphocheck R Immunoassay Plus Control (lot numbers: 40271, 40272).

IQC of AFP (α-feto protein), CA 125 (Cancer antigen 125), CA 15–3 (Cancer antigen 15–3), CA 19–9 (Cancer antigen 19–9), CEA (Carcinoembryonic antigen), and total PSA were performed using the BIO-RAD Lyphocheck R Tumor Marker Plus control (lot numbers: 54571, 54572). IQC of TG, ATG, ATA, C-peptide, IGF-1, insulin, PTH (lot numbers: 0212014111, 0212014112, 0212014113). CRP (C-Reactive protein), C3 (Complement 3), C4 (Complement 4), IGA (Immunoglobin A), IGG (Immunoglobin G), IGM (Immunoglobin M) were performed with Siemens IQC samples (lot numbers: 084742, 084744, 084745). Randox Maternal Control Material was used for IQC of maternal screening parameters AFP, HCG total, and PAPP-A (Pregnancy associated plasma protein A) (lot numbers: 1272015021, 1272015022). IQC of fibrinogen, aPTT (Activated partial thromboplastin time), PT (Prothrombin time) were performed with IQC samples from Stago (lot numbers: 108886, 111551). IQC of CK-MB (Creatine kinase muscle brain isoenzyme), myoglobin, NT -ProBNP (N terminale Pro-brain natriuretic peptide), Troponin T was performed with IQC samples from Roche (lot numbers: 173608, 173609).

External quality assurance (EQA) of biochemical tests, hemogram, HbA1c, specific proteins and immunoassays (except DHEA-S, TG, ATG, ATA, C-peptide, IGF-1, insulin, PTH) are performed with BIO-RAD EQAS (External Quality Assurance Services (USA)). EQA for immunoassay parameters, specific pro-

**Table 1. One-year average of CV% values of internal quality controls and bias% values of external quality control and process sigma values of Biochemistry and HbA1c tests**

| Test | Bias% | CV% | | Process sigma CLIA | | Process sigma RICOS (desirable) | | Process sigma EFLM BV (Desirable) | |
|---|---|---|---|---|---|---|---|---|---|
| | | L1 | L2 | L1 | L2 | L1 | L2 | L1 | L2 |
| Albumin (g/L) | 2.1 | 2.4 | 2.1 | 2.5 | 2.8 | 0.8 | 0.9 | 0.5 | 0.6 |
| ALP (U/L) | 1.6 | 5.1 | 4.4 | 3.6 | 4.2 | 2.0 | 2.4 | 1.7 | 2.0 |
| ALT (U/L) | 1.6 | 3.0 | 2.2 | 4.5 | 6.1 | 8.6 | 12.0 | 4.8 | 6.6 |
| AMY (U/L) | 1.7 | 2.1 | 1.9 | 4.0 | 4.4 | 6.1 | 6.7 | 5.5 | 6.1 |
| AST (U/L) | 2.3 | 2.6 | 2.1 | 4.9 | 6.0 | 5.5 | 6.8 | 4.3 | 5.4 |
| D.Bil (mg/dL) | 2.4 | 2.3 | 2.5 | – | – | 18.1 | 16.6 | 9.7 | 9.1 |
| T. Bil (mg/dL) | 2.9 | 2.9 | 2.4 | 5.9 | 7.1 | 6.3 | 7.1 | 7.6 | 9.1 |
| T. Calcium (mg/dL) | 1.5 | 1.8 | 2.1 | 5.7 | 5.1 | 0.6 | 0.5 | 0.4 | 0.4 |
| Cl (mEq/L) | 0.7 | 1.3 | 1.4 | 3.3 | 3.2 | 0.6 | 0.6 | 0.5 | 0.4 |
| HDL-C (mg/dL) | 2.4 | 3.7 | 3.0 | 4.8 | 5.9 | 2.5 | 3.1 | 2.4 | 2.9 |
| LDL-C (mg/dL) | 5.9 | 2.7 | 2.8 | 5.2 | 5 | 2.2 | 2.2 | 2.9 | 2.8 |
| T. Cholesterol (mg/dL) | 3.5 | 2.4 | 2.3 | 2.7 | 2.8 | 2.3 | 2.4 | 2.2 | 2.3 |
| CK (U/L) | 1.7 | 2.5 | 2.2 | 7.3 | 8.3 | 11.4 | 13.1 | 8.4 | 9.5 |
| Creatinine (mg/dL) | 4.9 | 3.2 | 2.6 | 1.6 | 2.0 | 1.2 | 1.5 | 0.8 | 1.1 |
| GGT (U/L) | 1.3 | 2.1 | 2.3 | 6.5 | 6 | 8.9 | 8.9 | 8.4 | 7.7 |
| Glucose (mg/dL) | 2.2 | 1.6 | 2.0 | 3.6 | 2.9 | 2.3 | 2.4 | 2.7 | 2.2 |
| Iron (µg/dL) | 3.6 | 1.6 | 1.9 | 7.1 | 6.0 | 10.4 | 14.2 | 14.4 | 12.2 |
| LDH (U/L) | 1.9 | 1.7 | 2.9 | 7.7 | 4.5 | 2.3 | 3.3 | 3.4 | 2.1 |
| Mg (mg/dL) | 2.2 | 2.3 | 2.2 | 5.6 | 5.8 | 1.0 | 1.2 | 0.8 | 0.8 |
| PO4 (mg/dL) | 2.3 | 2.4 | 3.4 | 3.2 | 2.3 | 2.5 | 2.3 | 3.1 | 2.2 |
| K (mEq/L) | 0.8 | 2.9 | 1.3 | 2.4 | 1.7 | 3.9 | 3.6 | 1.4 | 3.1 |
| T. protein (g/L) | 1.6 | 1.5 | 2.2 | 4.3 | 2.9 | 0.9 | 0.9 | 1.3 | 0.9 |
| Na (mEq/L) | 0.6 | 0.7 | 1.0 | 2.4 | 2.6 | 0.2 | 0.2 | 0.1 | 0.1 |
| TG (mg/dL) | 1.9 | 2.4 | 3.0 | 5.5 | 4.4 | 8.3 | 8.1 | 10.5 | 8.4 |
| Urea (mg/dL) | 2.2 | 3.3 | 2.9 | 2.1 | 2.3 | 4.1 | 4.6 | 4.7 | 5.4 |
| Uric acid (mg/dL) | 2.2 | 3.5 | 2.0 | 2.2 | 3.9 | 4.3 | 4.9 | – | – |
| HbA1c (%) | 2.4 | 4.9 | 1.2 | 1.6 | 6.3 | 0.3 | 0.5 | Negative | Negative |

CV: Coefficient of variation; CLIA: Clinical Laboratory Improvement Amendments; EFLM BV: European Federation of Clinical Chemistry and Laboratory Medicine Biological Variation; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase: AMY: Amylase; AST: Aspartate aminotransferase; D. Bil: Direct (Conjugated) bilirubin; T.Bil: Total bilirubin; T. Calsium: Total calcium; Cl: Chloride; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; T. Cholesterol: Total cholesterol; CK: Creatine kinase; GGT: γ- glutamyltransferase; LDH: Lactate dehydrogenase; Mg: Magnesium; PO4: Phosphate; K: Potassium; T.protein: Total protein; Na: Sodium; TG: Triglycerides; HbA1c: Glycated hemoglobin.

teins, prenatal screening tests, coagulation parameters and cardiac markers were performed with RIQAS (Randox International Quality Assessment Scheme (UK)) Control.

The one-year process sigma values of the relevant tests were calculated monthly, separately for each control level, and the annual average was taken. The process sigma was calculated according to equation-2. For the CV % value, the IQC data of each test were used and the arithmetic annual average was taken. Peer group bias % values obtained from the external quality control reports of each test were recorded regularly for each month and the annual arithmetic mean was calculated. The monthly EQA reports of the tests were accessed through the websites of the relevant EQA programs (www.qcnet.com and www.riqas.net) by the user code and password of the laboratory. Process sigma values were calculated separately based on the TEa according to CLIA (Clinical Laboratory Improvement Amendments) 2019, Ricos BV (Dr. Carmen Ricos Biological Variation) Desirable, and EFLM BV (European Federation of Clinical Chemistry and Laboratory Medicine Biological Variation) Desirable [6–8].

## Results

The 1-year target value averages and the CV% values of IQC of all tests included in the study, the bias% values obtained from the external quality control results, and the average of process sigma values for the internal quality control levels calculated according to CLIA 2019, RICOS BV desirable, and EFLM BV desirable criteria are shown in Tables 1-3.

**Table 2. One-year average of mean, standard deviation and CV% values of internal quality controls and bias % values of external quality control and process sigma values of hemogram, coagulation, cardiac marker, prenatal screening, specific protein tests**

| Test | Bias% | CV% | | | Process sigma CLIA | | | Process sigma RICOS (Desirable) | | | Process sigma EFLM BV (Desirable) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L1 | L2 | L3 | L1 | L2 | L3 | L1 | L2 | L3 | L1 | L2 | L3 |
| HCT (%) | 1.8 | 0.6 | 0.7 | 0.8 | 3.7 | 1.1 | 2.8 | 3.4 | 3.0 | 2.8 | 3.5 | 3.0 | 2.6 |
| HGB (g/dL) | 0.7 | 0.5 | 0.5 | 1.0 | 6.6 | 6.6 | 3.3 | 7.2 | 6.5 | 3.4 | 6.2 | 6.2 | 3.1 |
| MCH (pg/cell) | 2.0 | 0.6 | 0.8 | 1.0 | – | – | – | 0.8 | 0.6 | 0.5 | <0 | <0 | <0 |
| MCHC (g/dL) | 1.6 | 0.7 | 0.8 | 1.1 | – | – | – | <0 | <0 | <0 | <0 | <0 | <0 |
| MCV (fL) | 2.8 | 0.5 | 0.6 | 0.5 | – | – | – | <0 | <0 | <0 | <0 | <0 | <0 |
| MPV (fL) | 2.6 | 0.7 | 0.7 | 0.7 | – | – | – | 4.6 | 4.8 | 4.9 | 1.7 | 1.7 | 1.7 |
| PLT (K/µL) | 4.4 | 1.5 | 1.6 | 2.3 | 13.7 | 12.9 | 9.0 | 5.9 | 5.8 | 4.0 | 4.6 | 4.3 | 3.0 |
| RBC (M/µL) | 1.2 | 0.5 | 0.5 | 0.6 | 5.6 | 5.6 | 4.7 | 6.9 | 6.6 | 5.2 | 5.4 | 5.4 | 4.5 |
| RDW (%) | 1.7 | 1.2 | 1.3 | 1.4 | – | – | – | 2.4 | 2.1 | 2.0 | 0.8 | 0.7 | 0.6 |
| WBC (K/µL) | 2.5 | 1.2 | 1.4 | 2.3 | 2.1 | 1.8 | 1.1 | 10.6 | 9.4 | 5.7 | 9.4 | 8.1 | 4.9 |
| CK-MB (µg/L) | 3.7 | 4.5 | 4.7 | – | 4.7 | 4.5 | – | 5.9 | 5.6 | – | – | – | – |
| Myog (ng/mL) | 3.5 | 3.7 | 3.6 | – | – | – | – | 4.4 | 4.5 | – | – | – | – |
| NT-ProBNP (pg/mL) | 3.0 | 3.3 | 3.2 | – | 8.2 | 8.4 | – | 3.0 | 3.1 | – | – | – | – |
| Troponin T (ng/mL) | 9.4 | 4.6 | 2.5 | – | 4.5 | 8.2 | – | 8.6 | 15.8 | – | 1.8 | 3.3 | – |
| Fibrinogen (mg/dL) | 3.0 | 5.7 | 6.7 | – | 3.0 | 2.5 | – | 1.9 | 1.6 | – | – | – | – |
| a PTT (sn) | 6.0 | 3.2 | 3.6 | – | 2.8 | 2.5 | – | <0 | <0 | – | – | – | – |
| PT (sn) | 3.9 | 4.5 | 5.3 | – | 2.5 | 2.1 | – | 0.3 | 0.3 | – | – | – | – |
| AFP (IU/mL) | 4.9 | 4.7 | 4.8 | – | 3.2 | 3.1 | – | 3.6 | 3.6 | – | 2.7 | 2.6 | – |
| Total HCG | 7.2 | 4.1 | 4.9 | – | 2.6 | 2.2 | – | – | – | – | – | – | – |
| PAPP-A (mIU/mL) | 4.6 | 5.5 | 5.1 | – | – | – | – | 1.9 | 2.1 | – | – | – | – |
| C3 (g/L) | 5.5 | 5.0 | 4.5 | 5.0 | 1.9 | 2.1 | 1.9 | 0.6 | 0.6 | 0.6 | 0.5 | 0.5 | 0.5 |
| C4 (g/L) | 3.0 | 5.2 | 5.2 | 5.1 | 3.3 | 3.3 | 3.3 | 2.5 | 2.5 | 2.5 | 1.8 | 1.8 | 1.8 |
| IGA (g/L) | 5.4 | 4.8 | 4.3 | 5.0 | 2.0 | 2.2 | 1.9 | 1.7 | 1.9 | 1.6 | 0.9 | 1.0 | 0.9 |
| IGG (g/L) | 6.2 | 4.7 | 4.7 | 4.6 | 2.9 | 2.9 | 3.0 | 0.4 | 0.4 | 0.4 | 0.2 | 0.2 | 0.2 |
| IGM (g/L) | 6.1 | 4.3 | 5.4 | 5.1 | 3.2 | 2.6 | 2.7 | 2.5 | 2.0 | 2.1 | 2.6 | 2.0 | 2.2 |
| IgE (IU/mL) | 3.0 | 5.3 | 5.5 | – | 3.2 | 3.1 | – | – | – | – | – | – | – |

CV: Coefficient of variation; CLIA: Clinical Laboratory Improvement Amendments; EFLM BV: European Federation of Clinical Chemistry and Laboratory Medicine Biological Variation; HCT: Hematocrit; HGB: Hemoglobin; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; MCV: Mean corpuscular volume; MPV: Mean platelet volume; PLT: Platelet; RBC: Red blood cells; RDW: Red cell distrubition width; WBC: White blood cells; CK-MB: Creatine kinase muscle brain isoenzyme; Myog: Myoglobin; a PTT: Activated partial thromboplastin time; PT: Prothrombin time; AFP: α-feto protein; Total HCG: Total human chorionic gonadotropin; PAPP-A: Pregnancy associated plasma protein A; C3: Complement 3; C4: Complement 4; IGA: Immunoglobin A; IGG: Immunoglobin G; IGM: Immunoglobin M; IgE: Immunoglobulin E.

## Discussion

For many years, studies have been carried out and applied to evaluate and improve the quality of the analytical process. The Six Sigma protocol is one of the most effective and universal tools for evaluating clinical laboratory performance. The sigma value of a test is a numerically defined value for the quality measure of that test [9, 10].

For all tests in our study, unacceptable performance (<3 sigma) was observed in 54 of the 130 targets according to the CLIA criteria, 89 of the 172 targets according to the RICOS BV Desirable criteria, and 86 of the 133 targets according to the EFLM BV Desirable criteria. In this case, the success rate of the laboratory is 60% according to CLIA, 49% according to RICOS BV Desirable criteria, and 36% according to EFLM BV Desirable criteria. In the literature, there are studies that have similar results to our study, and it is possible to come across studies that have higher or lower performance than ours [11–19].

Process sigma analyses can be used effectively to evaluate analytical performance and govern internal quality control procedures, but some limiting factors should be considered when calculating the sigma value. The first of these factors is the method used to calculate the bias. As is well known, the best method for calculating bias is to use a reference material/method [12]. However, for many analytes, there is no reference method or reference material, and even if there is, it is very difficult for clinical laboratories to obtain. Therefore, it is very difficult to calculate the bias realistically. In practice, the percentage bias can be calculated using internal quality control data or external quality control data [9, 11].

**Table 3. One-year average of mean, standard deviation and CV% values of internal quality controls and bias % values of external quality control and process sigma values of hormon and tumor markers**

| Test | Bias% | CV% | | | Process sigma CLIA | | | Process sigma RICOS (Desirable) | | | Process sigma EFLM BV (Desirable) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L1 | L2 | L3 | L1 | L2 | L3 | L1 | L2 | L3 | L1 | L2 | L3 |
| AFP (ng/mL) | 5.7 | 5.3 | 5.6 | – | 2.7 | 2.6 | – | 3.1 | 2.9 | – | 2.2 | 2.1 | – |
| CA 125 (U/mL) | 4.5 | 4.4 | 4.7 | – | 3.5 | 3.3 | – | 7.0 | 6.5 | – | 2.1 | 2.0 | – |
| CA 15–3 (U/mL) | 3.7 | 4.6 | 4.6 | – | – | – | – | 3.7 | 3.7 | – | – | – | – |
| CA 19–9 (U/mL) | 4.2 | 4.5 | 4.8 | – | – | – | – | 9.2 | 8.8 | – | 3.0 | 2.9 | – |
| CEA (ng/mL) | 4.1 | 5.1 | 4.9 | – | – | – | – | 4.0 | 4.2 | – | 3.2 | 3.3 | – |
| Cortisol (µg/dL) | 2.9 | 7.1 | 5.3 | – | 2.4 | 3.2 | – | 2.8 | 3.8 | – | 3.3 | 4.4 | – |
| Estradiol (pg/mL) | 5.7 | 12.6 | 9.1 | – | 1.9 | 2.7 | – | 1.7 | 2.3 | – | 0.9 | 1.3 | – |
| Ferritin (ng/mL) | 9.6 | 5.6 | 5.2 | – | 1.9 | 2.0 | – | 1.3 | 1.4 | – | – | – | – |
| Folate (ng/mL) | 2.6 | 4.4 | 4.0 | – | 6.2 | 6.9 | – | 8.2 | 9.2 | – | – | – | – |
| FSH (mIU/mL) | 3.8 | 4.8 | 5.0 | – | 3.0 | 2.8 | – | 3.6 | 3.5 | – | 3.6 | 3.5 | – |
| HCG (mIU/mL) | 4.0 | 5.3 | 5.1 | – | 2.6 | 2.7 | – | – | – | – | – | – | – |
| LH (mIU/mL) | 4.2 | 6.7 | 6.3 | – | 2.4 | 2.5 | – | 3.6 | 3.8 | – | 3.6 | 3.8 | – |
| Prolactin (ng/mL) | 3.4 | 3.6 | 4.2 | – | 4.6 | 4.0 | – | 7.2 | 6.2 | – | 9.4 | 8.1 | – |
| Total PSA (ng/mL) | 3.7 | 8.2 | 4.5 | – | 2.0 | 3.6 | – | 3.6 | 6.7 | – | 1.5 | 2.8 | – |
| Free T3 (pg/mL) | 3.7 | 6.4 | 6.4 | – | – | – | – | 1.2 | 1.2 | – | 0.4 | 0.4 | – |
| Free T4 (ng/dL) | 4.3 | 5.8 | 4.6 | – | 1.8 | 2.3 | – | 0.6 | 0.8 | – | 0.3 | 0.4 | – |
| Testosterone (ng/mL) | 4.5 | 3.9 | 3.9 | – | 4.0 | 4.0 | – | 2.3 | 2.3 | – | 3.1 | 3.1 | – |
| TSH (µIU/mL) | 4.6 | 5.4 | 5.4 | – | 2.9 | 2.9 | – | 3.5 | 3.5 | – | 3.7 | 3.7 | – |
| CRP (mg/dL) | 4.1 | 4.7 | 4.5 | – | 5.5 | 5.8 | – | 11.9 | 12.5 | – | 9.9 | 10.4 | – |
| DHEA-S (µg/dL) | 2.0 | 5.8 | 5.5 | 4.8 | – | – | – | 1.9 | 2.0 | 2.3 | 1.4 | 1.5 | 1.8 |
| TG (ng/mL) | 9.9 | 8.3 | 5.3 | 5.3 | – | – | – | 1.4 | 2.3 | 2.3 | 2.2 | 3.5 | 3.5 |
| ATG (U/mL) | 11.6 | 8.1 | 7.7 | – | – | – | – | 2.0 | 2.1 | – | – | – | – |
| ATA (U/mL) | 8.6 | 8.9 | 6.6 | – | – | – | – | 4.2 | 5.7 | – | – | – | – |
| C peptide (ng/mL) | 8.7 | 4.3 | 3.8 | 4.3 | – | – | – | 2.8 | 3.2 | 2.8 | – | – | – |
| IGF-1 (ng/mL) | 11.6 | 5.5 | 4.9 | – | – | – | – | 2.3 | 2.5 | – | 0.6 | 0.7 | – |
| İnsulin (mU/L) | 22.1 | 4.0 | 3.3 | – | – | – | – | 2.7 | 3.2 | – | 2.4 | 2.8 | – |
| PTH (pg/mL) | 9.3 | 4.2 | 5.1 | – | – | – | – | 5.0 | 4.0 | – | 2.5 | 2.1 | – |

CV: Coefficient of variation; CLIA: Clinical Laboratory Improvement Amendments; EFLM BV: European Federation of Clinical Chemistry and Laboratory Medicine Biological Variation; AFP: α-feto protein; CA 125: Cancer antigen 125; CA 15-3: Cancer antigen 15-3; C A19-9: Cancer antigen 19-9; CEA: Carcinoembryonic antigen; FSH; Follicle- stimulating hormone; HCG: Human chorionic gonadotropin; LH: Luteinizing hormone; Total PSA: Total prostate spesific antigen; TSH: Thyroid stimulating hormone; CRP: C-Reactive protein; DHEA-S: Dehydroepiandrosterone sulfate; TG: Thyroglobulin; ATG: Anti-thyroglobulin; ATA: Anti- thyroperoxidase; IGF-1: Insulin-like growth factor 1; PTH: Parathyroid hormone.

While calculating the bias% value we used for each test in this study, we took the group mean value in the external quality control data as a basis. In similar studies in the literature, the bias value used for sigma calculation was obtained from the mean values of the external quality control group [9, 12, 15, 17, 19]. The point to be noted here is that the group mean values in the external quality control data are the mean values of the data, including all group participants. In other words, it is not a value determined by analyzing the reference method. Therefore, the bias value we calculated is not actually 'true bias', but maybe 'relative bias'. In addition, there are also studies using internal quality control data for bias calculation [11, 14, 16]. Some researchers argue that using IQC data will be more accurate than using EQA data, because the EQA group mean value is affected by the measurement uncertainty of all group participants, and they recommend using IQC data in the calculation of bias unless a comparison is made with the reference method [11]. We believe that using IQC bias will also be a biased approach and will not be sufficient to show objective and true bias. However, as part of routine laboratory operations, the use of reference material to calculate sigma values for all tests is not a cost-effective approach, so we preferred the use of EQA bias values. We believe that the use of bias from IQC is a barrier to comparing our results with those of other laboratories.

Another limiting factor is that the CV% values are related to the concentrations. In most cases, the CV% of a parameter is high at low concentrations and decreases as the concentration increases. Therefore, it is possible that different performances are obtained at different concentrations [20]. This

difference was also observed in our study. For example, as the CV% of the total PSA assay is high at low concentrations, the sigma value varies between 1.5 and 3.6, whereas its performance is at a world-class level at high concentrations. This is true for almost all parameters. In this case, the question of which CV % value to use for calculating performance can be answered by considering clinical decision limits.

One of the problems that cause limitations in calculating the sigma values of the tests is the use of control materials instead of patient samples. There is a matrix difference between control materials and real patient samples, so the analytical responses of control materials to the measurement of a test may not be uniformly matched to the patient sample [14]. On the other hand, stability can also be an issue in CV studies conducted with patient serum pools. Therefore, we recommend using internal quality control samples.

Another point to consider is that when calculating the analytical performance of a test, different sigma values are obtained according to the selected TEa reference (such as CLIA, RICOS BV, EFLM BV). Although the same Bias% and CV% values were used in our study, it was observed that different sigma values would be obtained according to the selected reference. For example, while the process sigma value of the Troponin T test was calculated with CLIA and RICOS, it had higher sigma values (between 4.5 and 15.8); when calculated with EFLM BV, the low concentration level showed unacceptable performance, and the sigma value of the high concentration level was on the borderline.

The TEa criteria of Biological Variation are often more stringent and their relevance is controversial. Tests such as electrolytes, HbA1c, and coagulation tests can be placed in this group. For example, the desirable limit for Sodium is 0.7% according to EFLM BV. Even if bias %=0 and CV %=0.5, the achievable sigma value is 1.4 and is considered unacceptable performance. Moreover, these values for bias% and CV% are too far away to be achieved in practical laboratory processes. Of course, parameters such as sodium must be controlled within a narrow range. Therefore, the targets of BV should be quite challenging but also realistic. In a study evaluating the process sigma performances of some clinical chemistry tests compared with laboratory data and manufacturer's brochure data, it was found that the goal of >3 sigma for laboratories cannot be achieved even when brochure data for parameters such as sodium, chloride, and calcium are used [12]. Manufacturers obtain precision under optimal conditions and bias from method comparison studies. Therefore, the data reported in the package insert may be considered quite optimistic compared with data obtained in routine laboratory practice [12]. For laboratory end users, trying to meet BV quality targets with current technologies is a waste of time, effort, and resources [12, 14]. In our opinion, many tests in our study had very low sigma values due to these very stringent criteria.

Although it seems reasonable to use the same TEa source for each test, it may be appropriate to use different TEa sources depending on the suitability for the test or the experience of the laboratory. Some TEa targets are quite generous and lead to a quality result of misleading optimism. Others, however, lead to a more pessimistic representation of quality performance, as in the case of BV. In the current situation, it is at the discretion of the laboratory director to choose the appropriate TEa criterion by making the most practical and appropriate decision.

## Conclusion

The process sigma values reported in this study reflect the time period in which the data were collected and thus represent a "snapshot" of sorts. Of course, performance can fluctuate for many reasons, including temperature, pH, and variations between different reagents. Regular calculation of the process sigma would be more useful in terms of testing and continuous monitoring of instrument quality. This is because when we look at the data from our study and other studies presented in the literature, test performance may increase or decrease due to changes in the process. Accordingly, process sigma can be used as a suitable quality assurance tool to determine test quality and monitor quality changes on a regular basis.

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## References

1. Taga Y, Aslan D, Güner G, Kutay ZF: Tıbbi Laboratuvarlarda Standardizasyon ve Kalite Yönetimi. Ankara: Türk Biyokimya Derneği Yayınları; 2000.
2. Bonini P, Plebani M, Ceriotti F, Rubboli F. Errors in laboratory medicine. Clin Chem 2002;48(5):691–8. [CrossRef]
3. Howanitz PJ. Errors in laboratory medicine: Practical lessons to improve patient safety. Arch Pathol Lab Med 2005;129(10):1252–61. [CrossRef]
4. Carraro P, Plebani M. Errors in a stat laboratory: Types and frequencies 10 years later. Clin Chem 2007;53(7):1338–42. [CrossRef]
5. Westgard JO, Klee GG. Quality management. In: Burtis CA, Ashwood ER, Bruns DE, editors. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. St. Louis: Elsevier Saunders; 2006. p. 485–529.
6. Departmant of Health and Human Services. Clinical Labora-

tory Improvement Amendments of 1988 (CLIA) proficiency testing regulations related to analytes and acceptable performance. Available at: https://www.federalregister.gov/documents/2022/07/11/2022-14513/clinical-laboratory-improvement-amendments- of-1988-clia-proficiency-testing-regulations-related-to. Accessed Jul 1, 2024.
7. Westgard QC. Quality requirements: Desirable biological variation database specifications. Available at: https://westgard.com/clia-a-quality/quality-requirements/238-biodatabase1.html. Accessed Jul 1, 2024.
8. European Federation of Clinical Chemistry and Laboratory Medicine (EFLM). EFLM biological variation database. Available at: https://biologicalvariation.eu/. Accessed Jul 1, 2024.
9. Schoenmakers CH, Naus AJ, Vermeer HJ, van Loon D, Steen G. Practical application of Sigma Metrics QC procedures in clinical chemistry. Clin Chem Lab Med 2011;49(11):1837–43. [CrossRef]
10. Westgard JO. Charts of operational process specifications ("OPSpecs charts") for testing performance criteria. Clin Chem 1992;38(7):1226–33. [CrossRef]
11. Aslan D, Sert S, Aybek H, Yılmaztürk G. Assessment of total clinical laboratory process perfromance: Normalized OPSpecs charts, six sigma and patient test results. Turk J Biochem [Article in Turkish] 2005;30(4);296–305.
12. Keleş M. Evaluation of the clinical chemistry tests analytical performance with Sigma Metric by using different quality specifications - comparison of analyser actual performance with manufacturer data. Biochem Med (Zagreb) 2022;32(1):010703. [CrossRef]
13. Westgard JO, Westgard SA. The quality of laboratory testing today: An assessment of σ metrics for analytic quality using performance data from proficiency testing surveys and the CLIA criteria for acceptable performance. Am J Clin Pathol 2006;125(3):343–54. [CrossRef]
14. Hens K, Berth M, Armbruster D, Westgard S. Sigma metrics used to assess analytical quality of clinical chemistry assays: Importance of the allowable total error (TEa) target. Clin Chem Lab Med 2014;52(7):973–80. [CrossRef]
15. Huysal K, Budak YU. Application of sigma metrics for the assessment of quality assurance using the MQ-2000 PT HbA1c analyzer. Biochem Med (Zagreb) 2015;25(3):416–20. [CrossRef]
16. Nar R, Emekli DI. The evaluation of analytical performance of immunoassay tests by using six-sigma method. J Med Biochem 2017;36(4):301–8. [CrossRef]
17. Çakırca G, Yazıcı T. Evaluation of analytical performance of clinical chemistry assays using six sigma. Turk Klin Biyokim Derg 2019;17(2):79–84.
18. Hollestelle MJ, Ruinemans-Koerts J, Idema RN, Meijer P, de Maat MPM. Determination of sigma score based on biological variation for haemostasis assays: Fit-for-purpose for daily practice? Clin Chem Lab Med 2019;57(8):1235–41. [CrossRef]
19. Ozdemir S, Ucar F. Determination of sigma metric based on various TEa sources for CBC parameters: The need for Sigma metric harmonization. J Lab Med 2022;46(2):133–41. [CrossRef]
20. Bozkurt Yavuz H, Karahan SC, Yaman H, Örem A, Aliyazıcıoğlu Y. Two approaches for measurement uncertainty estimation: Which role for bias? Complete blood count experience. Turk J Biochem 2021;46(6):639–45. [CrossRef]

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Research Article

# *In vitro* anticancer effect of theobromine in A549 non-small cell lung cancer cells

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## Abstract

**Objectives:** Theobromine, a naturally occurring alkaloid, is now recognized as a viable option due to its potential therapeutic benefit in many types of cancers. This preliminary study was undertaken to clarify how theobromine affects the viability, apoptosis, and migration of human non-small cell lung cancer (NSCLC) A549 cells.

**Methods:** Water-soluble tetrazolium-1 (WST-1) assay was utilized to measure the cytotoxicity of theobromine (5-200 μM) in A549 cells for 24 and 48 hours. Flow cytometry analysis was used to detect apoptosis. A wound healing assay was carried out to evaluate the migration of A549 cells. Cells incubated with the IC50 concentration of cisplatin for 24 hours served as the reference group.

**Results:** A time- and dose-dependent effect of theobromine on the inhibition of A549 cell viability was dramatically observed. Theobromine treatment led to an increased apoptotic cell population and caspase 3/7 activity ($p<0.0001$). Furthermore, the migratory capacity of A549 cells was reduced in the cells treated with theobromine.

**Conclusion:** The results suggested that theobromine potently reduced the cell growth and migration capacity of NSCLC A549 cells by inducing caspase 3/7-mediated apoptosis. Additional studies are necessary to comprehend the antitumor mechanisms of theobromine against NSCLC and to provide helpful perspectives for more research.

**Keywords:** Apoptosis, A549 cells, caspase 3/7, cytotoxicity, migration, NSCLC, theobromine, viability

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Non-small cell lung cancer (NSCLC) is one of the most commonly encountered subtypes of lung cancer [1]. Prevailing pharmacological options for NSCLC, including chemotherapy, targeted therapy, and immunotherapy alone or in combination with surgery/radiation, are not completely effective and well-tolerated options due to their side effects, low specificity, intrinsic and/or acquired resistance to chemotherapeutic agents, and the lack of major advancements in the survival rates of patients [2, 3]. Among several anticancer drugs applied to lung cancer, cisplatin has been largely used as a first-line therapy in combination with other compounds [4]. However, limited usage of cisplatin in clinical settings has been reported due to its well-known side effects and consequential chemoresistance [5].

Due to limitations in early diagnostic techniques and the absence of clinical signs in the early stage of cancer, a large number of lung cancer patients are detected at advanced stages of the disease [6]. Thus, alternative therapeutic options which could offer better prognostication of the patients at early stages of cancer are thought to be quite advantageous. There has been a noticeable rise in the number of naturally obtained anticancer drugs that have been officially accepted for lung cancer therapy [7, 8]. Therefore, the identification and presentation of natural products with anticancer activity have gained interest for the management of lung cancer due to their low toxicity, accessible, and inexpensive properties [9].

Apoptosis represents a programmed cell death that is executed through the activation of the caspase family and followed

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by a set of morphological changes [10]. Natural compounds have demonstrated antitumor activity via the induction of apoptotic cell signaling in lung cancer cells [4]. Previous studies demonstrated that methylxanthines have anticancer and anti-inflammatory properties [11, 12]. Among natural products, theobromine is a natural methylxanthine found in Theobroma cacao, such as chocolate, and other foods including tea [13]. Theobromine is a caffeine derivative that can also be absorbed into the body via caffeine metabolized by the cytochrome P450 oxidase enzyme system [14].

It has been previously shown that theobromine has multiple biological properties along with its antitumor activity, anti-inflammatory, and antioxidant effects [15, 16]. The primary action mechanism of theobromine as a phosphodiesterase (PDE) inhibitor is mainly through increasing intracellular cyclic adenosine monophosphate (cAMP) [17]. High cAMP levels are linked to the induction of apoptosis, invasion, and spread of cancer cells [18]. Specifically, theobromine triggers apoptosis in HT-29 colorectal cancer cells [19], and decreases cell growth in human glioblastoma cells [13]. Theobromine is also capable of the inhibition of cell proliferation along with its antiangiogenic properties in human glioblastoma, ovarian, and lung cancers [13, 20, 21]. Furthermore, theobromine is able to improve the cytotoxic activity of doxorubicin in human VoLo colorectal cancer cells [22]. Moreover, theobromine has demonstrated promising anticancer activities indicating its ability to act as a potential therapeutic agent towards a number of malignancies [21, 23, 24].

Herein, the present study firstly assessed the cytotoxic, apoptotic, and antimigratory effects of theobromine treatment in A549 lung cancer cells.

## Materials and Methods

### Cell culture and chemicals

The NSCLC A549 cells (ATCC) were cultured in DMEM (Capricorn Scientific) containing 10% FBS (Capricorn Scientific) and 1% Pen/Strep (Capricorn Scientific) at 37°C in 5% CO2 conditions. Theobromine was acquired from Sigma-Aldrich (catalog number: T4500) and dissolved in dimethyl sulfoxide (DMSO, Merck, Saint Louis, MO, USA) to have a 1 mM stock solution. Cisplatin (European Pharmacopoeia) was prepared with DMSO to obtain a 500 µM stock solution.

### WST-1 cell viability assay

The WST-1 cytotoxicity test was used to determine the cytotoxicity against the A549 cell line. The principle of this assay is that cellular mitochondrial dehydrogenases convert the tetrazolium salt WST-1 into formazan crystals. A549 cells were proliferated and incubated (96-well). After incubation, theobromine and cisplatin were prepared at concentrations of 0, 5, 10, 25, 50, 100, and 200 µM and applied to the cells. The measurements were analyzed at the 24$^{th}$ and 48$^{th}$ hours. To eliminate the effects from DMSO, the concentrations were added to the negative control wells at a rate where theobromine and cisplatin were dissolved along with the medium. After adding 10 µL of the WST-1 solution (Cayman Chemical, cat. no. 10008883), the cells were incubated for 4 hours. Following incubation, the appropriate inhibitor concentration for the cells was determined via measuring absorbance values at 450 nm. Cisplatin was used as a reference drug. The percentage of cell viability in theobromine-treated groups was expressed as the ratio of the control group, which was assumed to be 100%. Furthermore, GraphPad Prism 9.1.0 programme was used to calculate the %50 inhibitory concentrations ($IC_{50}$) of theobromine and cisplatin via regression analysis.

### Apoptosis detection by flow cytometry

The apoptosis induced by theobromine and cisplatin was examined with Annexin V-FITC and PI (Tonbo Biosciences, San Diego, CA). Adherent A549 cells ($1\times10^5$/well) were incubated with $IC_{50}$ values of theobromine (16.02 µM) or cisplatin (6.40 µM) for 24 hours at 37°C in 6-well plates. Then, the cells were stained with 5 µL of each of Annexin V-FITC and PI solution in binding buffer (15 minutes in the dark). Subsequently, the apoptosis rate was measured by ACEA NovoCyte flow cytometry (Agilent).

### Caspase 3/7 activity assay by flow cytometry

To measure caspase 3/7 levels, A549 cells were treated with the $IC_{50}$ concentration of theobromine (16.02 µM) or cisplatin (6.40 µM) and incubated overnight. After the 24-hour treatment period, the cells were trypsinized, collected, and analyzed for the detection of caspase 3/7 activity with Cell Meter™ Caspase 3/7 flow cytometry assay kit using ACEA NovoCyte flow cytometry instrument (Agilent).

### Wound-healing assay

A549 cells were cultured in medium for full confluence. A sterile plastic pipette tip was used to artificially create a gap, and then cells were incubated with DMEM containing the $IC_{50}$ values of theobromine and cisplatin for 24 hours. Subsequently, the wound area was photographed immediately at 0h with a light microscope (Olympus IX73), and the degree of wound healing was measured based on the area covered by cells at the indicated times (6, 12, and 24 hours) after the scratch.

### Statistical analysis

Statistical analysis was analyzed with GraphPad Prism 9.1.0 program using Student's t-test and two-way ANOVA. Statistical significance level was accepted as $p<0.05$.

## Results

### Antiproliferative effect of theobromine on human A549 cells

A concentration- and time-dependent reduction in cell survival was detected in A549 cells after 24 and 48 hours of exposure to theobromine (5–200 µM) and cisplatin (1–50 µM) according to the WST-1 test (Fig. 1a, b). After treatment with theobromine for 24 hours, cell viability dropped to 72.5% at 5 µM and 66.5% at 10 µM (Fig. 1a). The viability of A549 cells was less than 36.5% at concentrations higher than 25 µM

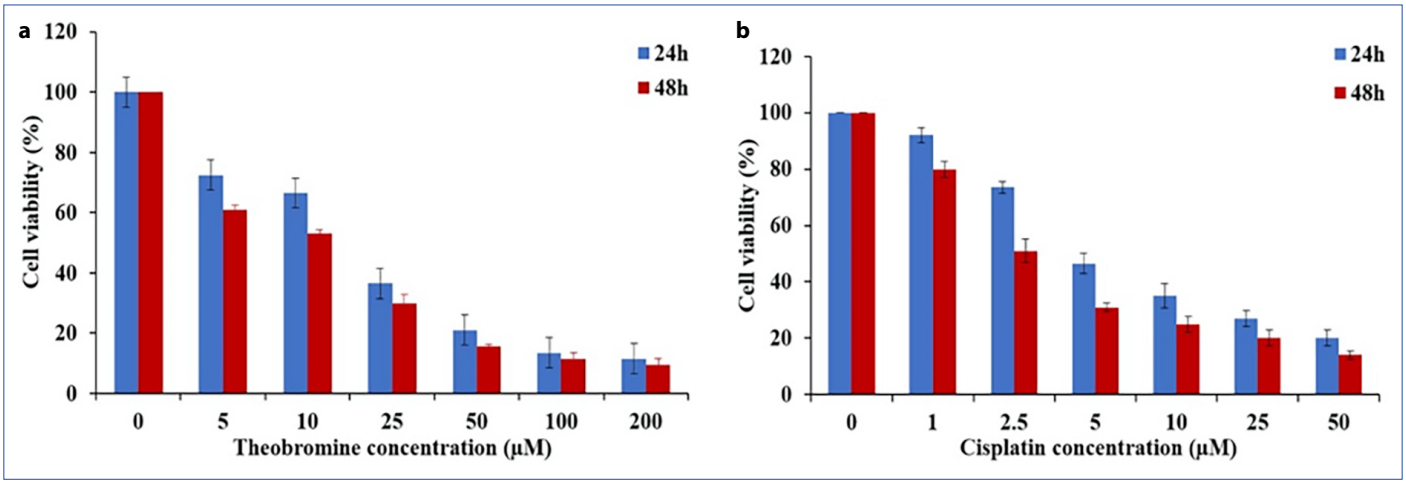


**Figure 1.** WST-1 assay showing cell viability of A549 after treatment with increasing doses of theobromine (a) and cisplatin (b) for 24 and 48 hours.
Data are displayed as the mean±SD. SD: Standard deviation.

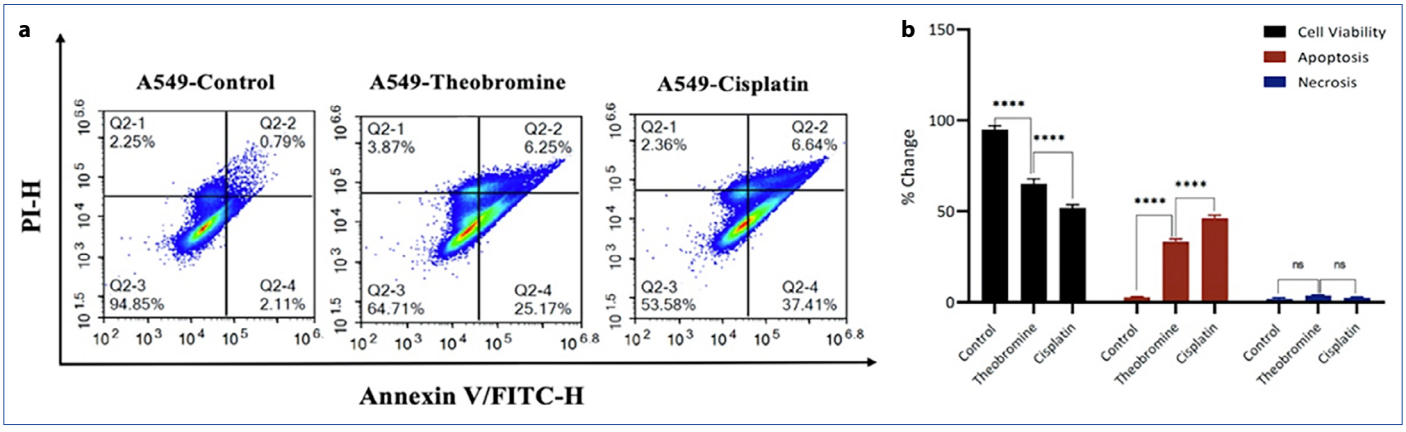


**Figure 2.** (a) Representative dot plots of A549 cells treated with theobromine and cisplatin at their $IC_{50}$ concentrations for 24 hours for determination of apoptotic cell death by Annexin V/FITC-PI double staining. (b) The percentages of viable, apoptotic, and necrotic cells were statistically analyzed. The values are the representative of three independent experiments.

Data are displayed as the mean±SD. Significances are shown in comparison to control cells and theobromine-treated cells (****: p<0.0001). SD: Standard deviation; PI-H: Propidium iodide; FITC-H: Fluorescein isothiocyanate.

theobromine. For 48 hours of treatment, this cytotoxic effect of theobromine was observed more severely than for 24 hours of treatment (Fig. 1a). $IC_{50}$ values of theobromine were calculated as 16.02 μM at 24 hours and 10.76 μM at 48 hours of treatment. Similar antiproliferative effects were also noted in cisplatin-treated A549 cells, as shown in Figure 1b, with $IC_{50}$ values of 6.40 μM at 24 hours and 3.84 μM at 48 hours. Regarding the $IC_{50}$ values of theobromine and cisplatin for 24 and 48 hours towards the A549 cell line, cisplatin, as the positive control, was more cytotoxic than theobromine.

### Theobromine promoted human A549 cell apoptosis

In agreement with the cytotoxicity results, the treatment of A549 cells with theobromine (16.02 μM) and cisplatin (6.40 μM) for 24 hours led to an increase in the rate of early and late apoptotic cells (31.42% and 44.05%, respectively) compared to control cells (2.9%) (Fig. 2a, b). The data indicated that theobromine has an antiproliferative effect on A549 lung cancer cells mediated by apoptosis induction. These results also indicated that the percentage of apoptotic cell death induced by cisplatin (44.05%) as a positive control was significantly higher than the theobromine-induced apoptosis rate (31.42%) ($p<0.0001$, Fig. 2b). In addition, A549 cells treated with theobromine and cisplatin exhibited a decreased population of viable cells by 64.71% and 53.58% in comparison to the non-treated group ($p<0.0001$, Fig. 2b).

### Theobromine induced caspase 3/7 activity in A549 cells

To understand the mechanism of theobromine, we measured the caspase 3/7 levels as the markers of apoptosis induction via flow cytometry analysis [25]. The effects of theobromine on apoptosis coincide with the results obtained by the caspase 3/7 assay. The treatment of A549 cells with $IC_{50}$ concentrations of theobromine for 24 hours resulted in augmented levels of caspase 3/7 activation when compared to the non-treated group ($p<0.0001$, Fig. 3a, b). As expect-

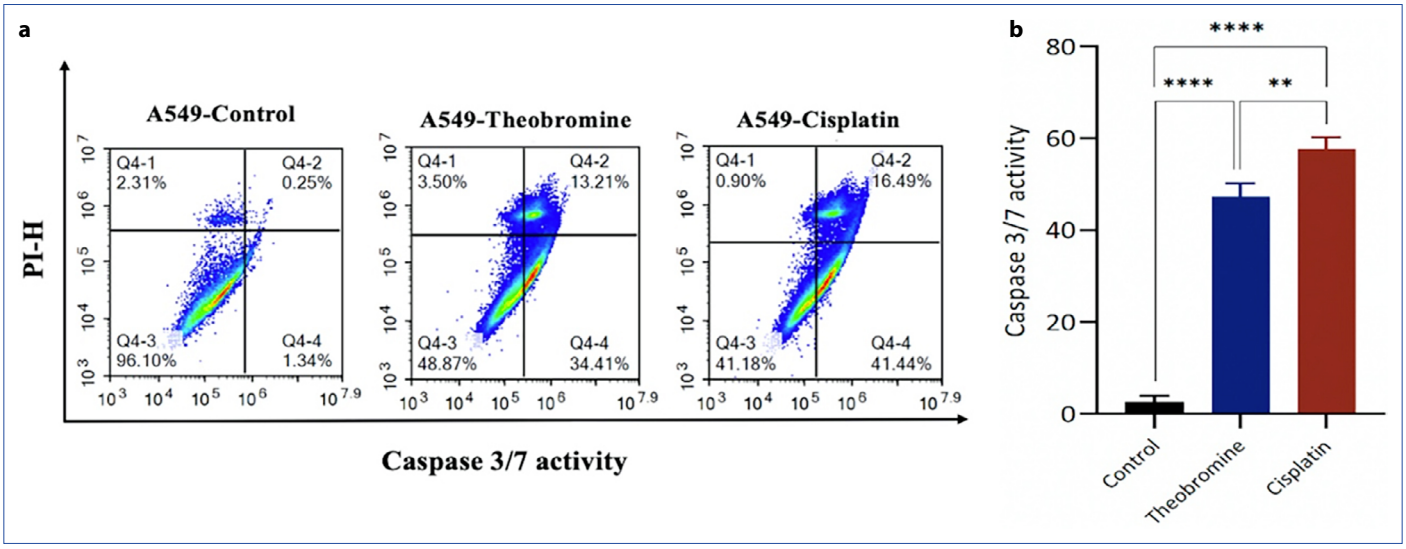


**Figure 3.** (a) Caspase 3/7 activity measured by flow cytometry in A549 cells treated with $IC_{50}$ concentrations of theobromine or cisplatin for 24 hours. (b) Comparison of caspase 3/7 activity among the groups.

Data are displayed as the mean±SD. Significances are shown in comparison to control cells and theobromine-treated cells (**: p<0.01; ****: p<0.0001). SD: Standard deviation; PI-H: Propidium iodide; $IC_{50}$: Half-maximal inhibitory concentration.

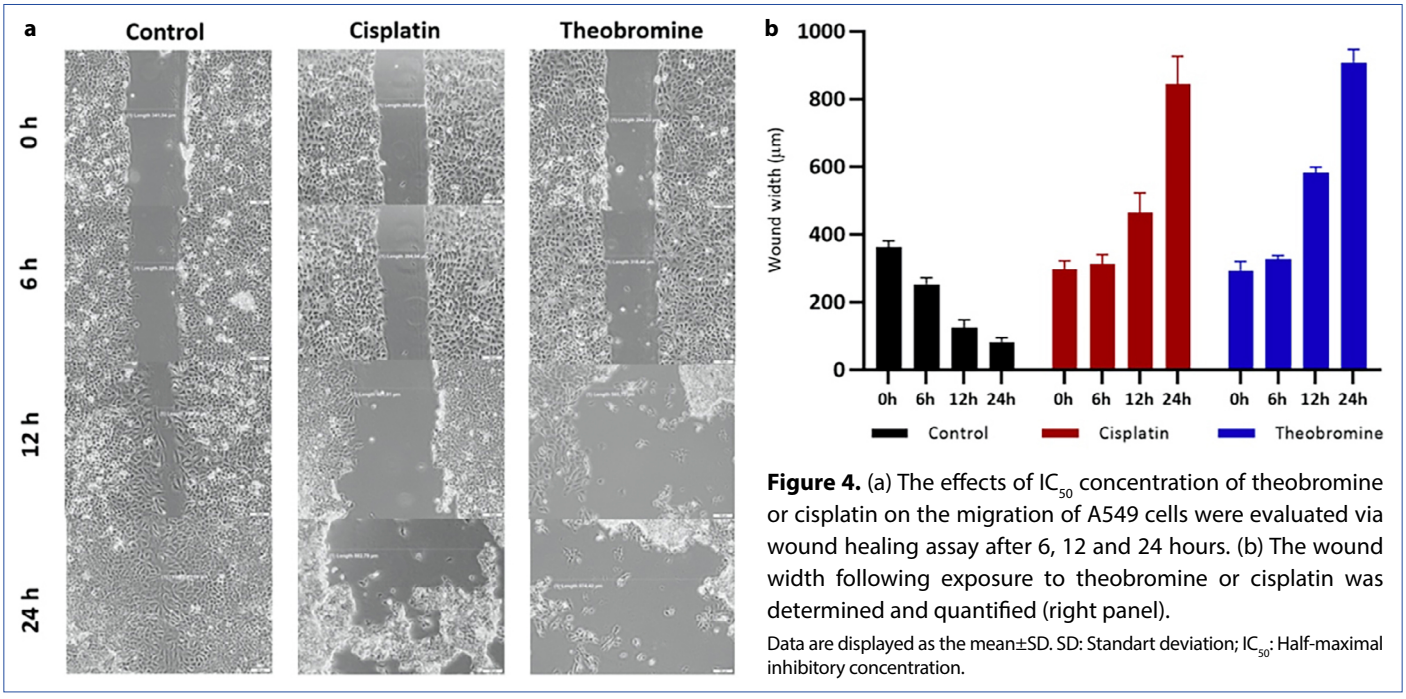


**Figure 4.** (a) The effects of $IC_{50}$ concentration of theobromine or cisplatin on the migration of A549 cells were evaluated via wound healing assay after 6, 12 and 24 hours. (b) The wound width following exposure to theobromine or cisplatin was determined and quantified (right panel).

Data are displayed as the mean±SD. SD: Standart deviation; $IC_{50}$: Half-maximal inhibitory concentration.

ed, the data indicated that caspase 3/7 activity was higher in the cisplatin group (57.93%) than the theobromine group (47.62%) (p<0.01, Fig. 3a, b), which was also supported by the result of the apoptosis assay.

### Theobromine reduced the migration ability of A549 cells

Theobromine prevented the migration of A549 cells. The migratory ability of A549 cells was significantly reduced with theobromine or cisplatin treatment for 24 hours as compared to control (p<0.0001, Fig. 4), suggesting that theobromine inhibited the migratory capacity of A549 cells. The relative wound width (area) following cisplatin treatment for 24 hours was smaller than that of theobromine-treated cells (p<0.01, Fig. 4).

## Discussion

Theobromine is a product of caffeine metabolism and possesses a significant role in the health benefits of cocoa as a more stable and safe compound than caffeine [26, 27]. The existing literature has already established that theobromine has the ability to suppress the growth of several cancer types [13]. A previous study demonstrated the anticancer effects

of theobromine by the suppression of the angiogenic potential of human lung cancer cells [20, 28]. However, there are no specific studies showing the apoptotic and antimigratory effects of theobromine on A549 lung cancer.

Our experiments showed the dose-dependent inhibitory effects of theobromine on the viability of A549 cells. The percentage of live cells after 48 hours of theobromine treatment tended to be lower for all concentrations compared to those within 24 hours. In previous studies, theobromine decreased proliferation of HT-29 colon cancer cells [19] and the malignant glioma U87-MG cell line [13]. Theobromine cytotoxicity was observed at higher concentrations (100–500 µg/mL) in RAW 264.7 macrophages [29]. In contrast to our finding, Eguchi et al. [30] reported that theobromine (50–200 µM) did not show cytotoxicity in human A549 lung cancer cells.

The controlled biological process of apoptosis controls the ratio of cell death to survival, and evading apoptosis is a characteristic of most forms of cancer [10]. The activation of the caspase family members is a key regulatory mechanism of apoptosis [25]. Annexin V-FITC and PI staining and the caspase 3/7 activity assay were employed to check whether this inhibitory impact of theobromine on cell viability was related to the induction of apoptosis. The results demonstrated a vital induction in caspase 3/7 activity and a marked increase in the percentage of apoptotic cells in the theobromine-treated group compared to the control cells. According to the findings, the apoptotic mechanism of theobromine in A549 cells is likely through the induction of caspase 3/7 activity. Similarly, Cadoná et al. [19] demonstrated the induction of apoptosis and enhanced sensitivity to chemotherapeutic drugs after theobromine treatment in HT-29 colon cancer cells. In addition, theobromine demonstrated anticancer activity in the malignant glioma cell line and an animal model of colon cancer through downregulation of the Akt/mTOR signaling pathway, which is a mechanism involved in the inhibition of apoptosis [13, 31]. In addition, theobromine-based derivatives induced apoptosis with its antiangiogenic properties in different cancer cell lines [32, 33]. Theobromine *in vitro* showed significant inhibitory activity on PARP-1, as a substrate of activated caspase 3/7, which is an important target for anticancer therapy [34]. In the 3T3-L1 preadipocytes, theobromine has been reported to induce G0/G1 arrest [35]. According to *in vivo* research, oral treatment with theobromine dramatically diminished the tumor weight in the Balb/c mouse with sarcoma cells [24], which could support the antiapoptotic effects of theobromine.

As well, the effects of caffeine are partly mediated by the products of its metabolism, such as theobromine, and the precise effects of theobromine vary greatly based on the type of cell and the amount of caffeine [26]. Numerous other investigations revealed that caffeine causes apoptosis, cell cycle arrest, and inhibition of viability in different cancer cells [36–38]. Caffeine also induces caspase 3/7 activity in human osteosarcoma cells [39]. Furthermore, caffeine has led to apoptosis in a skin epidermal cell line [36]. In human lung carcinoma cells, Qi et al. [38] showed that radiation-induced apoptosis was enhanced by 5 mM caffeine, but also caffeine alone caused apoptosis and a tumor suppressor protein p53-independent G1 phase arrest.

We further used a wound-healing assay to demonstrate the effect of theobromine on migration. Here, we observed that theobromine also suppressed the migration of the A549 lung cancer cell line in a time-dependent manner. Moreover, theobromine seemed to suppress migration more potently than cell viability at its $IC_{50}$ value, indicating that the antimetastatic action of theobromine might not depend only on cytotoxicity. In a study by Eissa et al. [32], a new theobromine derivative dramatically reduced the migratory capabilities of HepG2 cancer cells. Caffeine treatment (10 µg/mL) did not affect the migration ability of A549 and HeLa cancer cells [40].

## Conclusion

Our research revealed that theobromine induced apoptosis and enhanced caspase 3/7 activation, which in turn prevented A549 cells from proliferating and migrating. However, this article offers preliminary data that will be the basis for future studies. Thus, more fundamental researches are needed to verify favorable *in vivo* antitumor activity of theobromine to elucidate its exact mechanisms as an anticancer agent.

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

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## References

1. Molina JR, Yang P, Cassivi SD, Schild SE, Adjei AA. Non-small cell lung cancer: Epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc 2008;83(5):584–94. [CrossRef]
2. Guo Q, Liu L, Chen Z, Fan Y, Zhou Y, Yuan Z, et al. Current treatments for non-small cell lung cancer. Front Oncol 2022;12:945102. [CrossRef]
3. Liu J, Xiang Y, Fang T, Zeng L, Sun A, Lin Y, et al. Advances in the diagnosis and treatment of advanced non-small-cell lung cancer with EGFR exon 20 insertion mutation. Clin Lung Cancer 2024;25(2):100–8. [CrossRef]
4. Huang CY, Ju DT, Chang CF, Muralidhar Reddy P, Velmurugan BK. A review on the effects of current chemotherapy drugs and natural agents in treating non-small cell lung cancer. Biomedicine (Taipei) 2017;7(4):23. [CrossRef]

5. Dasari S, Njiki S, Mbemi A, Yedjou CG, Tchounwou PB. Pharmacological effects of cisplatin combination with natural products in cancer chemotherapy. Int J Mol Sci 2022;23(3):1532. [CrossRef]
6. Ruiz-Ceja KA, Chirino YI. Current FDA-approved treatments for non-small cell lung cancer and potential biomarkers for its detection. Biomed Pharmacother 2017;90:24–37. [CrossRef]
7. Naeem A, Hu P, Yang M, Zhang J, Liu Y, Zhu W, et al. Natural products as anticancer agents: Current status and future perspectives. Molecules 2022;27(23):8367. [CrossRef]
8. Spira A, Ettinger DS. Multidisciplinary management of lung cancer. N Engl J Med 2004;350(4):379–92. [CrossRef]
9. Talib WH, Alsayed AR, Barakat M, Abu-Taha MI, Mahmod AI. Targeting drug chemo-resistance in cancer using natural products. Biomedicines 2021;9(10):1353. [CrossRef]
10. Carneiro BA, El-Deiry WS. Targeting apoptosis in cancer therapy. Nat Rev Clin Oncol 2020;17(7):395–417. [CrossRef]
11. Miwa S, Sugimoto N, Shirai T, Hayashi K, Nishida H, Ohnari I, et al. Caffeine activates tumor suppressor PTEN in sarcoma cells. Int J Oncol 2011;39(2):465–72.
12. Saito T, Sugimoto N, Ohta K, Shimizu T, Ohtani K, Nakayama Y, et al. Phosphodiesterase inhibitors suppress Lactobacillus casei cell-wall-induced NF-κB and MAPK activations and cell proliferation through protein kinase A- Or exchange protein activated by cAMP-dependent signal pathway. ScientificWorldJournal 2012;2012:748572. [CrossRef]
13. Sugimoto N, Miwa S, Hitomi Y, Nakamura H, Tsuchiya H, Yachie A. Theobromine, the primary methylxanthine found in Theobroma cacao, prevents malignant glioblastoma proliferation by negatively regulating phosphodiesterase-4, extracellular signal-regulated kinase, Akt/mammalian target of rapamycin kinase, and nuclear factor-kappa B. Nutr Cancer 2014;66(3):419–23. [CrossRef]
14. Monteiro J, Alves MG, Oliveira PF, Silva BM. Pharmacological potential of methylxanthines: Retrospective analysis and future expectations. Crit Rev Food Sci Nutr 2019;59(16):2597–625. [CrossRef]
15. Azam S, Hadi N, Khan NU, Hadi SM. Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. Med Sci Monit 2003;9(9):BR325–30.
16. Fuggetta MP, Zonfrillo M, Villivà C, Bonmassar E, Ravagnan G. Inflammatory microenvironment and adipogenic differentiation in obesity: The inhibitory effect of theobromine in a model of human obesity *in vitro*. Mediators Inflamm 2019;2019:1515621. [CrossRef]
17. Yoneda M, Sugimoto N, Katakura M, Matsuzaki K, Tanigami H, Yachie A, et al. Theobromine up-regulates cerebral brain-derived neurotrophic factor and facilitates motor learning in mice. J Nutr Biochem 2017;39:110–16. [CrossRef]
18. Ahmed MB, Alghamdi AAA, Islam SU, Lee JS, Lee YS. cAMP signaling in cancer: A PKA-CREB and EPAC-centric approach. Cells 2022;11(13):2020. [CrossRef]
19. Cadoná FC, Machado AK, Azzolin VF, Barbisan F, Dornelles EB, Glanzner W, et al. Guaraná a caffeine-rich food increases oxaliplatin sensitivity of colorectal HT-29 cells by apoptosis pathway modulation. Anticancer Agents Med Chem 2016;16(8):1055–65. [CrossRef]
20. Skopińska-Różewska E, Sommer E, Demkow U, Chorostowska-Wynimko J, Bałan B, Rózycka B, et al. Screening of angiogenesis inhibitors by modified tumor-induced angiogenesis (TIA) test in lung cancer. Rocz Akad Med Bialymst 1997;42(Suppl 1):287–96.
21. Barcz E, Sommer E, Sokolnicka I, Gawrychowski K, Roszkowska-Purska K, Janik P, et al. The influence of theobromine on angiogenic activity and proangiogenic cytokines production of human ovarian cancer cells. Oncol Rep 1998;5(2):517–20. [CrossRef]
22. Środa-Pomianek K, Michalak K, Palko-Łabuz A, Poła A, Dzięgiel P, Puła B, et al. Cytotoxic and multidrug resistance reversal activity of phenothiazine derivative is strongly enhanced by theobromine, a phytochemical from cocoa. Eur J Pharmacol 2019;849:124–34. [CrossRef]
23. Lentini A, Kleinman HK, Mattioli P, Autuori-Pezzoli V, Nicolini L, Pietrini A, et al. Inhibition of melanoma pulmonary metastasis by methylxanthines due to decreased invasion and proliferation. Melanoma Res 1998;8(2):131–7. [CrossRef]
24. Bałan BJ, Siwicki AK, Pastewka K, Demkow U, Skopiński P, Skopińska-Różewska E, et al. Synergistic activity for natural and synthetic inhibitors of angiogenesis induced by murine sarcoma L-1 and human kidney cancer cells. Adv Exp Med Biol 2017;1020:91–104. [CrossRef]
25. Shim MK, Yoon HY, Lee S, Jo MK, Park J, Kim JH, et al. Caspase-3/-7-specific metabolic precursor for bioorthogonal tracking of tumor apoptosis. Sci Rep 2017;7(1):16635. [CrossRef]
26. Martínez-Pinilla E, Oñatibia-Astibia A, Franco R. The relevance of theobromine for the beneficial effects of cocoa consumption. Front Pharmacol 2015;6:30. [CrossRef]
27. Baggott MJ, Childs E, Hart AB, de Bruin E, Palmer AA, Wilkinson JE, et al. Psychopharmacology of theobromine in healthy volunteers. Psychopharmacology (Berl) 2013;228(1):109–18. [CrossRef]
28. Gil M, Skopińska-Różewska E, Radomska D, Demkow U, Skurzak H, Rochowska M, et al. Effect of purinergic receptor antagonists suramin and theobromine on tumor-induced angiogenesis in BALB/c mice. Folia Biol (Praha) 1993;39(2):63–8.
29. Lee HW, Choi IW, Ha SK. Immunostimulatory activities of theobromine on macrophages via the activation of MAPK and NF-κB signaling pathways. Curr Issues Mol Biol 2022;44(9):4216–28. [CrossRef]
30. Eguchi H, Kimura R, Onuma S, Ito A, Yu Y, Yoshino Y, et al. Elevation of Anticancer drug toxicity by caffeine in spheroid model of human lung adenocarcinoma A549 cells mediated by reduction in Claudin-2 and Nrf2 expression. Int J Mol Sci 2022;23(24):15447. [CrossRef]
31. Shojaei-Zarghani S, Yari Khosroushahi A, Rafraf M. Oncopreventive effects of theanine and theobromine on dimethylhydrazine-induced colon cancer model. Biomed Pharmacother 2021;134:111140. [CrossRef]
32. Eissa IH, Yousef RG, Elkady H, Elkaeed EB, Alsfouk AA, Husein DZ, et al. Identification of new theobromine-based derivatives as potent VEGFR-2 inhibitors: Design, semi-synthesis, biological evaluation, and in silico studies. RSC Adv 2023;13(33):23285–307. [CrossRef]

33. Eissa IH, Yousef RG, Elkady H, Elkaeed EB, Alsfouk AA, Husein DZ, et al. Design, semi-synthesis, anti-cancer assessment, docking, MD simulation, and DFT studies of novel theobromine-based derivatives as VEGFR-2 inhibitors and apoptosis inducers. Comput Biol Chem 2023;107:107953. [CrossRef]
34. Geraets L, Moonen HJ, Wouters EF, Bast A, Hageman GJ. Caffeine metabolites are inhibitors of the nuclear enzyme poly(ADP-ribose)polymerase-1 at physiological concentrations. Biochem Pharmacol 2006;72(7):902–10. [CrossRef]
35. Jang YJ, Koo HJ, Sohn EH, Kang SC, Rhee DK, Pyo S. Theobromine inhibits differentiation of 3T3-L1 cells during the early stage of adipogenesis via AMPK and MAPK signaling pathways. Food Funct 2015;6(7):2365–74. [CrossRef]
36. He Z, Ma WY, Hashimoto T, Bode AM, Yang CS, Dong Z. Induction of apoptosis by caffeine is mediated by the p53, Bax, and caspase 3 pathways. Cancer Res 2003;63(15):4396–401.
37. Ito K, Nakazato T, Miyakawa Y, Yamato K, Ikeda Y, Kizaki M. Caffeine induces G2/M arrest and apoptosis via a novel p53-dependent pathway in NB4 promyelocytic leukemia cells. J Cell Physiol 2003;196(2):276–83. [CrossRef]
38. Qi W, Qiao D, Martinez JD. Caffeine induces TP53-independent G(1)-phase arrest and apoptosis in human lung tumor cells in a dose-dependent manner. Radiat Res 2002;157(2):166–74. [CrossRef]
39. Igarashi K, Yamamoto N, Hayashi K, Takeuchi A, Kimura H, Miwa S, et al. Non-toxic efficacy of the combination of caffeine and valproic acid on human osteosarcoma cells *in vitro* and in orthotopic nude-mouse models. Anticancer Res 2016;36(9):4477–82. [CrossRef]
40. Xu H, Wang L, Shi B, Hu L, Gan C, Wang Y, et al. Caffeine inhibits the anticancer activity of paclitaxel via down-regulation of α-tubulin acetylation. Biomed Pharmacother 2020;129:110441. [CrossRef]

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Research Article

# Decreased Elabela level in the first 24 hours of ST Elevation Myocardial Infarction patients

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## Abstract

**Objectives:** Cardiovascular diseases are among the leading causes of death worldwide. ST Elevation Myocardial Infarction (STEMI) is one of the most important causes of cardiovascular mortality and morbidity. It has been determined that Elabela (ELA), a member of the apelinergic system, increases myocardial contractility and coronary vasodilation, and decreases blood pressure. The aim of this study was to evaluate the relationship between circulating ELA levels and various clinical, biochemical, and angiographic parameters in STEMI patients undergoing primary percutaneous coronary intervention (PCI).

**Methods:** Seventy-four patients hospitalized with the diagnosis of STEMI who underwent coronary angiography and primary PCI, and seventy-four patients with chest pain but no pathology detected in coronary angiography, were included in the study as the control group. Coronary lesion severity was measured using the SYNTAX score tool. Routine laboratory tests and ELA levels were measured.

**Results:** Plasma levels of ELA were significantly lower in patients with STEMI (0.68±0.68 ng/mL) than in controls (1.34±0.88 ng/mL, $p<0.001$). Glucose, cholesterol, LDL, CRP, troponin I, and SYNTAX score levels were statistically higher in the STEMI group, while ELA and HDL levels were lower. There was a high level of negative correlation between ELA and troponin I, SYNTAX score, cholesterol, LDL, and CRP.

**Conclusion:** In this study, it was determined that the level of ELA decreased in the first 24 hours of STEMI patients. In addition, a highly negative correlation was found between ELA and troponin I and SYNTAX scores.

**Keywords:** Coronary heart disease, Elabela (ELA), myocardial infarction, primary Percutaneous Coronary Intervention (PCI); ST Elevation Myocardial Infarction (STEMI)

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ST Elevation Myocardial Infarction (STEMI), which is included in Coronary Heart Disease (CHD), is one of the most important causes of cardiovascular mortality and morbidity, especially in developed countries. Although interventional procedures such as Percutaneous Coronary Interventions (PCI) and Coronary Artery Bypass Grafting contribute significantly to the reduction of clinical symptoms and mortality, acute heart failure, cardiogenic shock, and various complications are observed after the procedure. The clinic, comorbidity, echocardiographic findings, and biochemical results of the patients may affect the prognosis of STEMI. New biomarkers are needed for the diagnosis of STEMI patients with high mortality and morbidity risk [1, 2].

Approximately 200 hormones and neuropeptides show their activities via cardiac G protein-coupled receptors (GPCRs), such as endothelin-1 receptor (ET1R), A1 adenosine receptors (A1R), and beta adrenergic receptor kinase 1 (βARK1) [3]. In DNA sequencing studies, receptors that encode GPCR-like sequences but whose ligands are unknown, so-called "orphan GPCRs," have been identified [4]. After the

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discovery of Apelin, which can bind to Angiotensin Type 1 Receptor-Associated Proteins (APJ), defined as orphan GPCRs in 1998, a new endogenous peptide, Elabela (ELA, Toddler, and Apela) was found in the 2000s, which shares the same receptor [5]. APJ, Apelin, and ELA constitute the peptide family called the apelinergic system, and this family has been shown to have antihypertensive, cardiorenal protective, positive inotropic effects and regulate fluid homeostasis, vasodilation, angiogenesis, cellular differentiation, apoptosis, and oxidative stress [5, 6].

ELA has been shown mainly in human stem cells, kidneys, prostate, vascular endothelium, and plasma [7]. ELA has an antagonistic effect on the Renin-Angiotensin-Aldosterone system and lowers blood pressure. It has been determined that the effects of ELA to increase myocardial contractility and coronary vasodilation are stronger than Apelin [6, 8]. In addition, it has been found that the plasma level of ELA decreases in hypertensive patients and that this low level correlates with the risk of having hypertension [7]. Due to the positive inotropic effect of the apelinergic system and its upregulation of the remodeling phase after MI, the apelinergic system has begun to be emphasized in the treatment of cardiovascular diseases, and alternative treatment protocols are being developed over Apelin-Elabela Analogs and APJ agonists [6].

In this study, it was aimed to examine the circulating ELA levels in STEMI patients who underwent primary PCI. Therefore, the relationship between ELA levels and various clinical-biochemical and angiographic parameters in STEMI patients will be evaluated.

## Materials and Methods

### Study population

Patients who applied to the Cardiology Clinic and Emergency Department with the complaint of sudden onset chest pain, had no history of the disease, and had Acute Coronary Syndrome (ACS) findings in ECG changes and underwent coronary angiography were included in the study. The study was conducted in accordance with the Declaration of Helsinki. All patients were informed, and an informed consent form was signed. The study was conducted prospectively between the years 2021–2022. Ethics Committee approval was obtained for the study (No: 15/01/2021-616).

Patients with any chronic disease such as heart failure, cardiac arrhythmia, moderately advanced heart valve disease, chronic kidney disease, rheumatic or inflammatory disease were excluded.

According to the fourth universal definition of MI, patients with symptoms suggestive of ischemia and ST-segment elevation (at least two contiguous leads with ST-segment elevation ≥2.5 mm in men <40 years, ≥2 mm in men ≥40 years, or ≥1.5 mm in women in leads V2–V3 and/or ≥1 mm in the other leads) in at least two adjacent leads on the ECG were considered to have STEMI [9]. As a patient group, 74 patients who applied to the Cardiology Clinic and the Emergency Department with the complaint of chest pain, were diagnosed with Acute STEMI with ≥2 mm ST-segment elevation in any of the two adjacent leads in the first ECG and caused 100% narrowing in one or more coronary arteries in the coronary angiography and who underwent primary PCI due to thrombus were included. The SYNTAX (SYNergy between PCI with TAXUS and Cardiac Surgery) score of the included patients was evaluated [10]. Seventy-four patients who were hospitalized with a prediagnosis of ACS but whose biochemical parameters (Troponin I) were negative, coronary angiography was normal and the diagnosis of ACS was excluded because of no specific findings on ECG were included as the control group.

### Biochemical measurements

A second blood sample was taken for the ELA test and delivered to the laboratory during routine blood collection from patients who applied to the emergency clinic and had changes in their ECG and were referred for coronary angiography. These procedures were carried out within the first 6 hours. Clinical Chemistry Tests and CRP Spectrophotometric in ARCHITECT c16000 (Abbott Laboratories, USA) autoanalyzer, Hemogram measurements on Mindray BC 6800 (Mindray Building, High-Tech Industrial Park, Nanshan, Shenzhen China) device, HbA1c measurements in HA-8180 (ARKRAY, Inc. JAPAN) device by HPLC (High-Performance Liquid Chromatography) method, Serum Thyroid Stimulating Hormone (TSH), free T3, free T4, Folate, Vitamin B12, D Vitamin, Troponin I and Procalcitonin levels were studied with the electrochemiluminescence method in the Cobas e601 (Roche Diagnostics, Germany) device. For the ELA test, the blood was taken into standard clinical chemistry test tubes, centrifuged at 1500 g for 20 minutes, and the serum was separated and stored at -40°C. ELA test was performed with enzyme-linked immunosorbent assay (Human Elabela ELISA kit) method. Serum ELA levels were studied in accordance with the kit package insert (Sunred Biological Technology, Shanghai, China), and measurements were made in the Grifols Triturus Automated ELISA Analyzer device (Grifols Triturus, Grifols, S.A., Barcelona, Spain).

### Statistical analysis

For the statistical analysis of the data obtained in the study, SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) for Windows package program running on Windows was used. Data are presented as percentage (%), mean ± standard deviation (SD), median, interquartile range (IQR), correlation coefficient (r).

The conformity of the variables to the normal distribution was examined with the Kolmogorov-Smirnov test. Mean and standard deviation were used for normally distributed variables. The student t-test was used to compare the variables in normal distribution between the two groups, and the Mann-Whitney U-test was used for variables that did not fit the normal distribution. The Chi-square test was used to compare categorical variables, and the relationship between numerical variables was evaluated using Spearman's Correlation Analysis. $P<0.05$ was considered statistically significant.

**Table 1. Comparison of the baseline clinical and laboratory parameters**

| | STEMI n=74 | Controls n=74 | p |
|---|---|---|---|
| Age (years), median (IQR) | 54 (13) | 44 (9) | 0.001*** |
| Gender (n, %) | | | |
| Male | 59 (67.8) | 28 (32.2) | 0.001*** |
| Female | 15 (24.6) | 46 (75.4) | |
| Hypertension (n, %) | 10 (13.51) | 0 (0) | 0.001*** |
| Diabetes mellitus (n, %) | 9 (12.16) | 0 (0) | 0.003** |
| Glucose (mg/dL) median (IQR) | 96 (12) | 91 (18) | 0.003** |
| Urea (mg/dL) median (IQR) | 30 (12) | 24 (6) | 0.001*** |
| Creatinine (mg/dL) median (IQR) | 0.846 (0.28) | 0.72 (017) | 0.001*** |
| eGFR (mL/dk/1.73m$^2$) median (IQR) | 90 (2.75) | 90 | 0.001*** |
| Albumin (g/L) median (IQR) | 42 (5.45) | 46 (4) | 0.001*** |
| Total cholesterol (mg/dL) median (IQR) | 180 (53) | 151 (37.25) | 0.001*** |
| LDL-C (mg/dL) median (IQR) | 118 (42.25) | 89.5 (26) | 0.001*** |
| HDL-C (mg/dL) | 39.72±8.50 | 42.9±8.60 | 0.023* |
| TG (mg/dL), median (IQR) | 94 (85) | 82 (67.5) | 0.093 |
| Ca (mg/dL) | 9.05±0.41 | 9.68±0.39 | 0.001*** |
| Na (mmol/L) median (IQR) | 137 (3) | 139 (2) | 0.001*** |
| K (mmol/L) | 4.13±0.36 | 4.38±0.36 | 0.001*** |
| AST (U/L) median (IQR) | 52.5 (80) | 16.5 (6) | 0.001*** |
| ALT (U/L) median (IQR) | 26.5 (27) | 14 (11) | 0.001*** |
| LDH (U/L) median (IQR) | 351 (378) | 191 (54) | 0.001*** |
| WBC (10$^3$/uL) median (IQR) | 14.01 (5.16) | 7.33 (1.75) | 0.001*** |
| Hemoglobin (g/dL) | 14.67±1.80 | 14.13±1.69 | 0.062 |
| HCT (%) | 45.22±4.46 | 43.87±3.97 | 0.054 |
| PLT (10$^3$/uL) | 256±64 | 273±54 | 0.077 |
| TSH (mU/L) median (IQR) | 1.18 (1.26) | 1.67 (1.07) | 0.01** |
| T3 (pg/mL) | 3.17±0.65 | 3.64±0.43 | 0.001*** |
| T4 (ng/dL) median (IQR) | 1.29 (0.31) | 1.23 (0.24) | 0.142 |
| B 12 (ng/L) median (IQR) | 303 (134) | 308 (177) | 0.043* |
| Folat (ng/mL) median (IQR) | 5.7 (3.15) | 7.2 (3.95) | 0.001*** |
| D Vitamine (ng/mL) median (IQR) | 11.4 (5.35) | 10.3 (5.28) | 0.135 |
| HbA1c (%) median (IQR) | 5.6 (0.45) | 5.3 (0.2) | 0.001*** |
| CRP (mg/L) median (IQR) | 4.5 (4.33) | 2 | 0.001*** |
| PCT (ng/mL) | 0.24±0.06 | 0.28±0.05 | 0.001*** |
| Troponin I (ng/mL) median (IQR) | 12.92 (21.34) | 0.1 | 0.001*** |
| ELA (ng/mL) median (IQR) | 0.45 (0.4) | 0.88 (1.88) | 0.001*** |
| SYNTAX median (IQR) | 20.5 (7.75) | 0 | 0.001*** |

Data are presented as mean±SD or n (%), median. IQR. *: p<0.05 versus controls; **: p<0.01 versus controls; ***: p<0.001. IQR: Interquartile range; eGFR: Estimated glomerular filtration rate; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; TG: Triglyceride; Ca: Calcium; Na: Sodium; K: Potassium; AST: Aspartate aminotransferase; ALT: Alanine aminotransfease; LDH: Lactate dehydrogenase; WBC: White blood cell; HCT: Hematocrit; PLT: Platelet; TSH: Thyroid Stimulating Hormone; HbA1c: Hemoglobin A1c; CRP: C-Reactive protein; PCT: Procalcitonin; ELA: Elabela; SYNTAX: SYNergy between PCI with TAXUS and cardiac surgery.

## Results

Our study consists of 74 STEMI patient groups and 74 control groups. In demographic comparison, the mean age of the group with STEMI was higher (54.42±9.99), and the ratio of men 59 (67.8%) was higher than women 15 (24.5%). The frequency of DM (diabetes mellitus) and HT (hypertension) was higher in the STEMI group than in the control group. WBC (white blood cell), glucose, urea, creatinine, TC (total cholesterol), LDL-C (low-density lipoprotein cholesterol), CRP (C-reactive protein), Troponin I, HbA1c, and SYNTAX score levels were statistically higher in the STEMI group (p<0.001); ELA, TSH, B12, folate, fT3, PCT (Procalcitonin), Ca, K, HDL-C (high-density lipoprotein cholesterol) levels were significantly lower (Table 1). There was a negative correlation between ELA and age, gender, creatinine, TC, LDL-C, WBC, HbA1c, CRP, Troponin I, SYNTAX score, and a positive

**Table 2. Correlation between ELA and study variables in all subjects**

| | r | p |
|---|---|---|
| Age (years) | -0.183 | 0.026 |
| Gender | -0.167 | 0.042 |
| Diabetes mellitus | 0.185 | 0.025 |
| Creatinine (mg/dL) | -0.166 | 0.044 |
| eGFR (mL/dk/1.73m$^2$) | 0.019 | 0.019 |
| Total cholesterol (mg/dL) | -0.215 | 0.009 |
| LDL-C (mg/dL) | -0.254 | 0.002 |
| Ca (mg/dL) | 0.217 | 0.008 |
| K (mmol/L) | 0.199 | 0.015 |
| WBC ($10^3$/uL) | -0.341 | <0.001 |
| TSH (mU/L) | 0.181 | 0.027 |
| fT3 (pg/mL) | 0.177 | 0.031 |
| Folat (ng/mL) | 0.195 | 0.017 |
| HbA1c (%) | -0.327 | <0.001 |
| CRP (mg/L) | -0.341 | <0.001 |
| Troponin I (ng/mL) | -0.412 | <0.001 |
| SYNTAX | -0.417 | <0.001 |

ELA: Elabela; eGFR: Estimated glomerular filtration rate; LDL-C: Low density lipoprotein cholesterol; Ca: Calcium; K: Potassium; WBC: White blood cell count; TSH: Thyroid stimulating hormone; CRP: C-reactive protein; SYNTAX: SYNergy between PCI with TAXUS and cardiac surgery.

correlation between DM, eGFR (estimated glomerular filtration rate- calculated with CKD-EPI formula), Ca, K, TSH, fT3, and folate correlation was detected (Table 2).

ELA levels were significantly lower in patients with STEMI (0.68±0.68 ng/mL/0.45 (0.4) median (IQR)) than controls (1.34±0.88 ng/mL/0.88 (1.88) median (IQR), p<0.001) (Table 1, Fig. 1).

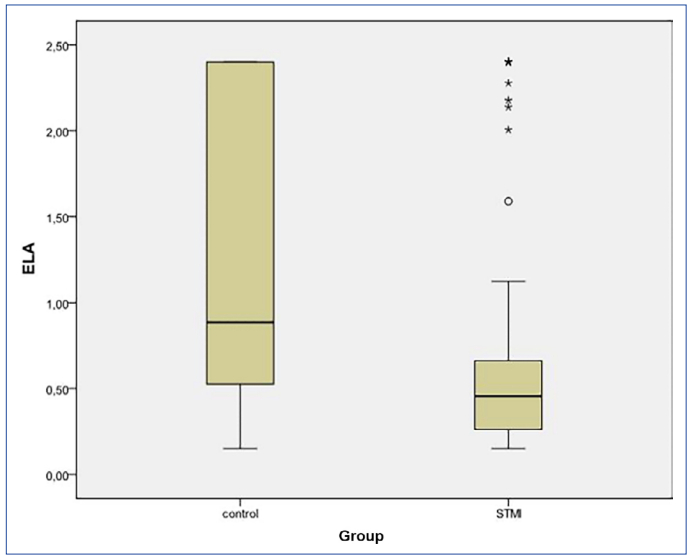


**Figure 1.** ELA levels on control and STMI groups.
ELA: Elabela; STMI: ST Elevation myocardial infarction.

## Discussion

The main finding of our study is that it is the first study showing a statistically significant decrease in ELA levels in the first 24 hours in patients with STEMI. In addition, a negative correlation was found between ELA and Troponin I and SYNTAX scores in the study (p<0.001).

Cardiovascular Diseases (CVD) account for 31% of deaths worldwide, and these diseases need to be diagnosed quickly, and treatment should be started [11]. The research that started with the discovery of APJ has revealed the importance of the regulatory role of Apelin and ELA on the cardiovascular system and fluid electrolyte homeostasis. ELA works like a hormone due to its expression in pluripotent stem cells and especially in kidneys and its homeostatic and cardioprotective effects on blood pressure-fluid-electrolyte balance [12]. Many studies have been done on cardiovascular, endocrine, and tumor-related diseases on ELA, as well as comparative studies with Apelin [13]. It has been observed that ELA plays an important role in ischemic cardiovascular diseases by activating APJ earlier than Apelin and stimulating angiogenesis [12, 13].

ELA affects intracellular signaling through APJ activation, resulting in a decrease in cAMP levels, stimulation of ERK, and an increase in intracellular calcium mobilization. Wang et al. [12] have shown that ELA induces a relaxation independent of the endothelium by determining that while ELA caused 73.7% relaxation in vessels with an intact endothelium, the relaxation in vessels with stripped endothelium was only 20% less in their study where they used mouse aortic vessels with intact endothelium and stripped endothelium. It was also examined whether this relaxation is NO dependent or not, and it was determined that ELA does not need NO mediation for relaxation. In the study, the effect of Apelin on vessels was also examined in parallel, and they determined that while Apelin caused 79% relaxation in vessels with intact endothelium, the relaxation in vessels with stripped endothelium was 48% to 31% less, and they determined that Apelin was more endothelial-dependent than ELA for vascular relaxation. Therefore, although they act through the APJ, there is a difference between ELA and Apelin in terms of vasodilation mechanisms.

Years of studies have shown that the apelinergic system is a good homeostasis regulator through the cardiovascular system and water-electrolyte balance. Detection of ELA, especially in the kidney, showed that it has an important function in maintaining fluid homeostasis and blood pressure together with the heart in RT-PCR analyzes [12]. In our study, in parallel with this information, a negative correlation was found between ELA and creatinine and a positive correlation with eGFR (p<0.05). It is predicted that ELA, which acts as a paracrine or endocrine hormone, can be used as a good diuretic agent. In addition, over the APJ axis; while Apelin activates the ACE2 promoter activity, ELA decreases the ACE promoter activity in a dose-dependent manner, thus neutralizing the negative effects of RAS and showing a cardioprotective effect [5].

Dönmez et al. [8] found that ELA levels increased, but this was not statistically significant in their study on STEMI patients, and found a moderately positive correlation between ELA and Troponin I, NT-ProBNP, but no correlation between Hs-CRP. They also found a moderate negative correlation between ELA and left ventricular ejection fraction (LVEF). Aydın et al. [14] found an increase in ELA and Apelin levels in the blood after MI in their study. It is thought that blood levels increase due to the release of ELA and Apelin into the circulation by damaged cardiomyocytes after ischemia. They also suggested that ELA and Apelin could be new indicators for clinically determining the severity of MI and for the diagnosis of MI. Yavuz et al. [15] on the other hand, found that the ELA levels of patients with Chronic Complete Occlusion in their coronary arteries were lower than the control group. They also stated that ELA levels of patients with good collateral development were higher than patients with low collateral development, and this was due to the positive effects of ELA on angiogenesis and arteriogenesis. Similar to the study of Yavuz et al. [15], in our study, ELA levels (0.68±0.68 ng/mL/0.45 (0.4)) in patients with STEMI were found to be lower than the control (1.34±0.88 ng/mL/0.88 (1.88), p<0.001) group. While ELA is detected in fibroblasts and intact endothelial cells in the heart, ELA production decreases in impaired endothelial function. This may be attributed to the decrease in ELA production due to impaired vascular endothelium in the acute phase of MI in STEMI patients who already show signs of endothelial dysfunction (HT, DM, high lipid profile, CRP, glucose, and low HDL-C). In addition, in our study, contrary to the findings of Dönmez et al. [8] a high level of negative correlation was found between ELA and CRP and Troponin I levels in the STEMI group (p<0.001).

Du et al. [16] found that ELA levels in patients with the ACS were significantly higher than in the control group (95.04±18.66 vs 71.90±8.93, p<0.01). Although they did not report a difference between the ELA level and the number of narrowed coronary arteries, they reported that the SYNTAX I score increased with the increase in ELA levels between 63.75 ng/mL and 85.49 ng/mL. In the study conducted by Tian et al. [17] with CHD, they divided into 3 groups as stable angina (SA), unstable angina (UAP), and acute myocardial infarction (AMI), ELA levels in patients with CHD were found to be 10.71% higher (p<0.05) compared to controls, and it was higher in the patients in the UAP and AMI subgroups than in the controls and SA subgroup (p<0.05). However, they could not detect a correlation between ELA concentration and SYNTAX score, LVEF, and other biochemical parameters in coronary heart patients. In our study, however, a high level of negative correlation was found between the SYNTAX score and ELA (p<0.001).

Myocardial cell necrosis can induce an increased inflammatory response and elevated CRP levels, and experimental studies suggest that inhibiting CRP in AMI may reduce myocardial damage [18]. In an experimental study by Rakhshan et al. [19] ELA peptide was administered intraperitoneally to rats with MI, and the development of infarction and myocardial necrosis after reperfusion were examined. They showed that the ELA peptide significantly reduced markers of myocardial damage, such as CK-MB and Troponin I, in the treatment groups. They also found an increase in LVIDd (Left Ventricular Internal Dimension At End-diastole) and LVIDs (Left Ventricular Internal Dimension At End-systole), and EF (Ejection Fraction) and FS (Fractional Shortening) decreased myocardial cell damage and improved cardiac function. In their study, Xi et al. [20] developed Fc-ELA-21 (longer half-life ELA analogue); found that this ELA peptide, which they infused continuously sc daily, significantly improved cardiac dysfunction by increasing angiogenesis and cardiomyocyte proliferation, reducing myocardial fibrosis and apoptosis in MI-induced rats. In our study, Troponin I and CRP levels, which are the ideal indicators of myocardial damage and inflammation, were found to be statistically higher and ELA levels lower in the STEMI group, and a high level of negative correlation was found between them and ELA (p<0.001). In addition, we found a positive correlation (p<0.001) between CRP and TC, LDL-C, and Troponin I, and a negative correlation (p<0.001) with ELA, which supports the view that inhibiting CRP can reduce myocardial damage. As a result of clinical studies to be conducted by administering ELA analogue to AMI patients in the future, the status of myocardial damage, inflammation markers (CRP, CK-MB, Troponin I, etc.) and necrosis area size can be determined.

Known risk factors for the development of AMI include high LDL-C and TG levels. It is known that LDL-C causes inflammation and formation of atherosclerotic plaques in the endothelium [21]. In addition, CRP is also stated to be a mechanical mediator of myocardial damage after STEMI, in addition to its involvement in cardiovascular risk factors [18]. In our study, TC, LDL-C, and CRP levels were found to be higher and HDL-C levels to be lower in patients with STEMI (p<0.001). Our negative correlation (p<0.01) between TC, LDL-C, CRP and ELA, which are risk factors for AMI, supports the prediction that ELA deficiency may be a risk factor for CVD.

### Study limitations

The main limitation of our study is that it is single-centered, and the sample size is relatively small. In addition, ELA levels were measured only in STEMI patients and in the first 6 hours of the study. We believe that it would be more meaningful to perform both STEMI and non-STEMI MI type and sequential measurements in larger patient groups to determine the course of ELA.

## Conclusion

In this study, it was determined that the level of ELA decreased in the first 6 hours of STEMI patients (compared to the control group). In addition, a highly negative correlation was found between ELA and Troponin I and SYNTAX scores.

ELA is expected to be a new biomarker for CVD and an effective drug in treatment. Therefore, it is important to comprehensively investigate how ELA affects CVD and its contribution to treatment.

**Ethics Committee Approval:** The study was approved by The University of Health Sciences Gazi Yasargil Training and Research Hospital Clinical Research Ethics Committee (No: 616, Date: 15/01/2021).

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## References

1. Liu HT, Chen M, Yu J, Li WJ, Tao L, Li Y, et al. Serum apelin level predicts the major adverse cardiac events in patients with ST elevation myocardial infarction receiving percutaneous coronary intervention. Medicine (Baltimore) 2015;94(4):e449. [CrossRef]
2. Barchielli A, Santoro GM, Balzi D, Carrabba N, Di Bari M, Gensini GF, et al. Long-term prognosis after primary PCI in unselected patients with ST-elevation myocardial infarction. J Cardiovasc Med (Hagerstown) 2012;13(12):819–27. [CrossRef]
3. Salazar NC, Chen J, Rockman HA. Cardiac GPCRs: GPCR signaling in healthy and failing hearts. Biochim Biophys Acta 2007;1768(4):1006–18. [CrossRef]
4. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochem Biophys Res Commun 1998;251(2):471–6. [CrossRef]
5. Ma Z, Song JJ, Martin S, Yang XC, Zhong JC. The Elabela-APJ axis: A promising therapeutic target for heart failure. Heart Fail Rev 2021;26(5):1249–58. [CrossRef]
6. Şahintürk S, İşbil N. Apelinergic system and myocardial contractility. J Uludağ Univ Med Fac [Article in Turkish] 2020;46(1):129–34. [CrossRef]
7. Ma Z, Zhao L, Zhang YP, Zhong JC, Yang XC. Declined ELABELA plasma levels in hypertension patients with atrial fibrillation: A case control study. BMC Cardiovasc Disord 2021;21(1):390. [CrossRef]
8. Dönmez Y, Acele A. Increased Elabela levels in the acute ST segment elevation myocardial infarction patients. Medicine (Baltimore) 2019;98(43):e17645. [CrossRef]
9. Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H, et al; ESC Scientific Document Group. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: The Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). Eur Heart J 2018;39(2):119–77.
10. Sianos G, Morel MA, Kappetein AP, Morice MC, Colombo A, Dawkins K, et al. The SYNTAX Score: An angiographic tool grading the complexity of coronary artery disease. EuroIntervention 2005;1(2):219–27.
11. Zhou S, Wang J, Wang Q, Meng Z, Peng J, Song W, et al. Essential role of the ELABELA-APJ signaling pathway in cardiovascular system development and diseases. J Cardiovasc Pharmacol 2020;75(4):284–91. [CrossRef]
12. Wang Z, Yu D, Wang M, Wang Q, Kouznetsova J, Yang R, et al. Elabela-apelin receptor signaling pathway is functional in mammalian systems. Sci Rep 2015;5:8170. [CrossRef]
13. Zhou M, Wu Y. Effects and signaling pathways of Elabela in the cardiovascular system. Peptides 2022;147:170674. [CrossRef]
14. Aydin S, Kuloglu T, Aydin Y, Yalcin MH, Ugur K, Albayrak S, et al. Effects of iloprost and sildenafil treatment on elabela, apelin-13, nitric oxide, and total antioxidant and total oxidant status in experimental enzyme-positive acute coronary syndrome in rats. Biotech Histochem 2020;95(2):145–51. [CrossRef]
15. Yavuz F, Kaplan M. Association between serum elabela levels and chronic totally occlusion in patients with stable angina pectoris. Arq Bras Cardiol 2021;117(3):503–10.
16. Du SL, Yang XC, Zhong JC, Wang LF, Fan YF. Plasma levels of Elabela are associated with coronary angiographic severity in patients with acute coronary syndrome. J Geriatr Cardiol 2020;17(11):674–9.
17. Tian QP, Liu ML, Zhang YR, Ji DR, Liu SM, Zhao J, et al. Plasma Level of elabela in patients with coronary heart disease and ıts correlation with the disease classification. Int Heart J 2021;62(4):752–5. [CrossRef]
18. Holzknecht M, Tiller C, Reindl M, Lechner I, Troger F, Hosp M, et al. C-reactive protein velocity predicts microvascular pathology after acute ST-elevation myocardial infarction. Int J Cardiol 2021;338:30–6. [CrossRef]
19. Rakhshan K, Azizi, Y, Naderi N, Afousi AG, Aboutaleb N. ELABELA (ELA) peptide exerts cardioprotection against myocardial infarction by targeting oxidative stress and the improvement of heart function. Int J Pept Res Ther 2019;25:613–21. [CrossRef]
20. Xi Y, Yu D, Yang R, Zhao Q, Wang J, Zhang H, et al. Recombinant Fc-Elabela fusion protein has extended plasma half-life and-mitigates post-infarct heart dysfunction in rats. Int J Cardiol 2019;292:180–7. [CrossRef]
21. Sia CH, Zheng H, Ho AF, Bulluck H, Chong J, Foo D, et al. The Lipid Paradox is present in ST-elevation but not in non-ST-elevation myocardial infarction patients: Insights from the Singapore Myocardial Infarction Registry. Sci Rep 2020;10(1):6799. [CrossRef]

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Research Article

# Effects of soybean seed on some biochemical parameters and pancreas weight in streptozotocin-induced diabetic rats

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## Abstract

**Objectives:** Diabetes mellitus is a group of chronic diseases in which the body has problems with the use or production of glucose, the main source of energy for metabolism. This condition can affect the metabolism of carbohydrates, fats, and proteins, potentially leading to chronic hyperglycemia (high blood sugar levels). The present study investigated the effects of soybean (Glycine max L.) seed extract on various biochemical parameters and pancreas weight in Wistar rats with experimental diabetes.

**Methods:** The study consisted of five groups of rats: control, diabetic, and treated (100, 200, and 400 mg/kg extract). To induce diabetes in rats, Streptozotocin (STZ) was administered through intraperitoneal injection (35 mg/kg). The rats were orally given water-soluble extracts at the indicated quantities once daily for four weeks. Venous blood was collected from the animals via heart puncture, after which the animals were sacrificed. Biochemical parameters, including blood glucose, were measured using an autoanalyzer.

**Results:** In diabetic rats, there was a significant increase in serum glucose concentration ($p<0.05$). However, treatment with soybean resulted in a significant reduction ($p<0.05$) of the elevated glucose concentration in the treated diabetic rats. It is noteworthy that the glucose concentrations were still significantly higher ($p<0.05$) than those of the control group. Biochemical parameters, including urea, AST, SGPT/ALT, cholesterol, triglycerides, glucose, LDL-cholesterol, and VLDL-cholesterol, were lower in the treated groups than in the diabetic control group and were considered significant ($p<0.05$). Moreover, it was observed that the HDL-cholesterol parameter was significantly higher than that of the diabetic control group ($p<0.05$), while the creatinine parameter was not found to be statistically significant ($p>0.05$).

**Conclusion:** The results suggest that soybean treatment may have a positive impact on certain biochemical parameters in diabetic rats. These findings are consistent with previous reports on the health benefits of soybean. It can be inferred that G. max may have a hypoglycemic effect in diabetic rats and could potentially mitigate the complications of diabetes mellitus.

**Keywords:** Biochemical parameters, diabetic rats, Glycine max (L), soybean seed

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Diabetes is a disease caused by lack of insulin secretion, insulin resistance, or both [1, 2]. It has been observed that disorders related to the metabolism of carbohydrates, fats, and proteins may arise due to either insufficient insulin secretion or decreased tissue sensitivity to insulin. As a result, chronic hyperglycemia is a commonly observed symptom [2]. Diabetes is a disease with acute and chronic complications. Indeed, in long-term complications, functional disorders or de-

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ficiencies are observed in organs such as eyes, heart, kidney, and brain [3]. Indeed, it is also associated with cardiovascular disorders resulting from changes in plasma lipid and lipoprotein profile [4].

Two types exist: type I diabetes mellitus, which requires insulin for treatment, and type II diabetes mellitus, which can be managed without insulin. The first is an autoimmune disorder resulting from the selective destruction of insulin-secreting beta (β) cells. The second is peripheral insulin resistance and impaired insulin secretion [5].

Diabetes mellitus is a very common disease affecting people living in developed and developing countries. WHO reports that the disease is a leading cause of death [1]. Within the next 25 years, it is predicted to become one of the world's leading causes of death [3].

Diabetes treatment is a global problem and an effective treatment method without side effects has not been developed yet [1, 6]. Over the past few years, plant-based drugs have become more preferred due to their fewer side effects and natural origin when compared to synthetic chemical drugs [1, 2, 7]. According to the literature, there are more than 400 medicinal plants used worldwide to treat diabetes mellitus. However, only a select few have undergone scientific authentication for their hypoglycemic activity [8, 9].

Soybean (Glycine max) is an annual plant that is commonly cultivated in many parts of the world and belongs to the Fabaceae family [10]. Millions of people take soybean products because of their high nutritional value. Soybean is high in protein, fat, and minerals like phosphorus, calcium, iron, and soluble fiber. It is therefore beneficial for diabetics and reduces the risk of developing diabetes. Thanks to the flavonoids it contains, it has also been reported to decrease the risk of cardiovascular problems by reducing triglycerides, blood cholesterol, low-density lipoprotein (LDL), and blood pressure [1, 9, 11].

Streptozotocin (STZ) is a kind of compound obtained from Streptomyces achromogenes bacteria and is toxic to beta cells producing pancreatic insulin, especially in mammals. It is used to initiate diabetes mellitus in experimental animals [12].

The aim of the study was to investigate the effects of soybean (Glycine max L.) seed extract on pancreatic weight and various biochemical indices, including urea, creatinine, AST, ALT, cholesterol, triglycerides, glucose, LDL, HDL, and VLDL, in Wistar rats with experimental diabetes.

## Materials and Methods

This research has been accepted by The Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (Resolution number: 2015/67).

### Plant material

Soybean seeds used in the experiment were obtained from a market in Konya, Turkey, and confirmed by Selçuk University Agriculture Faculty. Soybean seed extract was prepared with some modifications according to the method suggested by Badavi et al. [13]. Firstly, the seeds were powdered before use. The seed powder was soaked in 60% methanol for three days at room temperature and stirred three times daily. Subsequently, the mixture was filtered using a few layers of cloth, and the resulting filtrate was dried at room temperature (25–30°C) to evaporate the methanol, resulting in soybean extract in powder form. The extract was dissolved in distilled water according to treatment group doses and administered orally via gavage needle once a day.

### Animals

Twenty-five female Wistar rats weighing 210±10 g were obtained from KONÜDAM Animal Center in Necmettin Erbakan University in Konya, Turkey. The rats were accommodated in a controlled environment with a temperature of 22±1 ºC and 62% humidity, following a 12-hour light/12-hour dark schedule. An adaptation period of 15 days was allowed before the commencement of the experiment. For a period of 15 days, they were provided with water ad libitum and fed a basal diet.

### Experimental inducement of diabetes mellitus

Animals in all diabetic groups in the study (Group 2, 3, 4, 5) were fed a high-fat diet for 2 weeks [14]. Then, diabetes was induced in these animals, which had been fasted, with a single intraperitoneal injection of streptozotocin (STZ) (35 mg/kg body weight) dissolved in citrate buffer (pH 4.5). The high-fat diet was continued until the end of the experiment in these animals. Two weeks after STZ treatment, non-fasting rats with a blood glucose level of 300 mg/dL were diagnosed with type 2 diabetes. The chemicals were purchased from Sigma (MO, USA). Also, animals in the control group (Group 1) continued to be fed a basal diet.

### Experimental design and treatment

Rats were separated into five groups of 5 rats each. Group 1: Rats of the control group. Group 2: Control rats with diabetes. Group 3: Diabetic rats treated with 100 mg/kg dose of soybean extract. Group 4: Diabetic rats treated with soybean extract at a dose of 200 mg/kg. Group 5: Diabetic rats treated with 400 mg/kg dose of soybean extract. All treatments were given once a day for four weeks. When finished, the animals were euthanised and the next procedure was started.

### Biochemical analysis

Using a sterile syringe, blood samples were taken by cardiac puncture. The samples were then placed in the appropriate sterilized microcentrifuge tube and allowed to clot. Serum was recovered after centrifugation at 3000 rpm at 25°C for 15 minutes. Serum samples were immediately assayed for blood glucose, total cholesterol, HDL, LDL, VLDL, triglycerides, urea, creatinine, aspartate transaminase (AST), and alanine transaminase (ALT) using commercially available colorimetric diagnostic kits on an autoanalyzer (Biotecnica Instruments,

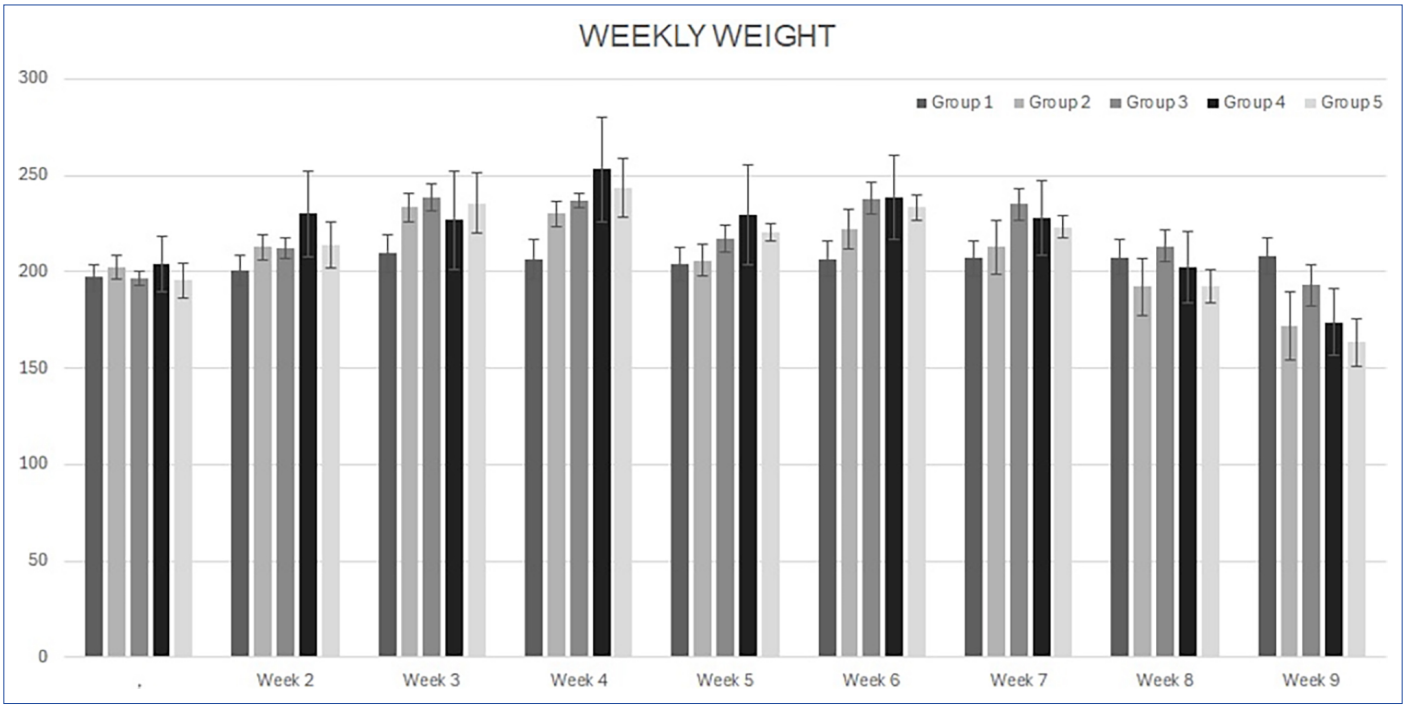


**Figure 1.** The average weight changes of the experimental animals.

BT3000 Plus, Italy).

### Determination of pancreas weights

After euthanasia, their pancreas was carefully removed, their weight was measured and noted.

### Statistical analysis

Results are expressed as mean±SD. One-way repeated measures analysis of variance (ANOVA) with Tukey's post hoc test was used to analyse differences between groups. The Statistical Package of Social Service (SPSS, version 22.0) was used for statistical analyses. A p-value of less than 0.05 was defined as statistically significant.

## Results

### Weight change results

The weights of the experimental animals used in the study were monitored weekly throughout the experiment, and the average weight changes are summarized in Figure 1. While there was not much difference in the average weight gain of the animals in the control group, there was a significant increase in the weights of the other groups until STZ application. It was determined that their weight decreased significantly in the week after STZ application ($p \leq 0.05$).

### Biochemical analysis results

Table 1 shows mean blood glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, triglyceride, urea, creatinine, aspartate aminotransferase (AST), and aminotransferase (ALT) in each of the five rat groups. Compared to the control group, the serum glucose concentration was significantly increased in the diabetic control group ($p<0.05$). This is an indication of an efficient induction of diabetes. Treatment with soybean significantly reduced glucose levels in the treated rats ($p<0.05$), but glucose levels were still significantly higher ($p<0.05$) than in the controls.

A statistically significant increase in serum cholesterol and serum triglycerides ($p<0.05$) was observed in the diabetic control group compared to the control group. After four weeks of treatment with soybeans, a reduction in cholesterol levels was noted, and surprisingly, the greatest reduction was seen at the dose of 200 mg/kg extract. Serum triglyceride levels were also observed to be very low ($p<0.05$) after soy treatment compared to the control group, which was very high in the diabetic control group.

LDL-cholesterol and VLDL-cholesterol levels were significantly increased ($p<0.05$), while HDL-cholesterol was decreased ($p<0.05$) in diabetic rats compared to controls. LDL cholesterol and VLDL cholesterol were significantly reduced, whereas HDL cholesterol was significantly increased ($p<0.05$) in diabetic rats treated with soybean for four weeks. However, this increase in the level of HDL cholesterol was not as great as the increase in the control group.

The parameters of ALT, AST, and Urea were significantly increased in the serum of diabetic groups ($p<0.05$). These values were found to be below the control group level in all treatment groups at the end of 4 weeks. The parameter creatinine, however, was not considered significant ($p>0.05$).

### Pancreas weights results

**Table 1. The effect of soybean extract on some serum biochemical levels of diabetic rats**

| Groups | Control Mean±SD | Diabetic control Mean±SD | 100 Mg/Kg extract Mean±SD | 200 Mg/Kg extract Mean±SD | 400 Mg/Kg extract Mean±SD | p | Range |
|---|---|---|---|---|---|---|---|
| Parameters | | | | | | | |
| Glucose (mg/dl) | 93.90±6.71 | 434.20±35.1 | 309.18±63.4 | 229.72±18.1 | 113.58±38.7 | <0.05 | 82.68–170.36 |
| Urea (BUN) (mg/dl) | 13.40±1.34 | 15.82±0.38 | 11.48±0.36 | 7.92±1.46 | 7.06±2.06 | <0.05 | 13.5–24.5 |
| Creatinine (mg/dl) | 0.40±0.10 | 0.54±0.89 | 0.60±0.10 | 0.43±0.30 | 0.42±0.14 | >0.05 | 0.30–0.60 |
| AST(UI/l) | 155.20±27.7 | 181.02±26.0 | 132.12±14.7 | 128.00±11.6 | 125.82±16.5 | <0.05 | 29.34–72.16 |
| ALT (U/l) | 67.22±16.08 | 131.00±18.8 | 55.48±8.63 | 43.96±3.76 | 46.72±9.63 | <0.05 | 62.75–126.65 |
| Triglyceride (mg/dL) | 88.38±4.98 | 335.40±16.7 | 285.26±12.6 | 52.28±20.21 | 49.12±6.63 | <0.05 | 26.78–65.88 |
| Cholesterol (mg/dl) | 54.96±7.75 | 71.80±2.04 | 47.58±9.24 | 37.40±5.54 | 59.40±7.60 | <0.05 | 62.47–104.13 |
| LDL (mg/dl) | 11.52±0.50 | 24.88±4.70 | 21.58±3.68 | 17.60±6.65 | 9.24±0.82 | <0.05 | 12.21–27.36 |
| HDL (mg/dl) | 72.78±9.96 | 30.96±4.35 | 22.18±3.03 | 39.86±14.56 | 39.12±11.46 | <0.05 | 37.00–68.73 |
| VLDL (mg/dl) | 26.11±6.75 | 67.32±22.89 | 45.56±23.42 | 25.94±13.66 | 24.31±1.85 | <0.05 | 5.05–13.16 |

SD: Standard deviation; AST: Aspartate transaminase; ALT: Alanine transaminase; LDL: Low-density lipoproteins; HDL: High-density lipoproteins; VLDL: Very low-density lipoprotein.

According to the data obtained, pancreatic weight (0.80±0.28 g) in diabetic rats was lower than that of control rats (1.12±0.23 g) ($p<0.05$). In addition, pancreas weight in all treatment groups was significantly higher than in the diabetic group. The biggest rise was observed in the 400 mg/kg extract group (1.40±0.07 g) (Fig. 2).

## Discussion

This study was designed to examine possible effects of soybean seed extract on several biochemical parameters in the serum of STZ-induced diabetic rats. STZ is a broad-spectrum antibiotic that has a cytotoxic effect on the insulin-producing beta cells in the pancreatic islets [12]. It is thought that STZ is a glucose analogue that enters the cell via the glucose transporter GLUT2 and causes DNA alkylation and apoptosis in β-cells [15]. It is, therefore, often used to induce diabetes mellitus in experimental animals. In this study, it was found that serum glucose values increased in STZ-treated animals. Soybeans contain high levels of isoflavones and fiber, which enhance metabolic processes [16]. In the studies presented by some researchers, it is reported that the isoflavones contained in soybeans are effective in reducing glucose levels [17–19]. However, it has also been reported that soluble fibers obtained from soybeans show resistance to digestion and absorption and can, therefore, be used to control glucose absorption and rising blood sugar levels in diabetes [20]. Gupta et al. [3] showed in their study that soybean extract orally administered to diabetic animals reduced blood glucose levels up to 5.97%. It has also been reported that isoflavone intake in the diet can prolong lifespan and increase glucose tolerance capacity in normal mice [21]. In this study, soybean treatment significantly reduced elevated serum glucose concentration, and this reduction was most effective in the high-dose group when evaluated among the administered doses. Based on these results, it seems that soy isoflavones may have a positive effect on the overall metabolism of diabetic animals. In dia-

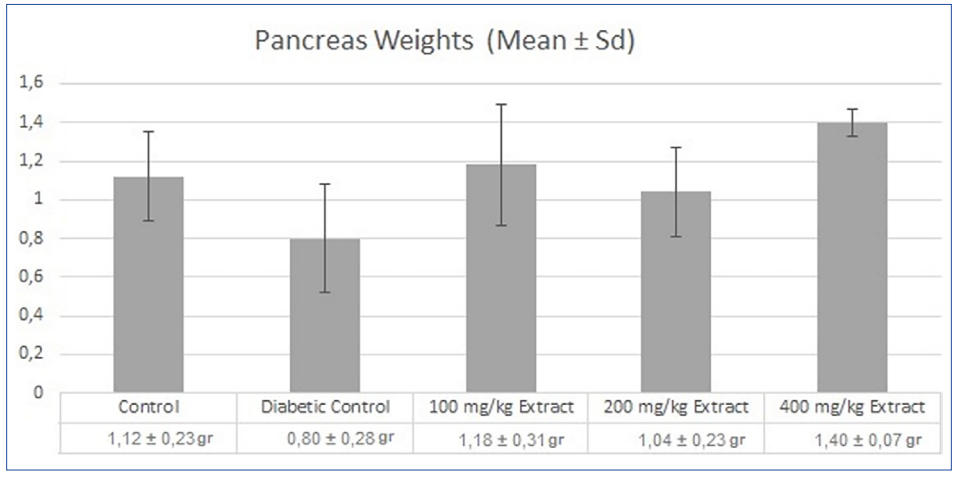


**Figure 2.** Mean pancreatic weight changes in the studied groups.
Sd: Standard deviation.

betes mellitus, not only carbohydrate metabolism is impaired, but also lipid and protein metabolism are significantly altered. Elevated serum total cholesterol and triglycerides are typical indicators of diabetes mellitus [22]. Insulin is essential for the inhibition of hormone-sensitive lipase and the activation of lipoprotein lipase. Therefore, mobilization of free fatty acids from peripheral fat stores increases in diabetes, and serum total triglyceride levels increase [23]. Total cholesterol and LDL levels increase in diets containing high levels of saturated fats [24]. There have been reports that the phytoestrogens and saponins in soybeans could be involved in the regulation of serum cholesterol levels [25–27]. Khushk et al. [28] assessed the non-diabetic potency of soybean extracts (chloroform and alcohol) in alloxan-induced diabetic rabbits. The extracts were shown to cause a substantial reduction in serum cholesterol levels. Consistent with these studies, in the present study, STZ-induced diabetic rats treated with soybean extract showed a significant decrease in serum cholesterol levels. This reduction was greatest at a dose of 200 mg/kg. Some studies have shown that there was no significant change in triglyceride levels after a soy-based diet [27, 29]. On the other hand, Ristić Medić et al. [30] found a 29% reduction in triglyceride levels after 12 weeks of treatment with soybeans in type 2 diabetics with hypertriglyceridemia. In this study, soybean extract was found to reduce triglyceride levels, and this reduction was less than in the control group at doses of 200 and 400 mg/kg.

Serum concentrations of LDL, HDL, and VLDL are essential for whole-body metabolism [31]. Dyslipidemia in type 2 diabetes is characterized by low levels of HDL, elevated LDL, and serum triglycerides associated with VLDL [32]. Soybean proteins, especially glycine and β-conglycinin, may be responsible for lower cholesterol concentrations by increasing bile acid production and excretion through feces [33]. This may lead to a shift in the hepatic cholesterol system to provide more cholesterol for bile acid deficiency and may also lead to increased LDL receptor activity by a mechanism that causes a decrease in total blood cholesterol concentration [34]. Previous studies have shown soy protein to lower cholesterol, triglycerides, and low-density lipoprotein (LDL) levels in people with diabetes and in healthy people and have shown similar effects in rats and rabbits [35–37]. Isoflavone compounds found in soybeans, such as genistein, cause less and smaller fat cells to be produced [9]. Lee [38] reported that a 60 mg/100 g genistein diet increased serum HDL cholesterol levels in diabetic rats. In this study, high levels of LDL were observed in the diabetic group after STZ administration, while low levels of HDL were detected. However, it was determined that there was a significant decrease in LDL and VLDL concentrations after different doses of soybean seed extract compared with the untreated diabetic control group. HDL concentration increased in the 200 and 400 mg/kg treatment groups.

Urea plays an important role in the metabolism of nitrogenous compounds and is a waste product of dietary protein. This organic compound is filtered into urine by the kidneys [39]. Uric acid is found in the cytosol and in soluble compounds found in the blood. It is synthesized mainly from purines based on adenine and guanine. Uric acid is found in all tissue compartments except the lipid phase [40]. Hyperuricemia is caused by increased uric acid. The uric acid is transported to the liver and is released into the bloodstream. Kidney function is related to glomerular filtration and tubular secretion of serum creatinine [41]. In health problems such as diabetes, kidney cells are damaged, urea levels in the blood increase, and creatinine levels increase. Therefore, the function of the kidney is reduced [42]. Soybean seeds contain phenolic compounds called flavonoids, and some studies have reported that soy protein helps prevent kidney disease in people with diabetes [27]. Kudou et al. [43] reported that in alloxan-induced diabetic rats, administration of soybean seeds reduced plasma creatinine and blood urea levels. In the present study, soybean seed extract significantly ($p<0.05$) decreased serum urea levels in STZ-induced diabetic rats, but serum creatinine levels were statistically insignificant ($p<0.05$).

Insulin clearance and the production of inflammatory cytokines take place mainly in the liver. It has an important role in the maintenance of normal fasting and postprandial glucose concentrations [44]. ALT and AST enzymes are well-known markers of liver injury. Serum levels are high because these enzymes are thought to leak from the cytosol into the bloodstream as a result of liver tissue damage [12]. Previous studies have shown increased serum AST and ALT levels in diabetic rats fed a high-fat diet and treated with low-dose STZ [45]. Bai et al. [46] have suggested that soybean may have a role to play in the enhancement of glucose and lipid homeostasis and liver function in T2DM. In this study, it was determined that soybean treatment decreased serum ALT and AST levels compared to control in all doses administered. Based on these data, it can be said that soybean has protective effects on hepatic dysfunction of type 2 diabetic rats.

One of the most important causes of diabetes is beta cell dysfunction in the pancreas [47]. In the studies conducted with various imaging methods, it was determined that the pancreas volume of patients with diabetes decreased [48, 49]. In this study, it was determined that the pancreas weight of the STZ-treated diabetes group decreased, while the pancreas weight increased in the soybean extract treatment groups. This increase was the highest at a dose of 400 mg/kg. Based on these results, it can be said that soybean may cause improvement in pancreatic cells.

## Conclusion

Soybeans have a very high concentration of lysine. They also contain abundant amounts of essential amino acids such as histidine, isoleucine, leucine, phenylalanine, tyrosine, tryptophan, and valine. For this reason, it is considered to be a cost-effective and easily accessible supply of protein. When all our results were evaluated in general, soybean treatment showed a positive effect on some biochemical parameters in diabetic rats. These findings lend support to reports of the

beneficial and health-promoting effects of soybeans. Furthermore, it can be concluded that G. max is hypoglycemic and reduces the complications of diabetes mellitus in diabetic rats.

**Ethics Committee Approval:** The study was approved by The Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (No: 2015/67, Date: 29/07/2015).

**Authorship Contributions:** Concept – E.A., H.A.; Design – E.A., H.A., E.G.M.; Supervision – E.A., H.A.; Fundings – E.A.; Materials – E.A., E.G.M.; Data collection &/or processing – E.G.M., D.E.K.; Analysis and/or interpretation – E.G.M.; Literature search – E.G.M.; Writing – E.G.M.; Critical review – E.G.M., E.A.

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

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## References

1. Aziz OH. Effect of soybean seeds alone or in combination with insulin or glibenclamide on serum lipid profiles in alloxan-induced diabetic rats. Iraqi J Vet Sci 2009;23(1):17–23.
2. Iqbal SS, Reddy RK, Borra SK, Kumar PP, Swathy R, Babu D, et al. Effect of polyherbal formulation in patients with Type 2 diabetes mellitus. J Med Sci Technol 2013;2(2):22–7.
3. Gupta R, Sharma SB. Effect of germinated Glycine max seeds on glycemic control in STZ+ NAD induced type 2 diabetic models: A preliminary study. J Exp Integr Med 2002;2(2):155.
4. Betteridge J. Lipid disorders in diabetes mellitus. In: Pickup JC, Williams G, editors. Textbook of Diabetes. 2nd ed. London: Blackwell Science; 1997. p. 1–35. [CrossRef]
5. Arora S, Ojha S, Vohora D. Characterization of streptozotocin induced diabetes mellitus in Swiss Albino Mice. Glob J Pharmacol 2009;3:81–4.
6. Galletto R, Siqueira VL, Ferreira EB, Oliveira AJ, Bazotti RB. Absence of antidiabetic and hypolipidemic effect of Gymnema sylvestre in nondiabetic and alloxan diabetic rats. Braz Arch Biol Techn 2004;47:545–51. [CrossRef]
7. Saeed MK, Deng Y, Dai R. Attenuation of biochemical parameters in streptozotocin-induced diabetic rats by oral administration of extracts and fractions of cephalotaxus sinensis. J Clin Biochem Nutr 2008;42(1):21–8. [CrossRef]
8. Sharma U, Sahu R, Roy A, Golwala D. *In vivo* antidiabetic and antioxidant potential of stephania hernandifolia in streptozotocin-ınduced-diabetic rats. J Young Pharm 2010;2(3):255–60.
9. Amer N. Effects of soybean seed on glucose levels, lipid profiles and histological structures of the liver in alloxan-induced diabetic albino rats. Tikrit J Pure Sci 2012;17(2):1–5.
10. Yoon MS, Lee J, Kim CY, Kang JH, Cho EG, Baek HJ. DNA profiling and genetic diversity of Korean soybean (Glycine max (L.) Merrill) landraces by SSR markers. Euphytica 2009;165:69–77.
11. Salmerón J, Manson JE, Stampfer MJ, Colditz GA, Wing AL, Willett WC. Dietary fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women. JAMA 1997;277(6):472–7.
12. Annadurai T, Muralidharan AR, Joseph T, Hsu MJ, Thomas PA, Geraldine P. Antihyperglycemic and antioxidant effects of a flavanone, naringenin, in streptozotocin-nicotinamide-induced experimental diabetic rats. J Physiol Biochem 2012;68(3):307–18.
13. Badavi M, Mehrgerdi FZ, Sarkaki A, Naseri MK, Dianat M. Effect of grape seed extract on lead induced hypertension and heart rate in rat. Pak J Biol Sci 2008;11(6):882–7. [CrossRef]
14. Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: A model for type 2 diabetes and pharmacological screening. Pharmacol Res 2005;52(4):313–20. [CrossRef]
15. Deeds MC, Anderson JM, Armstrong AS, Gastineau DA, Hiddinga HJ, Jahangir A, Eberhardt NL, et al. Single dose streptozotocin-induced diabetes: Considerations for study design in islet transplantation models. Lab Anim 2011;45(3):131–40.
16. Hermansen K, Søndergaard M, Høie L, Carstensen M, Brock B. Beneficial effects of a soy-based dietary supplement on lipid levels and cardiovascular risk markers in type 2 diabetic subjects. Diabetes Care 2001;24(2):228–33. [CrossRef]
17. Nilausen K, Meinertz H. Lipoprotein(a) and dietary proteins: Casein lowers lipoprotein(a) concentrations as compared with soy protein. Am J Clin Nutr 1999;69(3):419–25.
18. Anderson JW, Major AW. Pulses and lipaemia, short- and long-term effect: Potential in the prevention of cardiovascular disease. Br J Nutr 2002;88:S263–71. [CrossRef]
19. Janani C, Sundararajan B, Moola AK, Kumari BR. Antidiabetic activity of methanolic leaves extract of transformed soybean plantlets in streptozotocin (STZ) induced diabetic rats. J Stress Physiol Biochem 2021;17(2):66–78.
20. Naaz A, Yellayi S, Zakroczymski MA, Bunick D, Doerge DR, Lubahn DB, et al. The soy isoflavone genistein decreases adipose deposition in mice. Endocrinology 2003;144(8):3315–20.
21. Bartke A, Peluso MR, Moretz N, Wright C, Bonkowski M, Winters TA, et al. Effects of Soy-derived diets on plasma and liver lipids, glucose tolerance, and longevity in normal, long-lived and short-lived mice. Horm Metab Res 2004;36(8):550–8.
22. Shim JY, Kim YJ, Lee HS. Effects of soybean isoflavone extract on the plasma lipid profiles and antioxidant enzyme activity in streptozotocin-induced diabetic rats. Nutr Res Pract 2008;2(4):218–26.
23. Ali Hussain HE. Reversal of diabetic retinopathy in streptozotocin induced diabetic rats using traditional Indian anti-diabetic plant,Azadirachta indica (L.). Indian J Clin Biochem 2002;17(2):115–23. [CrossRef]
24. Öngün Yılmaz H. Hiperlipidemi ve beslenme. Türkiye Sağ Bil Araş Derg 2018;1(2):72–82.
25. Setchell KD. Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones. Am J Clin Nutr 1998;68:1333S–46S. [CrossRef]
26. Francis G, Kerem Z, Makkar HP, Becker K. The biological action of saponins in animal systems: A review. Br J Nutr 2002;88(6):587–605. [CrossRef]
27. Teixeira SR, Potter SM, Weigel R, Hannum S, Erdman JW Jr, Hasler CM. Effects of feeding 4 levels of soy protein for 3 and 6 wk on blood lipids and apolipoproteins in moderately hyper-

cholesterolemic men. Am J Clin Nutr 2000;71(5):1077–84.
28. Khushk I, Dahot MU, Baloach SA, Bhutto MA. The evaluation of soybean extracts in alloxan einduced diabetic rabbits. World Appl Sci J 2010;8:22–5.
29. Baum JA, Teng H, Erdman JW Jr, Weigel RM, Klein BP, Persky VW, et al. Long-term intake of soy protein improves blood lipid profiles and increases mononuclear cell low-density-lipoprotein receptor messenger RNA in hypercholesterolemic, postmenopausal women. Am J Clin Nutr 1998;68(3):545–51.
30. Ristić Medić D, Ristić V, Arsić A, Postić M, Ristić G, Blazencić Mladenović V, et al. Effects of soybean D-LeciVita product on serum lipids and fatty acid composition in type 2 diabetic patients with hyperlipidemia. Nutr Metab Cardiovasc Dis 2006;16(6):395–404. [CrossRef]
31. Wang W, Zhao L, He Z, Wu N, Li Q, Qiu X, et al. Metabolomics-based evidence of the hypoglycemic effect of Ge-Gen-Jiao-Tai-Wan in type 2 diabetic rats via UHPLC-QTOF/MS analysis. J Ethnopharmacol 2018;219:299–318. [CrossRef]
32. Schofield JD, Liu Y, Rao-Balakrishna P, Malik RA, Soran H. Diabetes dyslipidemia. Diabetes Ther 2016 ;7(2):203–19.
33. Beynen AC. Comparison of the mechanisms proposed to explain the hypocholesterolemic effect of soybean protein versus casein in experimental animals. J Nutr Sci Vitaminol (Tokyo) 1990;36(Suppl 2):S87–93. [CrossRef]
34. Tanaka K, Aso B, Sugano M. Biliary steroid excretion in rats fed soybean protein and casein or their amino acid mixtures. J Nutr 1984;114(1):26–32. [CrossRef]
35. Dewanjee S, Bose SK, Sahu R, Mandal SC. Antidiabetic effect of matured fruits of Diospyros peregrina in alloxan-induced diabetic rats. Available at: https://www.greenpharmacy.info/index.php/ijgp/article/view/37. Accessed Jul 4, 2024.
36. Azadbakht L, Shakerhosseini R, Atabak S, Jamshidian M, Mehrabi Y, Esmaill-Zadeh A. Beneficiary effect of dietary soy protein on lowering plasma levels of lipid and improving kidney function in type II diabetes with nephropathy. Eur J Clin Nutr 2003;57(10):1292–4. [CrossRef]
37. Sada NM, Tanko Y, Dikko AAU, Abdulrazak A, Mohammed A. Effects of fermented soya bean supplements on lipid profile and oxidative stress biomarkers in high fat diet-induced Type 2 diabetes mellitus in rabbits. J Afr Assoc Physiol Sci 2018;6(1):73–8.
38. Lee JS. Effects of soy protein and genistein on blood glucose, antioxidant enzyme activities, and lipid profile in streptozotocin-induced diabetic rats. Life Sci 2006;79(16):1578–84.
39. Ossman DH, Marouf BH, Ameen KH. Effect of extracorporeal unfractionated heparin on hematological and electrolyte markers in hemodialyzed patients J Photochem Photobiol. 2014;4:431–9. [CrossRef]
40. Reddy R, Keshavan M, Yao JK. Reduced plasma antioxidants in first-episode patients with schizophrenia. Schizophr Res 2003;62(3):205–12. [CrossRef]
41. Mohamed Ali MS, Babiker MA, Merghani LB, Ali FA, Abdulmajeed MH. Hematological changes post-hemo and peritoneal dialysis among renal failure patients in Sudan. Saudi J Kidney Dis Transpl 2008;19(2):274–9.
42. Nahdi AMTA, John A, Raza H. Elucidation of Molecular mechanisms of streptozotocin-ınduced oxidative stress, apoptosis, and mitochondrial dysfunction in Rin-5F pancreatic β-cells. Oxid Med Cell Longev 2017;2017:7054272. [CrossRef]
43. Kudou S, Fleury Y, Welti D, Magnolato D, Uchida T, Kitamura K,Okubo K. Malonyl isoflavone glycosides in soybean seeds (Glycine max Merrill). Agric Biol Chem 1991;55(9):2227–33.
44. Leclercq IA, Da Silva Morais A, Schroyen B, Van Hul N, Geerts A. Insulin resistance in hepatocytes and sinusoidal liver cells: Mechanisms and consequences. J Hepatol 2007;47(1):142–56. [CrossRef]
45. Sahin K, Tuzcu M, Orhan C, Sahin N, Kucuk O, Ozercan IH, et al. Anti-diabetic activity of chromium picolinate and biotin in rats with type 2 diabetes induced by high-fat diet and streptozotocin. Br J Nutr 2013;110(2):197–205. [CrossRef]
46. Bai Z, Huang X, Wu G, Zhou Y, Deng X, Yang J, et al. Hepatic metabolism-related effects of polysaccharides from red kidney bean and small black soybean on type 2 diabetes. Food Chem 2023;403:134334. [CrossRef]
47. Yagihashi S. Advances in pathology of diabetes from pancreatic islets to neuropathy - A tribute to Paul Langerhans. Pathol Int 2015;65(4):157–69. [CrossRef]
48. Alzaid A, Aideyan O, Nawaz S. The size of the pancreas in diabetes mellitus. Diabet Med 1993;10(8):759–63. [CrossRef]
49. Burute N, Nisenbaum R, Jenkins DJ, Mirrahimi A, Anthwal S, Colak E, et al. Pancreas volume measurement in patients with Type 2 diabetes using magnetic resonance imaging-based planimetry. Pancreatology 2014;14(4):268–74. [CrossRef]

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Research Article

# Evaluation of ethanol test analysis: The two years experience

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## Abstract

**Objectives:** Compounds with hydroxyl (-OH) attached to the carbon atom in their structure are generally defined as alcohols. People have been producing, consuming, and enjoying alcohol for a very long time. The prevalence of alcohol use disorder in the world is 5.1%. Alcohol toxicity can be life-threatening, and blood alcohol levels must be measured.

**Methods:** The 19568 ethanol analysis results were included in this study, performed between 01.01.2021 and 31.12.2022, and were obtained from Ankara Bilkent City Hospital's laboratory information system. Serum ethanol levels <10 mg/dL were accepted as negative. Ethanol analysis results according to positive serum ethanol levels were designed as follows; <10 mg/dL, between 10 mg/dL and ≤30 mg/dl, between 30 mg/dl> and ≤50 mg/dl, between 50 mg/dl> and ≤100 mg/dl, and above 100 mg/dL. Ethanol analysis results according to age were designed as follows ≤10 years, 11–20 years, 21–30 years, 31–40 years, 41–50 years, 51–60 years, and above 60 years. The ethanol analyses were performed by Atellica Chemistry- XPT ® (Siemens Healthineers, Erlangen, Germany) device.

**Results:** This study included a total of 19568 ethanol analysis results. The number of females and males was as follows: 5595 (%28.3), 13973 (%71.4) Ethanol positivity was higher in males 2735 (%76.5) than in females 839 (%23.5) (p<0.001). The results of the males were higher in each group (p<0.001) when evaluating the ethanol intervals among genders. When the ethanol intervals among age intervals were analyzed, the group aged between 21 to 30 years had the highest number while the group aged between 0 to 10 years had the least number (p<0.001).

**Conclusion:** Alcohol use is stated as a public health problem for the whole world. Our study, which evaluates alcohol analysis results according to gender, different age groups, and different ethanol intervals, may be helpful for future studies.

**Keywords:** Ethanol, ethanol toxicity, public health

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Compounds with a hydroxyl (-OH) group attached to a carbon atom in their structure are generally defined as alcohols. For a compound to be defined as an alcohol, no more than one -OH group should be bonded to the same carbon, and the carbon atom to which the -OH group is attached should not contain a pi bond [1].

People have been producing, consuming, and enjoying alcohol for a very long time [2]. The first evidence of alcohol use is pottery containing traces of wine, thought to date back to 7000 BC [3]. Views on the physical, mental, and social harms of alcohol use were put forward by Dr. Benjamin Rush in 1785. In the early 1900s, some anti-alcohol laws were passed [4].

It has been stated that approximately 5.1% of people over the age of 15 have an alcohol use disorder [5]. According to the World Health Organization (WHO) regions, alcohol use disorder is reported as 8.8% in the European region, 8.2% in the Americas, 4.7% in the Western Pacific region, 3.9% in the Southeast Asia region, 3.7% in Africa, and 0.8% in the Eastern Mediterranean region [6]. As reported by the Turkish Statistical Institute, there was an increase in the prevalence of alcohol use among individuals between the ages of 15 and 24 in Türkiye between 2010 and 2019. The rate of alcohol use was stated as 8.6% in 2010, 7.4% in 2012, 13.3% in 2014, 9.3% in 2016, and 11.4% in 2019 [7]. The rate of alcohol use is higher in men than in women [8].

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Some situations related to alcohol are stated in Articles 34/1, 34/2, and 57 of the Turkish Penal Code [9]. One per mille means the presence of 100 milligrams of alcohol in 100 milliliters of blood [10]. 0.2 per mille is the legal limit for vehicles other than private vehicles, and 0.5 per mille is the legal limit for private vehicles [11].

Alcohol is included under the heading "Substance-related disorders" in the Diagnostic and Statistical Manual of Mental Disorders [9]. Approximately 237 million men and 46 million women in the world suffer from alcohol use disorder [12]. Alcohol use may lead to alcohol addiction [13]. Long-term alcohol consumption causes various diseases, including both communicable and non-communicable ones [14]. Alcohol use plays a role in increased risks of tuberculosis and lower respiratory tract infections by suppressing the immune system; it also increases the likelihood of unprotected sexual intercourse, which may increase exposure to sexually transmitted infections [15]. Alcohol consumption causes approximately 4% of cancers in the world and increases the risk of colorectal, breast, liver, and digestive system cancers [16]. There is an association between alcohol and an increased risk of cardiovascular diseases [17], injuries, and some neurological conditions [18].

Foods taken with ethanol and medical procedures that delay gastric emptying slow down ethanol absorption. After absorption, 90% of the ethanol is eliminated from the body by enzymatic oxidation in the liver, and 5–10% is eliminated from the body through the kidneys, lungs, and sweating [19].

Alcohol toxicity can be life-threatening, and the best way to detect this condition is to measure blood alcohol levels [20]. As the blood alcohol level changes, different clinical findings occur. In individuals with blood alcohol levels between 20 and 30 mg/dL, motor skills slow down, thinking and judgment abilities are impaired, and motor and cognitive problems increase. Between 30 and 80 mg/dL, coordination and judgment disorders occur, and between 80 and 200 mg/dL, mood lability and impairment in cognitive functions are observed. Nystagmus, dysarthria, and memory disorders are observed in individuals with levels between 200 and 300 mg/dL, and confusion and stupor are observed in individuals with levels between 300 and 400 mg/dL. In individuals with blood alcohol levels between 400 and 500 mg/dL, coma may occur, and in individuals with blood alcohol levels >500 mg/dL, respiratory and circulatory centers may be affected, leading in some cases to death [5].

Ankara Bilkent City Hospital has one of the biggest biochemistry laboratories in Türkiye. In this study, we aimed to evaluate the ethanol test results of Ankara Bilkent City Hospital among different ethanol levels, genders, and different age groups, and to contribute to the literature.

## Materials and Methods

The 19568 ethanol analysis results included in this study, performed between 01.01.2021 and 31.12.2022, were obtained from Ankara Bilkent City Hospital's laboratory information system.

Internal and external quality controls are carried out for ethanol testing in our hospital. Hemolysis causes a decrease in the measured ethanol concentration [21]. While ethanol samples are accepted to the laboratory, their compliance with the sample acceptance and rejection criteria determined by our hospital is evaluated, and test analyses are performed on the appropriate samples.

Only the first results of patients whose ethanol analysis was performed more than once were included in the study. Groups were designed according to gender, age, and serum ethanol levels. Serum ethanol levels were stated as negative or positive. Serum ethanol levels <10 mg/dL were accepted as negative. Ethanol analysis results according to positive serum ethanol levels were designed as follows: <10 mg/dL, between 10 mg/dL and ≤30 mg/dL, between 30 mg/dL and ≤50 mg/dL, between 50 mg/dL and ≤100 mg/dL, and above 100 mg/dL. Ethanol analysis results according to age were designed as follows: ≤10 years, 11–20 years, 21–30 years, 31–40 years, 41–50 years, 51–60 years, and above 60 years. The ethanol analyses were performed using the Atellica Chemistry-XPT® (Siemens Healthineers, Erlangen, Germany) device. Ethanol measurement depends on the enzymatic reaction using the Emit II Plus reagent. The measurement kit includes two reagents. Reagent 1 contains the buffer system, and Reagent 2 contains alcohol dehydrogenase (ADH), coenzyme nicotinamide adenine dinucleotide (NAD), buffer, preservatives, and stabilizers. ADH catalyzes the oxidation of ethyl alcohol to acetaldehyde. During this reaction, NAD is reduced to NADH. The increase in absorbance at 340 nm is proportional to the alcohol concentration in the sample. At every stage of the present study, all researchers worked under the Declaration of Helsinki.

### Statistical analyses

Descriptive statistics were frequency, percent, median, 25$^{th}$, and 75$^{th}$ percentile (Interquartile ranges; IQR). A chi-square test was used for categorical comparisons. The Mann-Whitney U test was performed to compare the groups, and a p-value <0.05 was defined as statistically significant. IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp., Armonk, NY, USA), was used for statistical analyses.

## Results

A total of ethanol analysis results, including a two-year time period, were included in this study. Atellica Chemistry-XPT® (Siemens Healthineers, Erlangen, Germany) device kit insert.

In the ethanol kit insert of the Atellica Chemistry-XPT® (Siemens Healthineers, Erlangen, Germany) device, it was stated that ethanol concentrations below 10 mg/dL should be reported as <10 mg/dL; therefore, in our study, ethanol concentrations below 10 mg/dL were reported as negative. A total of 5,595 (28.3%) results belonged to females, and 13,973 results belonged to males (71.4%). In the present study, a chi-square test was performed, and statistical differences in ethanol positivity between males, 2,735 (76.5%), and females, 839 (23.5%), (p<0.001) were found.

A chi-square test was performed for the ethanol test analysis data among age intervals, and the 21–30 years age group had the highest number of positive ethanol results (p<0.001).

The quantitative variables (Table 1) and the comparison of ethanol test analysis data of age intervals among genders were presented (Table 2). The Mann-Whitney U test was performed to compare ages among the genders, and the median and IQR were as follows: 34 (25–46) for females and 35 (26–47) for males, with statistically significant differences found among the groups (p=0.013).

Ethanol test analysis data of age intervals among genders were evaluated with chi-square tests. No significant differences existed between genders in the 0–10 years, 11–20 years, and 21–30 years age groups. Males had a higher number of positive ethanol results in the following groups: 31–40 years (p<0.001), 41–50 years (p<0.001), 51–60 years (p<0.001), and over 60 years (p<0.001) (Table 2).

**Table 1. Quantitative variables**

| | n | % |
|---|---|---|
| Gender | | |
| Female | 5595 | 28.6 |
| Male | 13973 | 71.4 |
| Age (years) | | |
| 0–10 | 49 | 0.3 |
| 11–20 | 1478 | 7.6 |
| 21–30 | 6181 | 31.6 |
| 31–40 | 4525 | 23.1 |
| 41–50 | 3385 | 17.3 |
| 51–60 | 1993 | 10.2 |
| Over 61 | 1957 | 10 |
| Ethanol Intervals (mg/dL) | | |
| Negative | 15994 | 81.74 |
| >10 mg/dL–≤ 30 mg/dL | 274 | 1.40 |
| >30 mg/dL–≤ 50 mg/dL | 252 | 1.29 |
| >50 mg/dL–≤ 100 mg/dL | 553 | 2.83 |
| Over 100 mg/dL | 2495 | 12.75 |

## Discussion

Consuming alcohol is a worldwide public health problem [22]. Alcohol is an addictive substance and is used by many people for different reasons. More than 200 diseases are associated with alcohol use. It has been stated that 1 in every 3 people is an active alcohol drinker [23]. According to data, 30 to 45% of adults in the USA have experienced at least one temporary alcohol-related problem, and 3 to 5% of women and 10% of men have been diagnosed with alcohol addiction. The age of active alcohol use is 25–35 years, and alcohol use reaches its peak at the age of 35 [24].

As reported by a survey conducted on Kırıkkale University students with an average age of 20.9±1.8 years, 64% of the students have tried drinking alcohol, and the rate of alcohol use is 74.8% among male students and 54.8% among female students (p=0.000) [25]. In a study evaluating alcohol use among medical school students, with an average age of 21.03 years, it was found that alcohol use was higher in men [26].

According to a cross-sectional study held in Japan, a significant difference between males (74.9%) and females (59.6%) was reported [27]. As a result of a study whose sample consisted of individuals in the 15–18 age group in the rural area of Tekirdağ, it was stated that men consumed more alcohol than women [28].

**Table 2. Ethanol test analysis data of age intervals among genders**

| Age intervals (years) | Ethanol mg/dL | Gender | | | | p |
|---|---|---|---|---|---|---|
| | | Female | | Male | | |
| | | n | % | n | % | |
| 0–10 | Negative | 19 | 100 | 27 | 90 | 0.273 |
| | Positive | | | 3 | 10 | |
| 11–20 | Negative | 375 | 83.14 | 850 | 82.77 | 0.461 |
| | Positive | 76 | 16.86 | 177 | 17.23 | |
| 21–30 | Negative | 1429 | 79.34 | 3557 | 81.21 | 0.005 |
| | Positive | 372 | 20.66 | 823 | 18.79 | |
| 31–40 | Negative | 1087 | 84.39 | 2498 | 77.17 | <0.001* |
| | Positive | 201 | 15.61 | 739 | 22.89 | |
| 41–50 | Negative | 822 | 86.26 | 2498 | 77.17 | <0.001* |
| | Positive | 131 | 13.74 | 496 | 20.4 | |
| 51–60 | Negative | 506 | 93.19 | 1167 | 80.48 | <0.001* |
| | Positive | 37 | 6.81 | 283 | 19.52 | |
| Over 60 | Negative | 518 | 95.9 | 1203 | 84.9 | <0.001* |
| | Positive | 22 | 4.1 | 214 | 15.1 | |

*: p<0.05: Statistically significant. p: Chi-square test.

Alcohol use among adolescents is increasing. In a study conducted in Southern Ireland, including parents and adolescents, it was stated that 47% of parents and 34.2% of adolescents engaged in hazardous drinking [29]. In a study that retrospectively evaluated the data of adolescent patients with an average age of 16.50±0.70 years in a psychiatric ward, it was reported that alcohol use was higher in males than in females (p=0.01) [30]. It was reported that the prevalence of lifetime alcohol usage was 37.3%, while the prevalence of alcohol usage more than once was 24.3% in a study conducted with apprenticeship training students [31].

In a study that evaluated individuals aged 15–64 as young and those aged 65 and over as elderly, the ethanol positivity rate of young patients (24.4%) was found to be higher than that of older patients (5.5%) (p<0.0001) [32]. A study involving geriatric patients found no difference in alcohol use between men and women [33].

In the study by Akay et al. [34], it was stated that 341 (76.6%) of 445 patients included in the study had a blood alcohol level below 0.5 per mille (50 mg/dL), and 104 (23.4%) had a blood alcohol level of 0.5 per mille and above. In the study by Yılmaz et al. [35], which investigated the demographic and clinical features of blood ethyl alcohol level requests made from emergency services, it was reported that 63.6% of the results were below the legal limit and 36.4% were above the legal limit.

Our study includes a total of 19,568 ethanol analysis results. The number of females and males was as follows: 5,595 (28.3%) and 13,973 (71.4%) (Table 1).

The ethanol test analysis data among age intervals were revealed, and a statistical difference was observed; the 21–30 years age group had the highest number of positive ethanol results (p<0.001).

In Table 2, the ethanol test analysis data of age intervals among genders were evaluated. In all age groups, male results were higher in number. However, there were no statistical differences in the 0–10 years age group (p=0.273), 11–20 years age group (p=0.461), and 21–30 years age group (p=0.005); while there were statistical differences in the 31–40 years age group (p<0.001), 41–50 years age group (p<0.001), 51–60 years age group (p<0.001), and over 60 years age group (p<0.001).

The data of our study showed that ethanol positivity is higher in males than in females, in line with previous studies [25–28], and the 21–30 years age group had the highest number of ethanol-positive results.

### Strengths and limitations

The strengths of the present study are as follows: conducting our study in Ankara Bilkent City Hospital, one of the largest hospitals in Türkiye, allowed the use of a wide range of data. Ethanol results were evaluated in different ethanol intervals, according to gender, and in seven different age groups based on decades.

The limitation of our retrospective study was that we could not access data on the frequency of alcohol use, whether the alcohol use occurred with the free will of the individuals, and the clinical information of the patients related to alcohol through the laboratory information system.

## Conclusion

Many data in the literature are sufficient to explain that alcohol use has dangerous consequences. Alcohol use, which is stated as a public health problem for the whole world, is higher in developed countries than in developing countries. The frequency of alcohol use in our country is lower than in most countries [19].

The data of our study showed that ethanol positivity is higher in males than in females, and the 21–30 years age group had the highest number of positive ethanol results. Our study, which evaluates alcohol analysis results according to gender, different age groups, and different ethanol intervals, may be helpful for future studies. Studies on the problems caused by alcohol use should continue, and activities to prevent alcohol use should be carried out.

**Ethics Committee Approval:** The study was approved by The Ankara Bilkent City Hospital No 1 Clinical Research Ethics Committee (No: E1-23-3775, Date: 12/07/2023).

**Authorship Contributions:** Concept – G.G., T.T.; Design – G.G., T.T.; Supervision – G.G.; Funding – G.G.; Materials – G.G.; Data collection &/or processing – G.G.; Analysis and/or interpretation – G.G.; Literature search – G.G., T.T.; Writing – G.G., T.T.; Critical review – G.G., T.T.

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## References

1. Konukoğlu D, Turhan T. Laboratory in Ethanol Diagnosis and Follow-up [Etanol Tanı ve İzlemde Laboratuvar]. Ankara: Palme Yayınları; 2020.
2. Hendriks HF. Alcohol and human health: What is the evidence? Annu Rev Food Sci Technol 2020;11:1–21. [CrossRef]
3. Amstutz LJ. Alcohol. Minneapolis: Abdo Publishing; 2018.
4. Uzbay İT. Substance Addiction in All Its Dimensions Addiction and Addictive Substances [Madde Bağımlılığı Tüm Boyutlarıyla Bağımlılık ve Bağımlılık Yapan Maddeler]. İstanbul: İstanbul Tıp Kitabevi; 2015.
5. Glantz MD, Bharat C, Degenhardt L, Sampson NA, Scott KM, Lim CCW, et al. The epidemiology of alcohol use disorders cross-nationally: Findings from the World Mental Health Surveys. Addict Behav 2020;102:106128. [CrossRef]
6. GBD 2016 Alcohol Collaborators. Alcohol use and burden for 195 countries and territories, 1990–2016: A systematic analysis for the Global Burden of Disease Study 2016. Lancet 2018;392(10152):1015–35. [CrossRef]

7. İlhan MN, Arıkan Z, Kotan Z, Tunçoğlu T, Pinarci M, Taşdemir A, et al. Prevalence and socio-demographic determinants of tobacco, alcohol, substance use and drug misuse in general population in Turkey. Arch Neuropsychiatry 2016;53:205–12. [CrossRef]
8. Yılmaz G, Serkan İ, Aktuğ ZB. University students' use of tobacco products alcohol and drugs. Niğde Ömer Halisdemir Universitesi Sosyal Bilimler Enstitüsü Derg [Article in Turkish] 2020;2(2):73–80.
9. Tosun M, Çetin A, Konuk N. Forensic psychiatric aspects of alcohol and substance dependence. Turkiye Klinikleri J Psychiatry Special Topics [Article in Turkish] 2011;4(1):81–90.
10. Ayvaşik HB. Alcohol, learning processes and traffic accidents abstract. Türk Psikoloji Yazıları [Article in Turkish] 2002;5(9-10):83–103.
11. Bayram SD. Administrative and criminal sanctions for driving under the influence of alcohol. Yeditepe Üniversitesi Hukuk Fakültesi Derg [Article in Turkish] 2021;18(2):1711–46.
12. Hammer JH, Parent MC, Spiker DA. Mental Help Seeking Attitudes Scale (MHSAS): Development, reliability, validity, and comparison with the ATSPPH-SF and IASMHS-PO. J Couns Psychol 2018;65(1):74. [CrossRef]
13. Heilig M. Stress-related neuropeptide systems as targets for treatment of alcohol addiction: A clinical perspective. J Intern Med 2023;293(5):559–73. [CrossRef]
14. Gül A, Aygin D. Patients presenting to the emergency department due to alcohol consumption and its surgical dimension. Bağımlılık Derg [Article in Turkish] 2021;22(4):483–91.
15. Ding C, O'Neill D, Bell S, Stamatakis E, Britton A. Association of alcohol consumption with morbidity and mortality in patients with cardiovascular disease: Original data and meta-analysis of 48,423 men and women. BMC Med 2021;19:1–14. [CrossRef]
16. Rumgay H, Murphy N, Ferrari P, Soerjomataram I. Alcohol and cancer: Epidemiology and biological mechanisms. Nutrients 2021;13(9):3173. [CrossRef]
17. Biddinger KJ, Emdin CA, Haas ME, Wang M, Hindy G, Ellinor PT. Association of habitual alcohol intake with risk of cardiovascular disease. JAMA Netw Open 2022;5(3):e223849. [CrossRef]
18. Barbería-Latasa M, Gea A, Martínez-González MA. Alcohol, drinking pattern, and chronic disease. Nutrients 2022;14(9):1954. [CrossRef]
19. Vonghia L, Leggio L, Ferrulli A, Bertini M, Gasbarrini G, Addolorato G; Alcoholism Treatment Study Group. Acute alcohol intoxication. Eur J Intern Med 2008;19(8):561–7. [CrossRef]
20. Mirijello A, Sestito L, Antonelli M, Gasbarrini A, Addolorato G. Identification and management of acute alcohol intoxication. Eur J Intern Med 2023;108:1–8. [CrossRef]
21. Kazezoğlu C, Sutaşır YT. Effects of regulation changes on ethanol assay sample rejection rates in blood. Türk Klinik Biyokimya Derg [Article in Turkish] 2019;17(1):23–8.
22. Akvardar Y. Epidemiology of alcohol related disorders. Turkiye Klinikleri J Internal Med Sci [Article in Turkish] 2005;1(47):5–9.
23. Ilhan MN, Yapar D. Alcohol consumption and alcohol policy. Turk J Med Sci 2020;50(5):1197–202. [CrossRef]
24. İnce A, Doğruer Z, Türkçapar MH. Comparision of sociodemographic, clinic and psychopathologic features of the early and late onset alcohol dependent males. Klinik Psikiyatri [Article in Turkish] 2002;5(2):82–91.
25. Ulukoca N, Gökgöz Ş, Karakoç A. Prevalence of tobacco, alcohol, and substance use in Kirklareli University Students. Fırat Tıp Derg [Article in Turkish] 2013;18(4):230–4.
26. Şahin Y, Zengin MN, Yayla E, Sayin AD, Ünal MA, Assan L, et al. Evaluation of nicotine, alcohol, and caffeine use among medical school students. Muş Alparslan Üniversitesi Sağlık Bilimleri Derg [Article in Turkish] 2023;3(1):12–21.
27. Kawaida K, Yoshimoto H, Goto R, Saito G, Ogai Y, Morita N, et al. Reasons for drinking among college students in Japan: A cross-sectional study. Tohoku J Exp Med 2018;246(3):183–9. [CrossRef]
28. Arıcı A, Yaman ÖM. Alcohol use in youth and the effect of cultural structure on addiction. Sosyal Çalışma Derg [Article in Turkish] 2021;5(2):71–89.
29. Murphy E, O'Sullivan I, O'Donovan D, Hope A, Davoren MP. The association between parental attitudes and alcohol consumption and adolescent alcohol consumption in Southern Ireland: A cross-sectional study. BMC Public Health 2016;16:1–8. [CrossRef]
30. Egilmez O, Orum M. Evaluation of adolescent patients hospitalized in an adult psychiatry service: Data for 2015-2018. KOU Sag Bil Derg 2020;6(2):118–22. [CrossRef]
31. İlhan İÖ, Demirbaş H, Doğan YB. A study on alcohol use among working youth who are continuing apprentceship training. Türk Psikiyatri Derg [Article in Turkish] 2005;16(4):237–44.
32. Teichman A, Perea LL, San Roman J, Arya C, Suthar K, Johnson, et al. The effect of alcohol consumption on geriatric trauma outcomes. J Surg Res 2020;254:364–8. [CrossRef]
33. Helvik AS, Engedal K, Johannessen A. Elevated alcohol consumption among geriatric psychiatric in-patients. Nord Stud Alcohol Drugs 2020;37(4):400–10. [CrossRef]
34. Akay N, Çelik GK, Karakayalı O, Memiş M, Tanrıverdi F, Vural S, et al. The effects of blood alcohol levels on the severity of injury and on cost in the traumas caused by motor vehicle accidents. Ankara Med J 2014;14(1):5–10. [CrossRef]
35. Yılmaz İ, Bilgili S, Bozkaya G. Demographic and clinical characteristics of the blood ethyl alcohol requests from the emergency department and its relationship with concentration: The İzmir example. İzmir Eğitim ve Araştırma Hastanesi Tıp Derg [Article in Turkish] 2020;24(1):21–8.

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Research Article

# Evaluation of analytical phase performance of coagulation parameters by sigmametric methodology

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## Abstract

**Objectives:** This study aimed to evaluate the analytical performance of coagulation tests (prothrombin time (PT/INR), activated partial thromboplastin time (aPTT), fibrinogen, and D-dimer) using the Six Sigma methodology, focusing on identifying areas for improvement to enhance healthcare quality.

**Methods:** Internal quality control (IQC) and external quality control (EQC) data from September 2023 to February 2024 were collected for coagulation parameters analyzed by four Cobas T711 analyzers. Sigma values were calculated using IQC, EQC, and total allowable error (TEa) data. The sigma value for each parameter was calculated according to the formula "(TEa%–bias %)/CV%." The outpatient sample analyzers were labeled A1, A2, and A3. The one that analyzes samples from emergency and intensive care patients was labeled B.

**Results:** Across analyzers A1, A2, A3, and B, sigma values varied for different coagulation parameters. Notably, the D-dimer parameter consistently exhibited excellent performance (sigma >6) for all analyzers. In contrast, some analyzers showed poor performance for aPTT and PT parameters at level 1 (A1 and A3 for aPTT, B for INR). Fibrinogen performance varied, with some analyzers showing excellent performance (sigma >6) and others falling below acceptable levels.

**Conclusion:** By identifying areas of low performance, particularly in aPTT and INR parameters, this study highlights the importance of continuous quality improvement in laboratory testing. Addressing issues identified through the Six Sigma methodology can enhance the reliability of laboratory results and ultimately improve patient care. Further research and initiatives focused on analytical process improvement are needed to achieve higher quality standards in laboratory testing.

**Keywords:** Analytical performance, coagulation, quality control, Six Sigma

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The results of laboratory tests are very important for making healthcare decisions. Identifying and reducing laboratory errors is critical. Pre-analytical, analytical, and post-analytical stages of the laboratory procedure are all subject to error [1]. While errors frequently occur throughout the pre- and post-analytical phases, it is crucial to address the causes of analytical errors to guarantee patient safety. Enhancing analytical performance requires the implementation of both external quality control (EQC) and internal quality control (IQC) programs. Whereas EQC evaluates test trueness, IQC offers insights into test precision. The Six Sigma approach has grown in significance for assessing analytical performance since it integrates results from IQC and EQC [2].

Sigma metric methodology has become increasingly important in laboratory quality management, particularly in evaluating the performance of analytical methods and analyzers. This methodology incorporates the concepts of precision, bias, and analytical quality requirements into a comprehensive framework for assessing a laboratory's ability to produce accurate and reliable results [3]. Sigma metrics quantify assay performance by calculating the number of standard deviations between the mean of the target value and the nearest specification limit. The Sigma metric methodology allows laboratories to pinpoint areas needing improvement, establish achievable quality goals, and track the effectiveness of improvement efforts over time. Therefore, the Sigma metric methodology is

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important not only for identifying poor performance but also for offering practical guidance for improvement [4].

Sigma calculation entails utilizing the coefficient of variation (CV) data from IQC, bias data from EQC, and total allowable error (TEa) values established by international organizations. The corresponding Sigma values denote the following performance levels: poor performance (<3), indicating inadequate quality; appropriate quality necessitating stringent control measures (3–3.99); good quality (4–4.99); very good quality (5–5.99); and excellent (≥6), representing world-class performance [5]. Thus, the Six Sigma methodology is rapid, cost-effective, and provides information about analytical performance.

Coagulation parameters are particularly important in patients with bleeding and thrombosis conditions. Additionally, they are among the most requested test groups for emergency department patients. Given their critical role in patient care, reliable results from coagulation tests are crucial for ensuring patient safety and maintaining laboratory quality. In this study, we aimed to evaluate the analytical performance of the coagulation tests (prothrombin time (PT/INR), activated partial thromboplastin time (aPTT), fibrinogen, and D-dimer) on four coagulation analyzers using the Six Sigma methodology.

## Materials and Methods

This study was conducted at the Medical Biochemistry Laboratory of Ankara Etlik City Hospital after obtaining ethical approval (Decision number: AEŞH-BADEK-2024-364, Date: 24.04.2024). Between September 2023 and February 2024, sigma values were calculated using IQC and EQC data of coagulation parameters analyzed by Cobas T711 analyzers (Roche, Germany). All four coagulation analyzers in our laboratory were included, with three analyzing samples from outpatients (labeled as A1, A2, and A3) and one from emergency and intensive care patients (labeled as B).

Using the 6-month IQC (Roche, Germany) data, standard deviation (SD), mean, and CV values were calculated for each parameter and analyzer at both control levels (level 1 and level 2).

CV% = (SD/mean) ×100

EQC data were obtained from the EQC service provider (Bio-Rad, United States of America). Bias values were calculated from 6-month EQC data.

Bias (%) = [(laboratory mean - peer group mean) / peer group mean] ×100.

The TEa values of INR, aPTT, and fibrinogen parameters were obtained from the Clinical Laboratory Improvement Amendments (CLIA) 2019 database, and the D-dimer TEa value was obtained from the American Association of Bioanalysts (AAB) [6, 7].

CV% and bias% values were calculated each month for both control levels regarding aPTT, INR, fibrinogen, and D-dimer parameters. The averages of CV% and bias% for six months were calculated for each control level. The mean sigma value and total error (TE) were calculated using the 6-month mean CV% and bias% values for each control level. For levels with sigma values below 3, the quality goal index (QGI) was calculated to determine whether IQC, EQC, or both caused the problem. QGI scores of <0.8, >1.2, and 0.8–1.2 indicate imprecision, inaccuracy, and both imprecision and inaccuracy, respectively [8].

The formulations used are as follows:

Sigma (σ) = (TEa% – bias%)/CV%

TE = Bias% + 1.65×CV%

QGI = Bias% / (1.5×CV%)

## Results

Analyzer A1: The sigma values of <3 (level 1) and 3–3.99 (level 2) were determined for aPTT (Table 1). The sigma value for both control levels of INR was determined to be in the range of 4–4.99. Sigma values of 3–3.99 (level 1) and 5–5.99 (level 2) were determined for fibrinogen. For the D-dimer test, >6 sigma values were determined for both control levels (Fig. 1).

Analyzer A2: The sigma value for both control levels of aPTT was determined to be in the range of 3–3.99. Sigma values for INR were 3–3.99 (level 1) and 4–4.99 (level 2). Sigma values of 4–4.99 (level 1) and >6 (level 2) were determined for fibrinogen. For the D-dimer test, >6 sigma values were determined for both control levels (Fig. 2).

Analyzer A3: The sigma values of <3 (level 1) and 3–3.99 (level 2) were determined for aPTT. The sigma value for both control levels of INR was determined to be in the range of 4–4.99. Sigma values of 3–3.99 (level 1) and >6 (level 2) were determined for fibrinogen. For the D-dimer test, >6 sigma values were determined for both control levels (Fig. 3).

Analyzer B: The sigma value for both control levels of aPTT was determined in the range of 4–4.99. Sigma values for INR were <3 (level 1) and 3–3.99 (level 2). Sigma values of 3–3.99 (level 1) and 4–4.99 (level 2) were determined for fibrinogen. For the D-dimer test, >6 sigma values were determined for both control levels (Fig. 4).

## Discussion

Minimizing laboratory errors is very important for patient safety. Particularly, the Six Sigma metric methodology plays a pivotal role in evaluating laboratory analysis processes. The unique perspective provided by combining IQC and EQC programs used in monitoring analytical performance is noteworthy. In addition to identifying error sources, the Six Sigma metric offers recommendations for control measures, emphasizing the importance of proactive measures and error detection. In this study, we evaluated the analytical performance of coagulation parameters on four coagulation analyzers using the Six Sigma metric methodology. We observed that the sigma value of level 1 of the aPTT parameter was <3 in both analyzers A1 and A3. Moreover, in the B analyzer, the sigma value of the level 1 INR parameter was <3. We found that the sigma values for both control levels of the D-dimer parameter were >6 for all four devices.

**Table 1. Average six months data results of the four coagulation analyzers, regarding control levels 1 and 2**

| Analyzers | Tests | CV (%) | Bias (%) | TEa (%) | Sigma | Total error | Quality goal Index | Problem |
|---|---|---|---|---|---|---|---|---|
| A1 | aPTT-L1 | 2.92 | 6.39 | 15 | 2.95 | 11.2 | 1.46 | Inaccuracy |
| | aPTT-L2 | 2.32 | | | 3.70 | 10.3 | | |
| | INR-L1 | 2.31 | 5.24 | 15 | 4.23 | 9.04 | | |
| | INR-L2 | 2.12 | | | 4.61 | 8.73 | | |
| | Fibrinogen-L1 | 4.33 | 3.78 | 20 | 3.75 | 10.9 | | |
| | Fibrinogen-L2 | 2.71 | | | 5.99 | 8.24 | | |
| | D-dimer-L1 | 2.57 | 3.36 | 30 | 10.4 | 7.61 | | |
| | D-dimer-L2 | 2.60 | | | 10.2 | 7.66 | | |
| A2 | aPTT-L1 | 2.65 | 6.61 | 15 | 3.17 | 10.9 | | |
| | aPTT-L2 | 2.23 | | | 3.77 | 10.3 | | |
| | INR-L1 | 2.84 | 5.84 | 15 | 3.22 | 10.5 | | |
| | INR-L2 | 2.21 | | | 4.13 | 9.50 | | |
| | Fibrinogen-L1 | 4.04 | 1.37 | 20 | 4.61 | 8.03 | | |
| | Fibrinogen-L2 | 2.46 | | | 7.56 | 5.44 | | |
| | D-dimer-L1 | 3.67 | 1.54 | 30 | 7.75 | 7.59 | | |
| | D-dimer-L2 | 2.44 | | | 11.7 | 5.56 | | |
| A3 | aPTT-L1 | 2.91 | 6.86 | 15 | 2.80 | 11.7 | 1.57 | Inaccuracy |
| | aPTT-L2 | 2.27 | | | 3.58 | 10.6 | | |
| | INR-L1 | 2.21 | 4.85 | 15 | 4.60 | 8.49 | | |
| | INR-L2 | 2.24 | | | 4.53 | 8.55 | | |
| | Fibrinogen-L1 | 4.19 | 3.30 | 20 | 3.98 | 10.2 | | |
| | Fibrinogen-L2 | 2.69 | | | 6.21 | 7.74 | | |
| | D-dimer-L1 | 2.41 | 1.84 | 30 | 11.7 | 5.82 | | |
| | D-dimer-L2 | 2.71 | | | 10.4 | 6.31 | | |
| B | aPTT-L1 | 2.23 | 5.68 | 15 | 4.18 | 9.36 | | |
| | aPTT-L2 | 2.25 | | | 4.14 | 9.40 | | |
| | INR-L1 | 3.21 | 5.91 | 15 | 2.83 | 11.2 | 1.23 | Inaccuracy |
| | INR-L2 | 3.02 | | | 3.01 | 10.9 | | |
| | Fibrinogen-L1 | 4.65 | 3.14 | 20 | 3.63 | 10.8 | | |
| | Fibrinogen-L2 | 3.42 | | | 4.93 | 8.78 | | |
| | D-dimer-L1 | 2.82 | 1.48 | 30 | 10.1 | 6.13 | | |
| | D-dimer-L2 | 2.23 | | | 12.8 | 5.16 | | |

CV: Coefficient of variation; TEa: Total allowable error; aPTT: Activated partial thromboplastin time; INR: International normalized ratio; L1: Level 1; L2: Level 2.

Westgard's study, which assessed the performance of coagulation parameters on the Sysmex CS5100 analyzer using the Six Sigma metric methodology, found sigma values >6 for both control levels of the PT parameter. Additionally, for the aPTT parameter, sigma values were found to be <3 for level 1 and 5–5.99 for level 2 [7]. In Shaikh et al. [9]'s study assessing the performance of PT and fibrinogen parameters on the Sysmex CS-2000i analyzer using the Six Sigma metric methodology, sigma values <3 were identified for both control levels of PT. Moreover, for fibrinogen, sigma values were <3 for level 1 and 3–3.99 for level 2. In the study by Aksit et al. [10] on the Sysmex CS2500 analyzer evaluating the performance of coagulation parameters using the Six Sigma metric methodology and utilizing six months of data, sigma values <3 were identified for PT at level 2, and for fibrinogen, sigma values were <3 for both control levels. They observed a distribution ranging from 3 to 5.99 for other parameters and control levels. By calculating the Quality Goal Index (QGI) for parameters exhibiting low performance, they identified that the issues with these parameters were related to imprecision. In our study, the identified issues associated with parameters exhibiting low performance were attributed to inaccuracy. In the study conducted by Uge et al. [11], using three months of data to evaluate the performance of PT and aPTT parameters using the Six Sigma metric methodology, sigma values of 4–4.99 were determined for level 1 of PT and 3–3.99 for level 2. Furthermore, for aPTT, sigma values of 4–4.99 were identified for both control levels.

Variations in sigma values across studies may result from differences in instruments used, variations in internal quality control materials and calibrators, and the utilization of different external quality assurance programs. Moreover, considering the perceived influence of the number of partic-

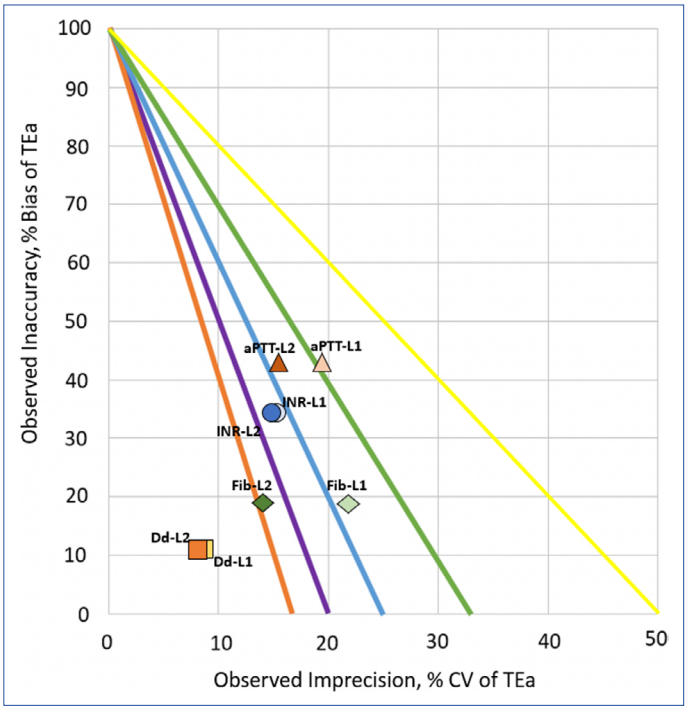


**Figure 1.** Normalized operating specifications chart for coagulation analyzer A1, showing 6 months average sigma values (September 2023-February 2024).

CV: Coefficient of variation; TEa: Total allowable error; aPTT: Activated partial thromboplastin time; INR: International normalized ratio; Fib: Fibrinogen; Dd: D-dimer; L1: Level 1; L2: Level 2. In the figure, the area between the orange line and the abscissa and ordinate indicates greater than 6 sigma, the area between the orange and purple lines indicates 5-6 sigma, the area between the purple and blue lines indicates 4-5 sigma, the area between the blue and green lines indicate 3-4 sigma and the area between the green and yellow lines indicates 2-3 sigma.

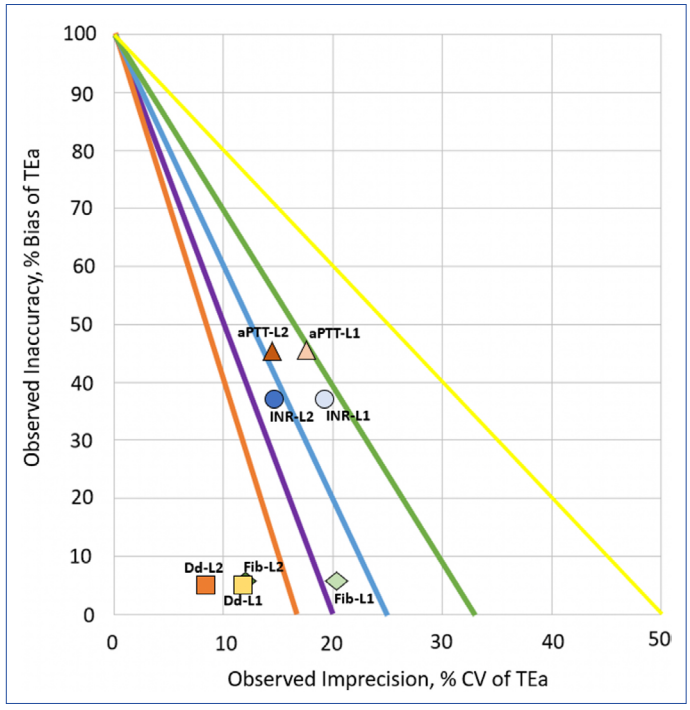


**Figure 2.** Normalized operating specifications chart for coagulation analyzer A2, showing 6 months average sigma values (September 2023-February 2024).

CV: Coefficient of variation; TEa: Total allowable error; aPTT: Activated partial thromboplastin time; INR: International normalized ratio; Fib: Fibrinogen; Dd: D-dimer; L1: Level 1; L2: Level 2. In the figure, the area between the orange line and the abscissa and ordinate indicates greater than 6 sigma, the area between the orange and purple lines indicates 5-6 sigma, the area between the purple and blue lines indicates 4-5 sigma, the area between the blue and green lines indicate 3-4 sigma and the area between the green and yellow lines indicates 2-3 sigma.

ipants in the EQC programs, the participant count holds significance. The acquisition of TEa data from different sources also contributes to variability. Sigma values calculated with higher TEa recommendations indicate better performance as a formulation necessity. In this context, El Sharkawy suggests harmonization for sigma calculations [12]. We used the TEa sources in our study because they have been used in studies in the current literature. In particular, the EFLM biological variation-based targets are very narrow and may be difficult to achieve under routine laboratory conditions. In addition, different implementations, such as using IQC or EQC data for bias calculation, may have contributed to the different results. Our study found that the control levels with poor performance (<3 sigma) were level 1. We also found that the CV% values of poorly performing level 1 controls were higher than level 2 controls. Level 1 control levels are normal control levels, and level 2 control levels are also pathological levels. Generally, analyses are performed at lower concentrations than pathological controls in normal level controls. The reason why we detected higher CV% values may be the possibility that those studied at low concentrations generally have higher CV% values than those studied at high concentrations.

Our study identified that the issues with parameters exhibiting low performance were attributed to inaccuracy, as revealed by the QGI. High bias in EQC results may be due to problems with the transport of the control material, improper storage of the control material, or random error during the analyzer's analysis of the control material. In addition, it is important in EQC programs to compare external quality results with a peer group using the same method and analyzer. The EQC program we use compares our external quality results according to the peer group when the number of peer groups is 9 or higher and with laboratories using the same measurement method when the number of peer groups is less than 9. Since the number of peer groups for aPTT and PT was insufficient, our external quality results were compared with laboratories with the same method (approximately 1000). We could have obtained lower bias results if the peer group was provided. Additionally, when evaluating parameters with high bias values, the acceptability of the Z score used in assessing EQC results within acceptable ranges (+2>x>-2) also supports the notion that the high bias is linked to the scarcity of participants in the peer group. Thus, despite Z scores falling within acceptable ranges, the presence of high bias values presents challenges for the Six Sigma metric methodology. In this context, there are also proposals that the Six Sigma methodology should be revised or that different formulations should be employed [13].

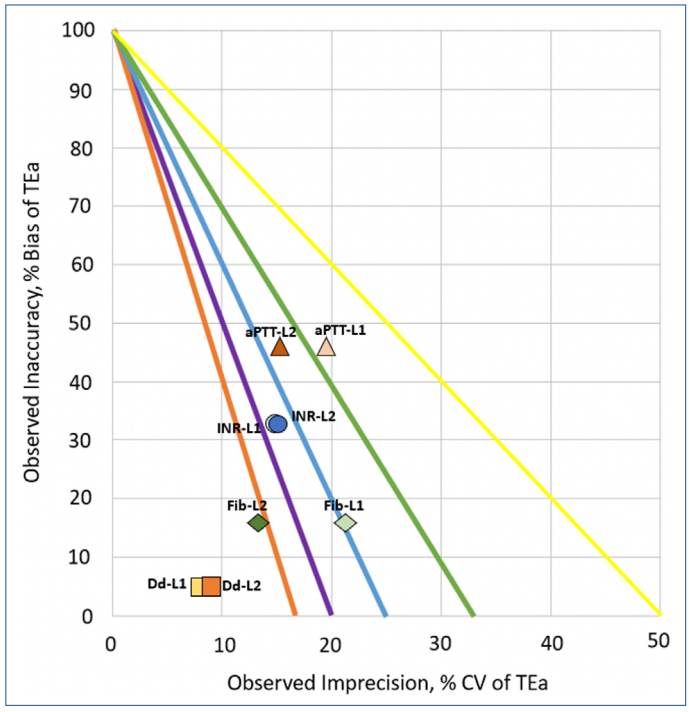


**Figure 3.** Normalized operating specifications chart for coagulation analyzer A3, showing 6 months average sigma values (September 2023-February 2024).

CV: Coefficient of variation; TEa: Total allowable error; aPTT: Activated partial thromboplastin time; INR: International normalized ratio; Fib: Fibrinogen; Dd: D-dimer; L1: Level 1; L2: Level 2. In the figure, the area between the orange line and the abscissa and ordinate indicates greater than 6 sigma, the area between the orange and purple lines indicates 5-6 sigma, the area between the purple and blue lines indicates 4-5 sigma, the area between the blue and green lines indicate 3-4 sigma and the area between the green and yellow lines indicates 2-3 sigma.

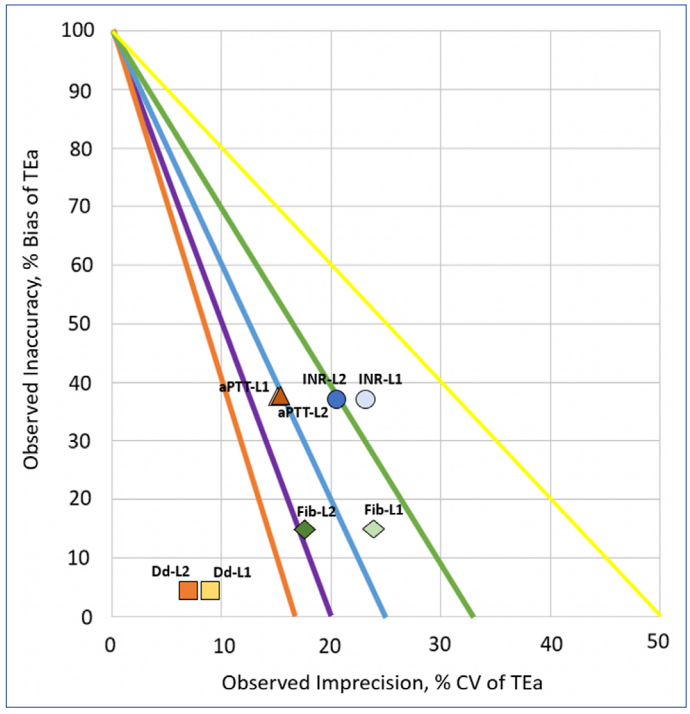


**Figure 4.** Normalized operating specifications chart for coagulation analyzer B, showing 6 months average sigma values (September 2023-February 2024).

CV: Coefficient of variation; TEa: Total allowable error; aPTT: Activated partial thromboplastin time; INR: International normalized ratio; Fib: Fibrinogen; Dd: D-dimer; L1: Level 1; L2: Level 2. In the figure, the area between the orange line and the abscissa and ordinate indicates greater than 6 sigma, the area between the orange and purple lines indicates 5-6 sigma, the area between the purple and blue lines indicates 4-5 sigma, the area between the blue and green lines indicate 3-4 sigma and the area between the green and yellow lines indicates 2-3 sigma.

According to the sigma levels obtained in our study, we will apply Westgard multirules in terms of IQC. In our study, we will apply Westgard multirule $1_{3s}/2_{2s}/R_{4s}/4_{1s}/8_{x}$ for control levels <4 sigma values, Westgard multirule $1_{3s}/2_{2s}/R_{4s}/4_{1s}$ for control levels between 4 and 4.99 sigma values, and Westgard multirule $1_{3s}/2_{2s}/R_{4s}$ for control levels between 5 and 5.99 sigma values.

The outstanding performance of the D-dimer parameter across all four instruments, reaching world-class standards at both control levels, reflects positively on our laboratory's performance. Additionally, the absence of poor performance in any device or control levels for fibrinogen is noteworthy and holds significant implications for our laboratory in terms of patient safety. Our laboratory works very intensively, so there are lot changes approximately once a month due to kit consumption. A potential limitation of our study may stem from inter-lot variations observed across months. Nevertheless, the satisfactory CV values for our internal quality suggest that this factor has minimal impact. In a multicenter study by Kitchen et al. [14] evaluating the performance of the Cobas T711 coagulation analyzer, they found that the Cobas T711 coagulation analyzer was reliable and accurate in routine practice for analyzing coagulation parameters. Our study is notable for being the first to employ the Six Sigma metric methodology to assess performance using the Cobas T711 coagulation analyzer. Furthermore, evaluating four different analyzers adds further interest to our findings.

In conclusion, we have identified areas in our laboratory that need improvement by evaluating the analytical performance of coagulation parameters using the Sigma metric method. Conducting assessments and improvement initiatives focused on the analytical process can help improve the reliability of laboratory results.

**Ethics Committee Approval:** The study was approved by The Ankara Etlik City Hospital Scientific Ethics Committee (No: 2024-364, Date: 24/04/2024).

**Authorship Contributions:** Concept – A.S., F.U., C.S.; Design – A.S., C.S., S.I.H., A.K.; Supervision – A.S., R.S., S.I.H., A.K.; Funding – A.S., S.T., S.I.H.; Materials – A.S., R.S., S.I.H.; Data collection &/or processing – A.S., C.S., S.T., A.K.; Analysis and/or interpretation – A.S.; Literature search – A.S., F.U., R.S., S.T.; Writing – A.S., F.U., S.I.H., A.K.; Critical review – A.S., F.U., C.S., R.S., S.T., S.I.H., A.K.

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

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## References


1. Plebani M. Errors in clinical laboratories or errors in laboratory medicine? Clin Chem Lab Med 2006;44(6):750–9. [CrossRef]
2. Mao X, Shao J, Zhang B, Wang Y. Evaluating analytical quality in clinical biochemistry laboratory using Six Sigma. Biochem Med Zagreb 2018;28(2):020904. [CrossRef]
3. Westgard S, Bayat H, Westgard JO. Analytical sigma metrics: A review of six sigma implementation tools for medical laboratories. Biochem Med Zagreb 2018;28(2):020502. [CrossRef]
4. Fasano T, Bedini JL, Fle PA, Jlaiel M, Hub K, Datta H, et al. Multisite performance evaluation and sigma metrics of 20 assays on the Atellica chemistry and immunoassay analyzers. Clin Chem Lab Med 2019;58(1):59–68. [CrossRef]
5. Ozdemir S, Ucar F. Determination of sigma metric based on various TEa sources for CBC parameters: The need for sigma metrics harmonization. J Lab Med 2022;46(2):133–41. [CrossRef]
6. Data Innovations. Allowable total error table. Available at: https://www.datainnovations.com/allowable-total-errortable. Accessed April 30, 2024.
7. Westgard JO. Sigma-metrics of a Sysmex Coagulation Analyzer cs5100. Available at: https://www.westgard.com/sysmex-cs5100.htm. Accessed April 30, 2024.
8. Kumar BV, Mohan T. Sigma metrics as a tool for evaluating the performance of internal quality control in a clinical chemistry laboratory. J Lab Physicians 2018;10(2):194–9. [CrossRef]
9. Shaikh MS, Moiz B. Analytical performance evaluation of a high-volume hematology laboratory utilizing sigma metrics as standard of excellence. Int J Lab Hematol 2016;38(2):193–7. [CrossRef]
10. Aksit M, Colak A, Basok BI, Zeytinli Aksit M, Fidan M, Kazar M, et al. Evaluation of analytical quality of coagulation parameters by sigmametric methodology. Int J Med Biochem 2023;6(2):84–9. [CrossRef]
11. Uge M, Koseoglu MH, Cuhadar S, Narin F. Six sigma management and evaluation in coagulation tests. J DEU Med 2022;36(1):1–8. [CrossRef]
12. El Sharkawy R, Westgard S, Awad AM, Ahmed AOI, Iman EH, Gaballah A, et al. Comparison between sigma metrics in four accredited Egyptian medical laboratories in some biochemical tests: An initiative towards sigma calculation harmonization. Biochem Med Zagreb 2018;28(2):020711. [CrossRef]
13. Badrick T, Theodorsson E. Six Sigma – is it time to re-evaluate its value in laboratory medicine? Clin Chem Lab Med 2024;2024:0377. [CrossRef]
14. Kitchen S, de Maat M, Nagler M, Jones R, Lowe A, Burden J, et al. System performance evaluation of the cobas t 711 and cobas t 511 coagulation analyzers in routine laboratory settings. Blood Coagul Fibrinolysis 2020;31(7):459–68. [CrossRef]

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Research Article

# Association of *rs121912724* polymorphism in *Apolipoprotein A1* gene with diabetic dyslipidemia and correlation with serum High-density lipoprotein Cholesterol, Triglycerides and Low-density lipoprotein Cholesterol levels

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## Abstract

**Objectives:** To identify the association of the *rs121912724* polymorphism in the *Apolipoprotein A1* gene with diabetic dyslipidemia and its correlation with serum High-density Lipoprotein Cholesterol (HDL), Triglycerides, (TG) and Low-density Lipoprotein Cholesterol (LDL-C) levels.

**Methods:** Two groups were taken, comprising 150 diabetic dyslipidemia (group I) patients and 150 healthy controls (group II). Demographic and biochemical data were analyzed using Statistical Package for Social Sciences 26 by applying the Student independent t-test. DNA samples of both groups were subjected to TETRA-Amplification Refractory System polymerase chain reaction, and allele A and C of the *rs121912724* polymorphism were amplified. The association of the *rs121912724* polymorphism with the disease was studied using Fisher's exact test and Cochran-Armitage test. The correlation between the polymorphism and levels of lipid levels was determined using Pearson correlation on SPSS version 27.

**Results:** The levels of HDL-C, LDL-C, and TG were significantly higher than in healthy group ($p<0.000$). The genotypic count of homozygous AA was 137, 12 of heterozygous AC, and 1 of homozygous CC in group I. The genotypic count of homozygous AA was 138, 12 of heterozygous AC in group II, with no homozygous CC. There was no association of *rs121912724* observed with the development of diabetic dyslipidemia, and a negative correlation of *rs121912724* with deranged levels of HDL-C, LDL-C, and TG was observed.

**Conclusion:** The *rs121912724* polymorphism in the APOA1 gene was not associated with diabetic dyslipidemia. There was no correlation found between the polymorphism and deranged levels of HDL-C, TG, and LDL-C.

**Keywords:** Anti-inflammatory, lecithin cholesterol acyltransferase, TETRA ARMS PCR, Type 2 diabetes

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Diabetic dyslipidemia is characterized by decreased levels of high-density lipoprotein (HDL-C), elevated levels of triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) [1]. Dyslipidemia is the most common metabolic abnormality linked with diabetes. Patients with diabetes are at greater risk of developing chronic coronary heart diseases than the general population due to increased cholesterol and triglyceride levels [2]. Diabetic dyslipidemia arises predominantly due to genetic predispositions, irregularities in metabolic processes, and various environmental factors. Multiple

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investigations have emphasized the significance of addressing diabetic dyslipidemia through a comprehensive approach that considers gene mutations or polymorphisms associated with the proteins and enzymes responsible for lipoprotein metabolism [3]. There has been an increased focus on identifying genetic variations linked to diabetic dyslipidemia due to growing apprehensions regarding the potential risks of micro- and macrovascular complications [4].

Apolipoprotein A-I (apoA-I) is a main structural protein of HDL particles and plays an essential role in cholesterol uptake. It collects cholesterol and phospholipids through its association with the ATP-binding cassette (ABCA1), which results in the formation of pre-1 HDL particles [5]. ApoA-I is a 29 kDa protein of 243 amino acids, encoded by the *APOA1* gene. The *APOA1* gene has 4 exons, and its cytogenetic location is on chromosome 11q23. A dysfunctional *APOA1* gene contributes to impaired reverse cholesterol transport in type 2 diabetes (T2D) patients [6]. The size and structure of HDL are determined by the apoA-I protein, which aids in the reverse transport of cholesterol by solubilizing its lipid component. It increases the flow of cholesterol from extrahepatic tissues into hepatic cells for metabolism by promoting lecithin cholesterol acyltransferase (LCAT) to generate cholesterol esters [7]. It has been noticed that patients with hyperglycemia or dyslipidemia for the same length of time or to the same degree can have significantly different susceptibilities to the cardiovascular and microvascular effects of metabolic syndrome [8].

It is considered that genetic mutations in the *APOA1* gene can lead to cardiac diseases [9]. ApoA-I, as a component of HDL, displays various cellular functions, including anti-inflammatory and anti-cancer [10, 11]. An *in vitro* study showed pro-inflammatory effects of oxidized apoA-I and HDL particles isolated from human atheroma, thus hampering cholesterol clearance [12]. A meta-analysis reported the protective role of the minor allele -75G/A polymorphism in *APOA1* against coronary artery disease [13]. The rs670 SNP in the *APOA1* gene demonstrated a decrease in LDL-cholesterol, fat mass, body weight, insulin resistance, and high levels of HDL cholesterol in A allele carriers.

The *rs121912724* SNP has not been previously studied in association with diabetic dyslipidemia, resulting in limited available literature on this particular genetic variant. The *APOA1* gene polymorphisms linked to diabetic dyslipidemia have not been previously reported in Pakistan. This study identifies *rs121912724* as a novel SNP associated with diabetic dyslipidemia in the Pakistani population. The genetic data from this research enhance our understanding of the molecular pathophysiology and genetic susceptibility to diabetic dyslipidemia, providing deeper insights into individual predisposition to this condition.

## Materials and Methods

### Study setting

This case-control study was conducted within the Department of Biochemistry and Molecular Biology, Army Medical College, Rawalpindi, from June 2023 to December 2023.

This study is an extension of an ongoing project in our lab on diabetic dyslipidemia. Ethical approval was obtained from the institutional ethical review committee (ERC/ID/271) before commencing the study, in compliance with established ethical standards.

A total of 300 DNA samples, already extracted from diabetic dyslipidemia patients and healthy controls, were collected via non-probability purposive sampling, based on ease of access to samples that were selected randomly from the targeted study population.

The study design was a Population Association Case-Control Study.

Written informed consent was obtained from patients. Participants were informed about the purpose of the research and the procedure. Two milliliters of whole blood were drawn by a phlebotomist via venipuncture using aseptic measures and poured into EDTA-containing vacutainers. A thermocole box containing ice packs was used to hold the sample tubes until their transportation to the CREAM lab of Army Medical College, Rawalpindi. An additional 2 mL blood sample was sent for serum lipid analysis using a Microlab Spectrophotometer, which ensures accurate and precise quantification of lipid concentrations through light absorption. The triglycerides were measured using the Randox Triglycerides Assay Kit, Lot Number 123456, while HDL cholesterol was determined with the Randox HDL-C Assay Kit, Lot Number 789012. LDL cholesterol was calculated using the Friedewald formula: LDL-C=Total Cholesterol – HDL-C – (Triglycerides/5), with total cholesterol measured using the Randox Total Cholesterol Assay Kit, Lot Number 345678. These methods and kits were selected to ensure reliable results for diagnosing and monitoring lipid-related conditions.

The diagnosis of dyslipidemia was made according to the guidelines of the National Cholesterol Education Program (National Cholesterol Education Program, 2001). The criteria included total cholesterol levels of ≥6.2 mmol/L, low-density lipoprotein cholesterol (LDL-C) levels of >4.15 mmol/L, triglycerides (TG) levels of ≥2.3 mmol/L, and high-density lipoprotein cholesterol (HDL-C) levels of <1.2 mmol/L.

Samples were stored at 4˚C for further molecular analysis. All samples were stored after proper labeling with identification numbers given to the subjects.

DNA extraction of these samples was done via the FavorPrep™ Blood Genomic DNA Extraction Kit marketed by Favorgen R (Cat No: Kit# FABGK001).

In this study, this stored DNA was taken for analysis of a novel SNP.

The threshold probability for rejecting the null hypothesis type I error rate was 0.05.

### Study subjects

A total of 150 diabetic dyslipidemia patients and 150 healthy controls were included in this study.

### Inclusion criteria

Newly diagnosed type 2 diabetic patients with dyslipidemia, aged 25–70 years, and both genders.

### Exclusion criteria

Type 1 diabetes mellitus patients, gestational diabetes patients, type 2 diabetes mellitus patients with other complications, patients on lipid-lowering therapy, and patients suffering from dyslipidemia-associated diseases, i.e., hypothyroidism, Cushing syndrome, polycystic ovarian syndrome, chronic liver disease, and cardiovascular diseases.

### Molecular analysis

#### *Gel electrophoresis*

DNA was analyzed both qualitatively and quantitatively using agarose gel electrophoresis. To prepare 1% agarose gel, 0.8 g of powdered agarose was dissolved in 40 mL of 1x TBE (0.89 M Tris-Borate and 0.032 M EDTA). Gel casting was preceded by the addition of 2–4 µL of ethidium bromide (EtBr) dye. The extracted DNA samples were visualized on a gel documentation system (BioRad).

### Primer designing

The sequence of the selected SNP was retrieved from the National Centre for Biotechnology Information (NCBI). The sequence was downloaded from the biological database "Ensembl". The SNP primers were generated using the bioinformatics web application tool "Primer 1". Primers specific to Tetra-ARMS PCR were developed. The sequence of the forward outer primer was 5´ACAGGGCCGAGCTGTTCGCACA3´, the reverse outer primer was 5´GCGTGACCTCCACCTTCAGCAAACG3´, the forward inner primer was 5´CCTTGCTCATCTCCTGCCTCAGGCC3´, and the reverse inner primer was 5´TGTCACCCAGGGCTCACCCCTGATA3´.

### Data analysis

Demographic and biochemical data were analyzed using SPSS version 26. The Student independent t-test was used to calculate the mean±standard deviation of continuous variables between two groups. DNA samples were analyzed on 1% agarose gel, and TETRA-ARMS polymerase chain reaction was performed to amplify the *rs121912724* A>C polymorphism in the *APOA1* gene. Fisher's exact test and Cochran-Armitage tests were applied to find the association of the polymorphism with the disease. The correlation between polymorphism and HDL, TG, and LDL levels was determined using the Pearson correlation test.

**Table 1. Comparison of Mean±SD of lipid profile and age between two groups**

| Variables | Diabetic dyslipidemia patients | Healthy controls | p |
|---|---|---|---|
| Age | 52.0±10.80 | 44.14±15.58 | 0.000** |
| Serum HDL | 0.79±0.25 mmol/L | 1.30±0.37 mmol/L | |
| Serum LDL | 2.9847±1.08 mmol/L | 2.31±0.70 mmol/L | |
| Serum TG | 3.22±1.75 mmol/L | 1.43±0.60 mmol/L | |

**: Highly significant. SD: Standard deviation; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TG: Triglycerides.

## Results

The mean age of diabetic dyslipidemia cases was 52±10.80 years, and that of healthy controls was 44.14±15.59 years. Out of 300 subjects, 37% were females, and 63% were males. Among 150 cases, 35% were females, and 65% were males, while in 150 healthy controls, 39% were females, and 61% were males. A significant difference was observed between levels of serum HDL, serum LDL, and serum TG in cases and controls, where the p-value was 0.000 (p≤0.05), as shown in Table 1.

The *rs121912724* polymorphism in the *APOA1* gene involves an A>C transition according to the SNPdb database, with allele C considered less common and the risk allele in this study. The genotypic distribution for homozygous AA was 275, heterozygous AC was 24, and homozygous CC was 1 (n=300). There was an insignificant difference observed in genotypic distribution between group I and group II for AA, AC, and CC. The AA genotype was the most common in both group I and group II compared to the AC and CC genotypes. There was also no association of the CC genotype of *rs121912724* with the development of diabetic dyslipidemia, and the AA homozygous genotype was displayed as protective (Table 2).

An insignificant correlation coefficient (r) value was observed between the *rs121912724* polymorphism and levels of HDL-C, LDL-C, and TG in the diabetic dyslipidemia group I (Table 3). The p-value was greater than 0.05 and was observed as insignificant.

**Table 2. Association of *rs121912724* with diabetic dyslipidemia**

| Groups | Distribution of *rs121912724* | | | Cochran Armitage trend test | Fisher Exact value |
|---|---|---|---|---|---|
| | AA | AC | CC | | |
| Diabetic dyslipidemia patients (n=150) | 137 | 12 | 1 | $X^2$=0.1554 0.6935 | F=257 0.586 |
| Healthy controls (n=150) | 138 | 2 | 0 | | |

**Table 3. Correlation between *rs121912724* polymorphism and levels of HDL-C, LDL-C and TG**

| | HDL-C | LDL-C | TG |
|---|---|---|---|
| Diabetic dyslipidemia patients | | | |
| *rs121912724* | | | |
| Pearson correlation coefficient | -0.152 | 0.052 | -0.057 |
| Sig. (2-tailed) | 0.063 | 0.530 | 0.486 |
| n | 150 | 150 | 150 |
| Healthy controls | | | |
| *rs121912724* | | | |
| Pearson correlation coefficient | 0.150 | 0.112 | 0.002 |
| Sig. (2-tailed) | 0.067 | 0.171 | 0.076 |
| n | 150 | 150 | 150 |

**: Correlation is significant at the 0.01 level (2-tailed). HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; TG: Triglycerides; Sig.: Significant.

## Discussion

The International Diabetes Federation stated a 26.7% prevalence of diabetes in Pakistan in 2022 [14]. The genetic predisposition associated with dyslipidemia in type 2 diabetes is not well documented in the Pakistani population. This study found a novel single nucleotide polymorphism (SNP), not previously documented in diabetic dyslipidemia patients in Pakistan. This study showed that *rs121912724* with genotype AA was not associated with diabetic dyslipidemia, as the p-value was less than 0.005. Moreover, an insignificant correlation was observed between deranged levels of HDL-C, LDL-C, TG, and AA+AC genotypes. A study reported an association of rs5072 in the *APOA1* gene with dysregulated TG metabolism and increased susceptibility to dyslipidemia in schizophrenia patients [15]. The *APOA1/C3/A4/A5-ZPR1-BUD13* gene cluster was studied in dyslipidemia patients and found an association between polymorphism of the gene cluster and dyslipidemia [16]. Hypertriglyceridemia was observed as the most common abnormality in diabetic dyslipidemia, either due to poor clearance or increased production [17]. These studies augment the generation of a pool of genetic data in our population to understand the molecular etiology of dyslipidemia. In the current study, an equal frequency of the AA genotype in cases and controls has proposed a protective role of *rs121912724* against diabetic dyslipidemia. A previous study on apolipoprotein AI transgenic diabetic mice suggested the beneficial impact of an increased ratio of *APOA1/HDL* in diabetic people having reduced cardiovascular disease [18]. A study conducted on 135 type 2 diabetic patients reported an association of 83 C>T polymorphism in the *APOA1* gene with deranged levels of TG, HDL-C, and LDL-C, and predicted susceptibility to myocardial infarction [19].

The genetic data obtained from this research offered significant insights and expanded our comprehension of the molecular mechanisms and genetic predisposition to diabetic dyslipidemia in our population. This information enriched our knowledge base, enabling more thorough and detailed assessments of individual susceptibility to this condition. The insignificant association observed in this study may be due to the small sample size and needs to be studied in a larger population to gain better insight. Moreover, ethnicity is an important factor to be taken into account for polymorphism studies, as it contributes to the inconclusive effect of genotype.

## Conclusion

The study concludes an insignificant association of *rs121912724* in the *APOA1* gene with diabetic dyslipidemia and a negative correlation between the SNP and levels of HDL-C, TG, and LDL-C.

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**Ethics Committee Approval:** The study was approved by The Army Medical College Ethics Committee (No: ERC/ID/271, Date: 21/06/2023).

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## References

1. Hedayatnia M, Asadi Z, Zare-Feyzabadi R, Yaghooti-Khorasani M, Ghazizadeh H, Ghaffarian-Zirak R, et al. Dyslipidemia and cardiovascular disease risk among the MASHAD study population. Lipids Health Dis 2020;19(1):42. [CrossRef]
2. Schofield JD, Liu Y, Rao-Balakrishna P, Malik RA, Soran H. Diabetes dyslipidemia. Diabetes Ther 2016;7(2):203–19.

3. Sanghera DK, Hopkins R, Malone-Perez MW, Bejar C, Tan C, Mussa H, et al. Targeted sequencing of candidate genes of dyslipidemia in Punjabi Sikhs: Population-specific rare variants in GCKR promote ectopic fat deposition. PLoS One 2019;14(8):e0211661. [CrossRef]
4. Tetik Vardarlı A, Harman E, Bozok Çetintaş V, Kayıkçıoğlu M, Vardarlı E, Zengi A, et al. Polymorphisms of lipid metabolism enzyme-coding genes in patients with diabetic dyslipidemia. Anatol J Cardiol 2017;17(4):313–21.
5. Femlak M, Gluba-Brzózka A, Ciałkowska-Rysz A, Rysz J. The role and function of HDL in patients with diabetes mellitus and the related cardiovascular risk. Lipids Health Dis 2017;16(1):207.
6. Athyros VG, Doumas M, Imprialos KP, Stavropoulos K, Georgianou E, Katsimardou A, et al. Diabetes and lipid metabolism. Hormones (Athens) 2018;17(1):61–7. [CrossRef]
7. Mangaraj M, Nanda R, Panda S. Apolipoprotein A-I: A molecule of diverse function. Indian J Clin Biochem 2016;31(3):253–9.
8. Zhang Z, Wang Q, Yao J, Zhou X, Zhao J, Zhang X, et al. Chemokine receptor 5, a double-edged sword in metabolic syndrome and cardiovascular disease. Front Pharmacol 2020;11:146. [CrossRef]
9. Chistiakov DA, Orekhov AN, Bobryshev YV. ApoA1 and ApoA1-specific self-antibodies in cardiovascular disease. Lab Invest.2016;96(7):708–18. [CrossRef]
10. Catapano AL, Pirillo A, Bonacina F, Norata GD. HDL in innate and adaptive immunity. Cardiovasc Res 2014;103(3):372–83.
11. Neyen C, Mukhopadhyay S, Gordon S, Hagemann T. An apolipoprotein A-I mimetic targets scavenger receptor A on tumor-associated macrophages: A prospective anticancer treatment? Oncoimmunology 2013;2(6):e24461. [CrossRef]
12. Huang Y, DiDonato JA, Levison BS, Schmitt D, Li L, Wu Y, et al. An abundant dysfunctional apolipoprotein A1 in human atheroma. Nat Med 2014;20(2):193–203. [CrossRef]
13. Xu LB, Zhou YF, Yao JL, Sun SJ, Rui Q, Yang XJ, et al. Apolipoprotein A1 polymorphisms and risk of coronary artery disease: A meta-analysis. Arch Med Sci 2017;13(4):813–9. [CrossRef]
14. International Diabetes Federation. IDF diabetes atlas - 10th edition. Available at: https://diabetesatlas.org/idfawp/resource-files/2021/07/IDF_Atlas_10th_Edition_2021.pdf. Accessed Aug 21, 2024.
15. Fan L, You Y, Fan Y, Shen C, Xue Y. Association between ApoA1 gene polymorphisms and antipsychotic drug-induced dyslipidemia in schizophrenia. Neuropsychiatr Dis Treat 2021;17:1289–97. [CrossRef]
16. Bai W, Kou C, Zhang L, You Y, Yu W, Hua W, et al. Functional polymorphisms of the APOA1/C3/A4/A5-ZPR1-BUD13 gene cluster are associated with dyslipidemia in a sex-specific pattern. PeerJ 2019;6:e6175. [CrossRef]
17. Jialal I, Singh G. Management of diabetic dyslipidemia: An update. World J Diabetes 2019;10(5):280–90. [CrossRef]
18. Barrett TJ, Distel E, Murphy AJ, Hu J, Garshick MS, Ogando Y, et al. Apolipoprotein AI) promotes atherosclerosis regression in diabetic mice by suppressing myelopoiesis and plaque inflammation. Circulation 2019;140(14):1170–84. [CrossRef]
19. Casillas FA, Martínez Fernández DE, Valle Y, Aceves Ramírez M, Parra-Reyna B, Sarabia Pulido S, et al. APOA1 (-75 G>A and 83 C>T) and APOB (2488 C>T) polymorphisms and their association with myocardial infarction, lipids and apolipoproteins in patients with type 2 diabetes mellitus. Arch Med Sci 2021;18:1438–45. [CrossRef]

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Research Article

# CD3/CD28 costimulation-induced NF-KB activation, is not mediated by protein metallothionein 2A and FAS associated death domain

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## Abstract

**Objectives:** The immune response depends on T cell activation, which is triggered by signals from receptors such as CD28 and TCR/CD3, resulting in T cell proliferation and programmed cell death (AICD). This control avoids disorders like immunity and cancer. These receptors are stimulated by Antigen-Presenting Cells (APCs), which set off a signaling cascade that activates the important transcription factor NF-κB. Degrading inhibitory proteins is necessary for NF-κB activation, which permits it to reach the nucleus and regulate gene expression.

**Methods:** Semi-quantitative RT-PCR was used to assess the levels of metallothionein 2A mRNA in primary T cells to validate the results of the microarray analysis. Five- to six-week-old male C57BL/6 wild-type mice were used in the investigation. Mouse primary T cells from lymph nodes were suspended aseptically, and anti-CD3/CD28 was used to activate the cells. The cells were transfected with plasmid DNA using a Gen Pulser, and the T cells were separated by magnetic cell sorting. Following the synthesis of cDNA from total RNA, microarray analysis was used to assess variations in gene expression. The microarray results were validated by RT-PCR. Western blotting was used to confirm protein expression, and flow cytometry with CD69 was used to measure cell death. Immunoreactive bands were visible in co-immunoprecipitation assays using monoclonal anti-MT2A and FADD antibodies.

**Results:** This work focused on FAS-associated Death Domain (FADD), MT2A, and NF-κB gene expression profiles in mouse primary T cells before and after anti-CD3/CD28 stimulation, utilizing microarray analysis. The results shed light on these genes' functions in AICD and T cell activation. The cascade triggers the activation of transcription factors necessary for T cell proliferation and cytokine production, such as NF-κB. Degradation of inhibitory IκB proteins is necessary for NF-κB activation, which permits NF-κB to reach the nucleus and control gene transcription. The involvement of PLC-γ1 in CD3/CD28-induced NF-κB activation has been highlighted by recent studies.

**Conclusion:** The processes that determine whether T cells divide or undergo apoptosis are still unknown, despite advances. To compare the gene expression of FADD, MT2A, and NF-κB in mouse primary T cells before and after anti-CD3/CD28 stimulation, this work used microarray analysis. The purpose of our research is to shed light on these genes' functions in T cell activation and activation-induced cell death (AICD).

**Keywords:** Apoptosis, CD28, NF-KB, T cell activation, T-cell receptor (TCR/CD3)

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The process of activating T cells is intricate and requires close collaboration. It is among the immunological response's most significant occurrences. In a physiologically normal state, lymphocytes are at rest. The potency of signals received by the T-cell receptor (TCR/CD3) and a series of co-stimulatory receptors, the most notable of which is CD28, determines the capacity of naïve T cells to multiply and acquire potential roles [1]. Concurrent ligation of these receptors sets off signals that

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promote T cell differentiation and proliferation. Recent data have shown that the signals naïve T cells respond to when T cell receptors are ligated not only cause effector cell production and differentiation, but also cause immature thymocytes to undergo apoptosis and mature T cells to experience activation-induced cell death (AICD) [2, 3].

Apoptotic cell death is a crucial process that regulates cellular homeostasis during development, differentiation, and other pathophysiological conditions; dysregulation of this process is linked to a number of diseases, such as cancer, stroke, autoimmunity, tumors that develop drug resistance, and the advancement of some degenerative diseases [4, 5]. After activation and multiplication, T cells must reduce in number to mount an effective immune response. This is because unregulated T cell proliferation increases the risk of cancer and several autoimmune disorders [6].

Antigen-presenting cells (APCs) simultaneously stimulate costimulatory receptors like CD28 and the T-cell receptor (TCR)/CD3 complexes, which activate T cells. Lck, ZAP70, and Syk are examples of cytosolic tyrosine kinases that get activated in response to TCR/CD3 stimulation [7]. LAT, SLP76, Vav, and Grb2 are among the adaptor proteins that these tyrosine kinases phosphorylate in turn [8–10]. The effector proteins, which include small GTPases, phospholipase C-γ1 (PLC-γ1), and protein kinases/phosphatases, are further recruited by the phosphorylated adapter proteins. This results in the activation of several transcription factors, such as NF-AT, AP-1, and NF-κB, which ultimately regulate the transcription of cytokines and T-cell proliferation. Crucially, activation of NF-κB in T cells requires more than just TCR/CD3 complex stimulation. For the best activation of CD28, costimulation through its ligand, B7, is necessary for NF-κB activation, leading to T cell activation and optimal production of interleukin-2 (IL-2) and other cytokines [11].

The Rel homology DNA-binding domains found in the NF-κB family of transcription factors can exist as different homo- or heterodimers [12]. Their interactions with a group of cytoplasmic inhibitory proteins known as IκB govern their function. IκB proteins sequester NF-κB in the cytoplasm by hiding NF-κB's nuclear localization signal. IκB kinase (IKK) is activated when cells are treated with different stimuli, such as tumor necrosis factor alpha (TNF-α), IL-1ε, phorbol myristate acetate (PMA), or costimulation of TCR/CD3 and CD28 (CD3/CD28 costimulation). After IKK phosphorylates IκB, IκB in the 26S proteasome complex is quickly ubiquitinated and proteolyzed [13]. The nuclear localization signal of NF-κB is then revealed by the degradation of IκB. After making a quick translocation into the nucleus, NF-κB dimers interact with cognate κB enhancer regions to alter the transcription of several genes related to the immunological and inflammatory responses [14]. While the exact mechanism by which CD3/CD28 costimulation-induced signaling pathways result in NF-κB activation remains unclear, current research suggests that PLC-γ1 is critical for this process [15].

The signal transduction pathways responsible for T cell activation or apoptosis induced by TCR/CD3 have been better understood in recent times. Nevertheless, the switch that dictates whether primary T cells undergo activation-induced proliferation or apoptosis remains poorly understood, despite being of great interest. In the meantime, a number of significant findings on the post-activation gene expression profiles of human T cells were obtained by the use of microarray analysis [16, 17]. Therefore, in this work, we report the use of an Affymetrix Mouse Genome 430 2.0 Array to evaluate the variations in the gene expression patterns of mouse primary T cells prior to and following stimulation with anti-CD3/CD28 for either 0 or 18 hours. The changes in FADD, MT2A, and NF-κB expression before and after T cell activation are of particular interest to us. The results of this study might provide more molecular evidence for understanding the function of FADD, MT2A, and NF-κB in T cell activation and its associated AICD.

## Materials and Methods

BD Pharmingen (San Diego, CA) supplied the antibodies against CD3, CD28, Th1 (FITC-conjugated), and CD69 (FITC-conjugated). Trizol came from Invitrogen, whereas the antibody against mouse MT2A was acquired from Zymed (Zymed, CA). The supplier of CFSE was Sigma in St. Louis, MO.

### Cell culture and plasmid transfection

Five- to six-week-old male wild-type C57BL/6 mice were acquired from SIPPR-BK Experimental Animal Centre in Shanghai, China, and were kept in an environment devoid of pathogens. Fresh RPMI 1640 medium (Gibco, USA) supplemented with 20% FBS, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 1% non-essential amino acids, penicillin (100 U/ml), and streptomycin (100 mg/ml) was used to prepare an aseptic suspension of mouse primary T cells from lymph nodes, which were then diluted to a density of $1\times10^7$ cells/ml. The cells were then kept in an incubator set at 37°C with 95% room air and 5% $CO_2$. The primary T cells were activated by the addition of anti-CD3/CD28. At 37°C and 5% $CO_2$, Jurkat cells were grown in RPMI 1640 medium enhanced with 10% fetal calf serum [18]. Transfection was done using a Gene Pulser (Bio-Rad, Hercules, CA) at 250 V (950 μF) with pRK5-MT2A plasmid DNA.

### Preparing an aseptic suspension of mouse primary T cells from lymph nodes

To prepare an aseptic suspension of mouse primary T cells from lymph nodes, start by euthanizing the mouse using an approved method and sterilize the area with 70% ethanol. Carefully dissect the lymph nodes, typically from the cervical, axillary, and inguinal regions, using sterile instruments. Place the lymph nodes in a Petri dish containing sterile, cold PBS (phosphate-buffered saline). Mince the lymph nodes into small pieces and gently grind them using a sterile glass slide or plunger to release the cells into the PBS. Filter the cell suspension through a 70 μm cell strainer to remove debris and obtain a single-cell suspension. Centrifuge the suspension at 300xg for 5–10 minutes at 4°C, discard the supernatant, and resuspend the cell pellet in a suitable volume of sterile culture medium. Count the cells using a hemocytometer and adjust the concentration as

needed for downstream applications. Maintain aseptic techniques throughout the process to prevent contamination.

### Western blot analysis

The protocol for the immunoblot analysis was followed. On 12% SDS-PAGE gels, equal volumes of protein (50 µg) were separated. Next, the protein was blotted onto PVDF (polyvinylidene fluoride) membranes using an electrophoretic technique. The membrane was exposed to Kodak X-Omat Blue film (NEN Life Science Products) after being developed using enhanced chemiluminescence reagent (Amersham Life Science Inc.).

### Isolation of T cells by macs magnetic cell sorting

Following the manufacturer's instructions, MACS magnetic cell sorting was used to isolate T cells (Miltenyi Biotec, Germany). In short, biotinylated anti-Thy1.2 antibody was used to mark the primary cells after they were extracted. MicroBeads containing streptavidin were used for secondary labeling. MACS LS columns were used to separate the cell suspension following magnetic labeling. To isolate RNA, cell pellets were again put in Trizol reagent.

### Preparation of biotin-labeled CDNA probes and hybridization

Primary T cells were treated with an RNeasy Kit (Qiagen Crawley, UK) to extract total cellular RNA. According to the manufacturer's instructions (Genetech Inc., CA), double-stranded cDNA probes were made from total RNA, purified using Phase Lock Gel (PLG)-phenol/chloroform extraction, and then concentrated using the ethanol precipitation method. Using a BioArray High Yield RNA Transcript Labeling Kit (Affymetrix, P/N 900182), probes were biotin-labeled. Following labeling, the probes were once again quantified and purified before being hybridized for 16 hours with Mouse Genome 430 2.0 Array chips (Affymetrix). Following hybridization, the array chips underwent staining, cleaning, and scanning using an Affymetrix G2500A GeneArray Scanner. Software called Affymetrix Microarray Suite (MAS) was used to analyze the data.

### RT-PCR Analysis

Using oligo (dT)18 as a primer and AMV Reverse Transcriptase (Promega), total cellular RNA from primary T cells was reverse transcribed into cDNA. The cDNA products were amplified using PCR, ensuring the templates were equal. The sequence of the oligonucleotide primers was as follows: (MT2A) antisense, 5'-GTCGCGTTTCTACATC-3', and sense, 5'-ATGACTGGTGGACAGCAA-3'.

### Detection of CD69 expression level and cell death

After being stimulated with anti-CD3/anti-CD28 for a predetermined amount of time, the cells were collected, twice-washed in staining buffer (PBS+1% BSA+0.2% Sodium Azide+1% HEPES), and simultaneously labeled with FITC-anti-CD69 on ice for 30 minutes to measure CD69. Afterwards, flow cytometry (Becton Dickinson, USA) was used to examine the cells. For every sample, a total of 10,000 events were analyzed.

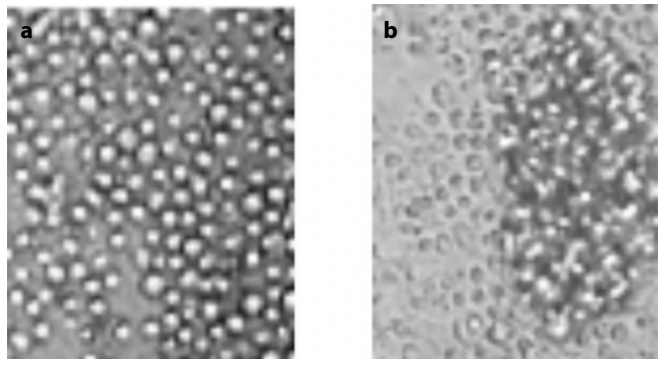


**Figure 1.** Activation of primary T cells with anti-CD3/CD28. Primary lymphocytes were aseptically taken from mice lymph nodes, separated and diluted into $1\times10^7$ cells/ml, further cultured in RPMI 1640 for 18 h with (a) or without anti-CD3/CD28 (b), cells were photographed with a KODAK camera (DX4330) (magnification 100×).

### Co-immunoprecipitation

After 18 hours of maintenance in 1640 Medium, isolated B6 mouse primary T cells, CD3+, and CD28+ cells were incubated for an additional 18 hours in a cell incubator with regulated $CO_2$. Following this, a lysis buffer was used to lyse the cells. After 30 seconds of sonication, cell lysates were centrifuged for 10 minutes at 4°C at 14,000 rpm. After mixing 5 µl of anti-FADD antibody (Cell Signaling Technology, Beverly, MA) with protein lysate in 500 µl lysis buffer, which served as the precipitating antibody, the mixture was incubated at 4°C for two hours on a rocking platform. Protein was immunoprecipitated using Protein A agarose beads. Following SDS-PAGE protein separation and immunoblotting with a monoclonal anti-MT2A antibody (Zymed, USA), the membrane was cleaned and subjected to horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using enhanced chemiluminescence reagents (Pierce, Rockford, IL).

### Electrophoretic mobility shift assay

The DNA binding activity of MT2A was measured in the primary T cell extract from lymphosarcoma cells following a published protocol [19].

## Results

### Activation of primary T cells

The primary lymphocytes from mouse lymph nodes exposed to anti-CD3/CD28 for 18 hours formed large cell aggregates (Fig. 1), which is characteristic of T cell proliferation. In contrast, the control cells did not exhibit this change (Fig. 1), indicating that anti-CD3/CD28 was successful in activating primary lymphocytes.

### Differential gene expression profiles in mouse T cells after stimulation for 18 H

Using biotin-anti-Thy1.2 labeling and MACS magnetic cell sorting, naïve T cells (purity >90%) were separated from cul-

**Table 1. The differentially expressed MT, FADD and NFKB genes in primary T cells after stimulation for 18 H (at least 2-fold)**

| Gene name | Genebank ID | Ratio – 18h |
|---|---|---|
| 1 Metallothionein 2A | NM_005953 | 28.5 |
| 2 Caspase 8 and FADD like apoptosis regulator | NM_009805 | 4.3 |
| 3 NFkB p105 | NM_003998 | 20.2 |

MT: Metallothionein; FADD: FAS-associated Death Domain; NFKB: Nuclear factor kappa B.

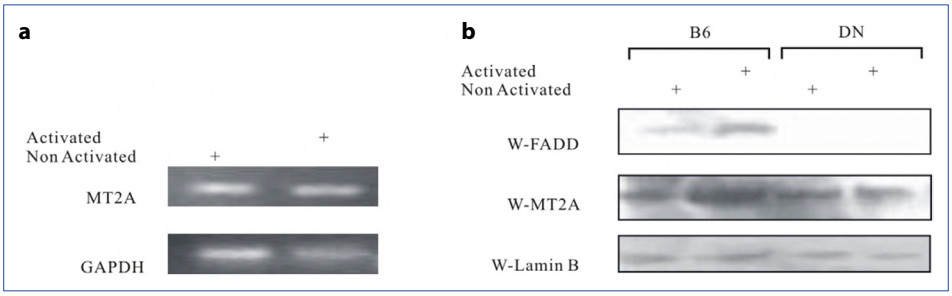


**Figure 2.** Activation-related genes differently expressed in primary T cells after stimulation. Cells were stimulated with anti-CD3/CD28 for 18 h. Verification of microarray results with semi-quantitative RT-PCR (a), and western blot (b) At least 3 independent experiments were performed.

MT: Metallothionein; FADD: Fas-associated death domain protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RT-PCR: Reverse transcription polymerase chain reaction.

tured lymph node cells (data not shown). After 18 hours of activation, the gene expression pattern in T cells was evaluated using the mouse Genome 430 2.0 Array (Affymetrix), which includes over 39,000 transcripts. After stimulation for eighteen hours, a significant variation in the gene expression patterns of T cells was discovered. Specifically, in T cells activated for 18 hours, 4543 genes were found to be up-regulated and 5797 genes down-regulated. Table 1 provides an overview of the general data about genes that were differentially expressed in T cells during an 18-hour stimulation period.

### Genes related to activation of primary T cells

Consistent with previous studies [20–22], our findings demonstrated that numerous genes, including Metallothionein 2A, directly implicated in the activation of primary T cells had notable alterations in their expression levels. Metallothionein 2A mRNA levels in primary T cells were measured using semi-quantitative RT-PCR to confirm the microarray analysis findings. Western blot was used to confirm protein expression. The outcome, as shown in Figure 2, aligned with the microarray findings. The current work suggests that one of the most important steps in the activation of T cells is the disruption of MT2A in naïve T cells. Figure 2 shows the alterations in these genes in primary T cells during an 18-hour stimulation period.

### FADD-DN inhibits mitogen-induced proliferation of mature T cells

Flow cytometry analysis in Figure 3 indicated that CD69 was up-regulated in a time-dependent manner. The precise roles of these two genes—MT and NF-κB—in primary T cell activation are still unknown, despite their being widely documented as functionally significant to cell development.

To explore this, control and pure T cell stimulation via CD3+CD28 cross-linking were examined. Mitogenic antibodies against CD3 and CD28 were used *in vitro* to activate control and FADD-DN transgenic animals. When exposed to CD3/CD28 stimuli, T cells from FADD-DN transgenic mice reacted inappropriately. DN had approximately 28% of CD69 activation compared to B6's 85%. All things considered, these findings demonstrate that disruption of FADD/MORT1's regular function results in a malfunction in the mitogen-induced proliferation of T cells.

### FADD-DN downregulates NF-κB activation

We recently discovered that MT interacts directly with NF-κB in our study. According to the data, there is a correlation between the downregulated NF-κB activity of DN T cells and their lower activation and proliferation (Fig. 4).

Based on the aforementioned findings, it is evident that dominant negative significantly impairs FADD's ability to promote T cell proliferation in FADD-DN T cells. Dysregulation of NF-κB activation and MT2A expression in activated T cells coexist with this phenomenon.

## Discussion

Physiologically, T cell activation is a prolonged process that takes at least a few days, even though the early stage event is crucial for both T cell activation and the initiation of the immune response. Therefore, it is biologically significant to clarify the late phase of T cell activation. Regretfully, there

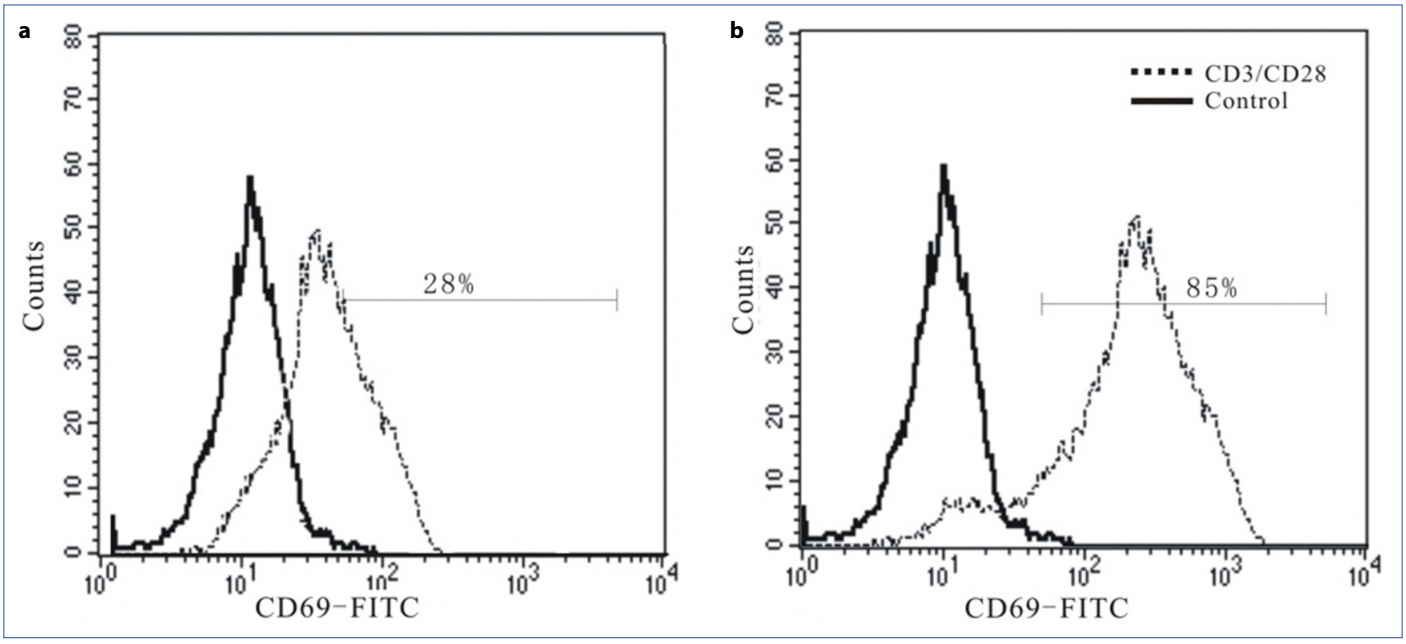


**Figure 3.** Primary T cells were stimulated with anti-CD3/CD28-FITC 0h (a), or 18h (b). At least 3 independent experiments were performed.
FITC: Fluorescein isothiocyanate.

have not been many reports on this issue until now. In the current study, we compared the differentially expressed genes in mouse primary T cells after stimulation for 0 and 18 hours using a Mouse Genome 430 2.0 Array. The results showed that the gene expression profiles of primary T cells were completely different after stimulation for 18 hours. Lastly, a few genes, whose functions are unknown in T cell proliferation, were found to be preferentially regulated in activated T cells, such as FADD.

Three reports on the interaction between MT and NF-κB have recently been published [23–25]. According to Abdel-Mageed et al. [23] MT and NF-κB interact directly, as shown by EMSA supershift analysis with an anti-MT antibody. However, Sakurai et al. [24] found that MT inhibits the degradation of IκB produced by tumor necrosis factor and suppresses the expression of genes that are dependent on NF-κB. Crowthers et al. [25] observed that activated splenocytes from MT-null animals had increased NF-κB activity. To bind zinc, NF-κB's cysteine residues need to be free of thioredoxin [26], although the exact mechanism is yet unknown.

MT2A's mRNA level increased threefold *in vitro* after eighteen hours. A transcription factor called NF-κB is activated by TCR signaling and may control the transcription machinery that activates Fas L [27]. Throughout T cells' lifetime, the two opposing biological processes of cell growth and death are frequently combined, though they sometimes occur separately. In actuality, numerous genes, including JNK and FADD, are involved in the transmission of both proliferation and death signals. Although FADD's phosphorylated form is increasingly understood to be a crucial regulator of cell proliferation, it has long been known to function as an adapter in the traditional CD95-mediated cell apoptosis [28]. Thus, these molecules, including p-FADD and MT2A, may be the crucial factors at the crossroads that will either save or destroy primordial T cells after activation. Our study identified that, after activation, these molecules produce a high amount of proteins. These differentially expressed genes are important for T cell proliferation because they have been linked to a number of cellular functions, such as anti-apoptotic response, cell cycle regulation, and carcinogenesis.


**Figure 4.** NFKB binding of primary T cells after stimulation with CD3/CD28. Primary T cells culture were stimulated by anti-CD3 Abs (CD3) and anti-CD28 Abs (CD28) for 18h. Nuclear extracts were subjected to EMSA using 32P-labeled oligonucleotides containing an NF B binding site. Left two lanes indicate B6 mice and right two lanes for FADD-DN transgenic mice.
NFKB: Nuclear factor kappa B; EMSA: Electrophoretic mobility shift assay; FADD-DN: Fas-associated death domain protein C-terminal death domain.

The results of the analysis of the primary T cell proliferation process using FACS protein level, gene expression mRNA level, and EMSA all pointed to the correlation between FADD, MT2A, and NF-κB in primary T cells and their necessity for the process of T cell activation and proliferation.

## Conclusion

The processes governing whether T cells divide or undergo apoptosis are still unknown, despite advances. Through the use of microarray analysis, the gene expression profiles of FADD, MT2A, and NF-κB in mouse primary T cells were compared before and after anti-CD3/CD28 stimulation in an effort to shed light on these processes. Important discoveries emphasize these genes' functions in T cell activation and activation-induced cell death (AICD). Degradation of inhibitory IκB proteins is necessary for the activation of transcription factors such as NF-κB, which are critical for T cell proliferation and cytokine production. The involvement of PLC-γ1 in CD3/CD28-induced NF-κB activation has also been highlighted by recent investigations. By shedding light on the molecular processes underlying T cell responses, our work advances knowledge of immune control and possible targets for treatment.

**Ethics Committee Approval:** The study was approved by The State Key Laboratory of Pharmaceutical Biotechnology, College of Life Sciences, Nanjing University, Nanjing, China Ethics Committee (No: RP/S/2022/10, Date: 22/09/2022).

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## References

1. Acuto O, Michel F. CD28-mediated co-stimulation: A quantitative support for TCR signalling. Nat Rev Immunol 2003;3(12):939–51. [CrossRef]
2. Wang H, Han P, Qi X, Li F, Li M, Fan L, et al. Bcl-2 Enhances chimeric antigen receptor T cell persistence by reducing activation-ınduced apoptosis. Cancers (Basel) 2021;13(2):197.
3. Donohoe C, Senge MO, Arnaut LG, Gomes-da-Silva LC. Cell death in photodynamic therapy: From oxidative stress to anti-tumor immunity. Biochim Biophys Acta Rev Cancer 2019;1872(2):188308. [CrossRef]
4. D'Arcy MS. Cell death: A review of the major forms of apoptosis, necrosis and autophagy. Cell Biol Int 2019;43(6):582–92.
5. Peng F, Liao M, Qin R, Zhu S, Peng C, Fu L, et al. Regulated cell death (RCD) in cancer: Key pathways and targeted therapies. Signal Transduct Target Ther 2022;7(1):286. [CrossRef]
6. Hatzioannou A, Boumpas A, Papadopoulou M, Papafragkos I, Varveri A, Alissafi T, et al. Regulatory T cells in autoimmunity and cancer: A duplicitous lifestyle. Front Immunol 2021;12:731947. [CrossRef]
7. Mørch AM, Bálint Š, Santos AM, Davis SJ, Dustin ML. Coreceptors and TCR signaling - the strong and the weak of it. Front Cell Dev Biol 2020;8:597627. [CrossRef]
8. Shah K, Al-Haidari A, Sun J, Kazi JU. T cell receptor (TCR) signaling in health and disease. Signal Transduct Target Ther 2021;6(1):412. [CrossRef]
9. Ashouri JF, Lo WL, Nguyen TTT, Shen L, Weiss A. ZAP70, too little, too much can lead to autoimmunity. Immunol Rev 2022;307(1):145–60. [CrossRef]
10. Chen Y, Lu D, Churov A, Fu R. Research progress on NK cell receptors and their signaling pathways. Mediators Inflamm 2020;2020:6437057. [CrossRef]
11. Kolios AGA, Tsokos GC, Klatzmann D. Interleukin-2 and regulatory T cells in rheumatic diseases. Nat Rev Rheumatol 2021;17(12):749–66. [CrossRef]
12. Wang B, Chen C, Liu X, Zhou S, Xu T, Wu M. The effect of combining PD-1 agonist and low-dose Interleukin-2 on treating systemic lupus erythematosus. Front Immunol 2023;14:1111005.
13. Dadwal N, Mix C, Reinhold A, Witte A, Freund C, Schraven B, et al. The multiple roles of the cytosolic adapter proteins ADAP, SKAP1 and SKAP2 for TCR/CD3 -mediated signaling events. Front Immunol 2021;12:703534. [CrossRef]
14. Bacher S, Meier-Soelch J, Kracht M, Schmitz ML. Regulation of transcription factor NF-κB in its natural habitat: The nucleus. Cells 2021;10(4):753. [CrossRef]
15. Marikar FM, Jin G, Sheng W, Ma D, Hua Z. Metallothionein 2A an interactive protein linking phosphorylated FADD to NF-κB pathway leads to colorectal cancer formation. Chin Clin Oncol 2016;5(6):76. [CrossRef]
16. Kumar V, Rosenzweig R, Asalla S, Nehra S, Prabhu SD, Bansal SS. TNFR1 contributes to activation-induced cell death of pathological CD4+ T lymphocytes during ischemic heart failure. JACC Basic Transl Sci 2022;7(10):1038–49. [CrossRef]
17. Rosetti F, Madera-Salcedo IK, Rodríguez-Rodríguez N, Crispín JC. Regulation of activated T cell survival in rheumatic autoimmune diseases. Nat Rev Rheumatol 2022;18(4):232–44.
18. van der Donk LEH, Ates LS, van der Spek J, Tukker LM, Geijtenbeek TBH, et al. Separate signaling events control TCR downregulation and T cell activation in primary human T cells. Immun Inflamm Dis 2021;9(1):223–38. [CrossRef]
19. Marikar FM, Sun QM, Hua ZC. Production of the polyclonal anti-human metallothionein 2A antibody with recombinant protein technology. Acta Biochim Biophys Sin (Shanghai) 2006;38(5):305–9. [CrossRef]
20. Nah J, Seong RH. Krüppel-like factor 4 regulates the cytolytic effector function of exhausted CD8 T cells. Sci Adv 2022;8(47):eadc9346. [CrossRef]
21. Yang K, Kallies A. Tissue-specific differentiation of CD8+ resident memory T cells. Trends Immunol 2021;42(10):876–90.
22. Lee E, Cheung J, Bialkowska AB. Krüppel-like factors 4 and 5 in colorectal tumorigenesis. Cancers (Basel) 2023;15(9):2430.
23. Abdel-Mageed AB, Zhao F, Rider BJ, Agrawal KC. Erythropoietin-induced metallothionein gene expression: role in proliferation of K562 cells. Exp Biol Med (Maywood) 2003;228(9):1033–9. [CrossRef]

24. Sakurai A, Hara S, Okano N, Kondo Y, Inoue J, Imura N. Regulatory role of metallothionein in NF-kappaB activation. FEBS Lett 1999;455(1-2):55–8. [CrossRef]
25. Crowthers KC, Kline V, Giardina C, Lynes MA. Augmented humoral immune function in metallothionein-null mice. Toxicol Appl Pharmacol. 2000 Aug 1;166(3):161–72. [CrossRef]
26. Khan MA, Rabbani G, Kumari M, Khan MJ. Ellagic acid protects type II collagen induced arthritis in rat via diminution of IKB phosphorylation and suppression IKB-NF-kB complex activation: *In vivo* and in silico study. Inflammopharmacology 2022;30(5):1729–43. [CrossRef]
27. Huo J, Xu S, Lam KP. ASK1 mediates Nur77 expression in T-cell receptor mediated thymocyte apoptosis. Cells 2020;9(3):585.
28. Lin Y, Liu J, Chen J, Yao C, Yang Y, Wang J, et al. FADD phosphorylation modulates blood glucose levels by decreasing the expression of insulin-degrading enzyme. Mol Cells 2020;43(4):373–83.

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Research Article

# Association of NLR, MLR, PLR, SII, and SIRI with the stages of chronic kidney disease - A cross-sectional study

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## Abstract

**Objectives:** Chronic Kidney Disease (CKD) is a long-standing metabolic disease manifested by renal impairment, high morbidity and mortality, and causing a huge financial burden. Systemic inflammation and local intrarenal inflammation are found to exacerbate this irreversible condition. White blood cells, platelets, and their derived indices may aid in the assessment of the progression of CKD. This study aimed to assess the alterations of complete blood count and their derived indices in the various stages of chronic kidney disease.

**Methods:** The retrospective cross-sectional study was conducted in the Department of Biochemistry at a tertiary care hospital, Chennai, India. The data were collected from the Medical Records Department from July 2022 to June 2023. The study included chronic kidney disease patients aged 35 to 70 years of both genders. Children, pregnant women, and patients with heart and liver diseases were excluded. The data of the renal profile and complete blood count were collected. Statistical analysis was performed using SPSS software version 16. A $p \leq 0.05$ was considered statistically significant.

**Results:** Among the study participants, 65% were male and were more than 50 years of age. All the derived inflammation index parameters, such as neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lympho- cyte ratio (PLR), systemic immune-inflammation index (SII), and systemic inflammation response index (SIRI). were significantly increased in stage 5 of CKD. Also, SII and SIRI were found to be correlated with other inflammatory variables.

**Conclusion:** Chronic inflammation is considered to be prevalent among CKD patients. Inflammatory markers such as SII and SIRI are simple and cost-effective parameters to routinely assess the staging of CKD and thus initiate appropriate management to improve the quality of life.

**Keywords:** Chronic kidney disease, eGFR, MLR, NLR, PLR, SII, SIRI

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Chronic kidney disease (CKD) affects more than 850 million adults across the globe. CKD refers to the gradual loss of kidney function to the extent that the estimated glomerular filtration rate (eGFR) falls below 60 mL/min/1.73 $m^2$; this pathology should have been present for at least three months, without regard to the etiology [1]. In India, the prevalence of CKD is 1–13% across different regions of the country. The prevalence as given by the International Society of Nephrology's Kidney Disease Data Centre Study is 17% [2]. CKD is insidious in nature and is asymptomatic. CKD equally involves developed and developing countries. In a developing country where the literacy rate is poor, people are unaware of the disease. Hence, early diagnosis of CKD is rarely done, and it is highly challenging. By the time they are diagnosed, the disease has almost reached an irreversible state.

Many factors are found to contribute to the initiation of inflammation, including increased synthesis and release of proinflammatory mediators, release of reactive oxygen

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species and reactive nitrogen species from oxidative stress, metabolic acidosis, alteration in gut microbiota, and altered metabolism of adipose tissue [3]. Risk factors of CKD include diabetes mellitus, hypertension, glomerulonephritis, polycystic kidney disease, and prolonged use of certain medications [4]. GFR is considered to be the marker for the assessment of the extent of kidney damage. Many equations are available to calculate GFR, called eGFR, but the one which has gained wide acceptance is the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI). Based on eGFR, CKD is classified into various stages [5].

In CKD, chronic inflammation is associated with increased levels of proinflammatory markers such as C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor-alpha (TNF-α). Additionally, there is increased malondialdehyde (MDA), which is an oxidative stress marker [6]. Cells such as neutrophils, monocytes, T lymphocytes, and B lymphocytes are part of the immune system. All the immune cells, upon activation, release various inflammatory mediators [7]. Platelets participate in hemostasis, thrombosis, and wound healing, thus contributing to the pathological processes in CKD and associated cardiac complications [8]. In addition to individual white blood cells (WBCs) in circulation, composite inflammatory indices calculated based on the individual WBC counts and platelets serve as easy and cost-effective tools in assessing systemic inflammation. These indices include neutrophil-to-lymphocyte ratio (NLR), monocyte-to-lymphocyte ratio (MLR), platelet-to-lymphocyte ratio (PLR), systemic immune-inflammation index (SII), and systemic inflammation response index (SIRI). SII and PLR values are higher in low-grade inflammation patients who have elevated CRP. Additionally, the platelets reflect a systemic inflammatory response [9]. All the indices—NLR, SIRI, SII, PLR, and MLR—independently have positive predictive values for inflammation [10]. The current study was designed to elucidate the association between eGFR and the inflammatory markers in the various stages of CKD.

## Materials and Methods

### Study design and site

The retrospective cross-sectional study was conducted in the Department of Biochemistry, SRIHER, Chennai, India. The data were collected from the Medical Records Department from July 2022 to June 2023. Two milliliters each of fluoride, citrate, and EDTA plasma and serum samples were collected for the analysis of glucose, glycated hemoglobin (HbA1c), blood urea nitrogen (BUN), creatinine, and complete blood count (CBC). Plasma glucose was analyzed by hexokinase, serum blood urea nitrogen by urease, and serum creatinine by the modified Jaffe's method (Beckman Coulter AU 5800, Beckman Coulter, Inc., California, USA) and HbA1c by ion exchange chromatography method (Tosoh Automated Glycohemoglobin Analyzer HLC-723 G8, Tosoh Corporation, Japan). Among CBC, hemoglobin (Hb) was analyzed by spectrophotometry; white blood cell (WBC), red blood cell (RBC), and platelet counts were analyzed by the impedance method; and differential count by fluorescence flow cytometry (Sysmex XN-3100 six-part CBC analyzer, Sysmex Corporation, Japan). Derived indices were calculated as follows:

NLR = Neutrophil count / Lymphocyte count

MLR = Monocyte count / Lymphocyte count

PLR = Platelet count / Lymphocyte count

SII = (Platelet × Neutrophil) / Lymphocyte [10]

SIRI = (Monocyte × Neutrophil) / Lymphocyte [10]

The laboratory data were obtained from the patient records at the Medical Records Department. eGFR was calculated using the CKD-EPI formula. CKD-EPI stands for Chronic Kidney Disease Epidemiology Collaboration. CKD-EPI formula:

$eGFRcr=142\times\min(Scr/\kappa, 1)\alpha\times\max(Scr/\kappa, 1)^{-1.200}\times0.9938\text{^}Age\times 1.012$ [if female]

### Inclusion criteria

The study included chronic kidney disease individuals aged 35 to 70 years of both genders. Individuals with hypertension, type 2 diabetes mellitus, and obesity were included. Since all the patients were either diabetic or hypertensive, they were on regular antidiabetic or antihypertensive drugs. In addition, they were on maintenance hemodialysis in stage 5 of CKD.

### Exclusion criteria

Children, pregnant women, and patients with heart and liver diseases were excluded.

### Ethics committee approval

Ethics approval was obtained from the Institutional Ethics Committee of the institute (CSP-MED/23/NOV/96/288, dated 28-11-2023). The study was carried out according to the principles outlined in the Declaration of Helsinki. A waiver of informed consent was obtained since the patients were treated and discharged from the hospital.

### Statistics

The obtained data were subjected to checking for normality of distribution. Since the data were found to follow a normal distribution, the continuous variables were expressed as mean and standard deviation. One-way ANOVA with Tukey's HSD post-hoc test was used to compare the variables across the groups. The Pearson correlation coefficient was used to compare the variables. Statistical analysis was performed using SPSS software version 16. A $p\leq0.05$ was considered statistically significant.

## Results

The retrospective study was conducted among 418 CKD patients belonging to stages 1 to 5. Around 65% were male, and 35% were female. Approximately 34.2% of study participants were 61–70 years old, and 34.9% of study partici-

**Table 1. Demographic details of the study participants**

| Variables | Frequency | |
|---|---|---|
| | n | % |
| Gender | | |
| Male | 272 | 65.1 |
| Female | 146 | 34.9 |
| Age (years) | | |
| 35–40 | 41 | 9.8 |
| 41–50 | 88 | 21.1 |
| 51–60 | 146 | 34.9 |
| 61–70 | 143 | 34.2 |
| Distribution of participants according to CKD stages | | |
| CKD 1 | 6 | 1.4 |
| CKD 2 | 2 | 0.4 |
| CKD 3a | 12 | 2.8 |
| CKD 3b | 16 | 3.9 |
| CKD 4 | 100 | 23.9 |
| CKD 5 | 282 | 67.6 |
| Duration of T2DM (years) | | |
| 0–5 | 215 | 51.4 |
| 6–10 | 94 | 22.5 |
| 11–15 | 64 | 15.3 |
| 16–20 | 30 | 7.2 |
| >20 | 15 | 3.6 |
| Duration of HTN (years) | | |
| 0–5 | 241 | 57.7 |
| 6–10 | 107 | 25.6 |
| 11–15 | 36 | 8.6 |
| 16–20 | 24 | 5.7 |
| >20 | 10 | 2.4 |

Expressed as frequency and percentage. CKD: Chronic kidney disease; T2DM: Type 2 diabetes mellitus; HTN: Hypertension.

pants were 51–60 years old. Around 51.1% and 57.7% of the individuals had T2DM and HTN, respectively, for less than five years (Table 1).

The biochemical variables were compared from stages 3a to stage 5 of CKD. Renal parameters were significantly altered across the stages of CKD. There was a consistent decrease in blood hemoglobin level from stage 3a to stage 5. There were statistically significant alterations among the groups. The inflammatory indices, such as NLR, MLR, PLR, SII, and SIRI, were significantly increased in stage 5 compared to other stages of CKD (Table 2).

The study participants were grouped into five based on the duration (in years) of T2DM and HTN: group A (0–5 years), group B (6–10 years), group C (11–15 years), group D (16–20 years), and group E (>20 years) (Tables 3, 4). There was a statistically significant increase in creatinine across the groups based on the duration of T2DM. When compared among groups, there was a statistically significant difference between groups D and E. eGFR was statistically significant in both diabetes mellitus and hypertension (Table 3).

There was a statistically significant difference in the total WBC count across the duration of T2DM. Within the groups, statistically significant differences were obtained between groups D (16–20 years) and E (>20 years). There was a statistically significant difference in absolute neutrophil count (ANC) across the duration of T2DM. Among the groups, there was a statistically significant difference between groups A (<5 years) and B (6–10 years) as well as between groups D (16–20 years) and E (>20 years) (Table 4).

All the composite indices showed a consistent increase in levels across the groups with increasing duration of either T2DM or HTN. NLR showed a statistically significant increase with the advancing duration of T2DM and HTN. MLR showed a statistically significant increase with the advancing duration of T2DM and HTN. Among diabetics, MLR showed a statistically significant difference between groups A (<5 years) and E (>20 years) as well as between groups B (6–10 years) and E (>20 years). Among hypertensives, MLR showed statistically significant differences between groups B (6–10 years) and E (>20 years). PLR showed statistically significant differences across the groups of duration of T2DM and HTN. SII showed statistically significant differences across the groups of duration of T2DM and HTN. Among the diabetics, there were statistically significant differences between groups A and E as well as between groups B and E. SIRI showed statistically significant differences across the groups of duration of T2DM and HTN. Among the diabetics, there were statistically significant differences between groups A and B as well as between groups A and E (Table 5).

BUN was positively correlated with SII. Hb was positively correlated with ALC and negatively correlated with NLR, PLR, and SII. MLR was positively correlated with NLR. PLR was positively correlated with NLR and MLR. SII was positively correlated with NLR, MLR, and PLR. SIRI was positively correlated with NLR, MLR, PLR, and SII (Table 6).

## Discussion

The present retrospective study was conducted among 418 CKD patients belonging to stages 1 to 5 to assess the association of inflammatory markers with the progression of CKD. Since the number of participants in CKD stages 1 and 2 was few, only stages 3a to 5 were included for further analysis and discussion. Similar to other studies, most of the participants were male. This could probably be due to the high predisposition of males to comorbid conditions such as hypertension or diabetes mellitus. Approximately 34.2% of study participants were 61–70 years old, and 34.9% of study participants were 51–60 years old. Around 51.1% and 57.7% of the individuals had T2DM and HTN, respectively, for less than five years (Table 1). The findings in the present study were similar to the study by Aneez et al. [4], where around

**Table 2. Distribution of biomarkers according to the stages of CKD**

| Markers | Stages of CKD | | | | p |
|---|---|---|---|---|---|
| | Stage 3a (n=12) | Stage 3b (n=16) | Stage 4 (n=100) | Stage 5 (n=282) | |
| BUN (mg/dL) | 20.16 (7.94) | 25.86 (13.96) | 29.87 (12.38) | 50.19 (20.79) | <0.001** |
| Creatinine (mg/dL) | 1.55 (0.12) | 2.19 (0.35) | 3.38 (0.69) | 7.56 (2.89) | <0.001** |
| eGFR (mL/min) | 50.5 (4.12) | 34.06 (4.11) | 19.81 (4.34) | 8.15 (2.98) | <0.001** |
| Hb (g/dL) | 11.08 (3.43) | 9.17 (2.76) | 9.05 (1.97) | 8.45 (1.85) | 0.001** |
| | Significance between groups: p1=0.004, p2=0.002, p3=0.003** | | | | |
| FPG | 162.1 (70.1) | 189.2 (66.2) | 154.9 (80.3) | 153.2 (72.8) | 0.33 |
| HbA1c | 7.3 (1.3) | 7.0 (1.7) | 6.8 (1.6) | 6.8 (1.6) | 0.72 |
| PLT ($10^5$ cells/ µL) | 2.39 (1.18) | 2.37 (1.03) | 2.49 (0.97) | 2.29 (1.03) | 0.431 |
| WBC-total (cells/µL) | 7367.5 (1261.99) | 7705.33 (1914.22) | 9147.45 (3636.08) | 8955.60 (3554.19) | 0.201 |
| ANC (cells/µL) | 4833.83 (1060.75) | 5434.06 (1851.93) | 6869.07 (3520.71) | 6744.39 (3290.36) | 0.093 |
| AMC (cells/µL) | 503.25 (143.87) | 393.6 (139.12) | 426.33 (195.56) | 473.11 (267.85) | 0.249 |
| ALC (cells/µL) | 1687.33 (481.12) | 1467.53 (730.04) | 1468.82 (669.12) | 1347.66 (710.74) | 0.198 |
| NLR | 3.05 (0.98) | 4.11 (2.30) | 5.79 (4.58) | 6.67 (5.88) | 0.033* |
| MLR | 0.26 (0.06) | 0.27 (0.11) | 0.35 (0.21) | 0.45 (0.39) | 0.018* |
| PLR | 114.61 (35.65) | 138.86 (38.89) | 191.35 (107.05) | 216.06 (163.75) | 0.037* |
| SII | 123.2 (30.76) | 129.70 (38.76) | 248.44 (92.08) | 385 (123.76) | 0.036* |
| SIRI | 134.91 (47.60) | 139.44 (69.27) | 232.12 (95.40) | 308.62 (99.14) | 0.005** |

Expressed as mean and SD, ANOVA and Tukey post-hoc tests were used. p: p value for comparing between the studied groups; p1: Groups 3a & 3b; p2: Groups 3a & 4; p3: Groups 3a & 5; p4: Groups 3b & 4; p5: Groups 3b & 5; p6: Groups 4 & 5. *: p value statistically significant; **: p value statically highly significant. CKD: Chronic kidney disease; BUN: Blood urea nitrogen; eGFR: Estimated glomerular filtration rate; Hb: Hemoglobin; FPG: Fasting plasma glucose; HbA1c: Glycated haemoglobin; PLT: Platelet; WBC: White blood cell; ANC: Absolute neutrophil count; AMC: Absolute monocyte count; ALC: Absolute lymphocyte count; NLR: Neutrophil to lymphocyte ratio; MLR: Monocyte to lymphocyte ratio; PLR: Platelet to lymphocyte ratio; SII: Systemic immune inflammation index; SIRI: Systemic inflammation response index; SD: Standard deviation.

**Table 3. Distribution of renal markers according to the duration of T2DM and HTN among CKD patients**

| Markers | T2DM/HTN | Duration (years) | | | | | p |
|---|---|---|---|---|---|---|---|
| | | Group A (0–5) | Group B (6–10) | Group C (11–15) | Group D (16–20) | Group E (>20) | |
| BUN (mg/dL) | T2DM | 41.86 (21.00) | 44.85 (23.44) | 46.06 (22.30) | 33.56 (17.24) | 49.38 (19.93) | 0.052 |
| | HTN | 42.09 (21.50) | 44.74 (21.99) | 42.83 (22.83) | 40.25 (20.38) | 45.4 (17.73) | 0.807 |
| Creatinine (mg/dL) | T2DM | 6.44 (3.41) | 5.98 (3.56) | 5.71 (2.74) | 4.46 (2.19) | 5.66 (2.36) | 0.027* |
| | | Significance between groups: p9=0.001** | | | | | |
| | HTN | 5.95 (3.34) | 6.61 (3.45) | 4.98 (2.21) | 6.18 (2.89) | 6.13 (3.01) | 0.119 |
| eGFR (mL/min) | T2DM | 14.13 (12.70) | 11.83 (6.46) | 10.37 (4.69) | 9.08 (2.02) | 8.14 (2.47) | 0.04* |
| | HTN | 15.16 (14.54) | 12.16 (10.40) | 11.61 (4.55) | 8.90 (3.79) | 7.85 (3.48) | 0.037* |

Expressed as mean and SD, ANOVA and Tukey post-hoc tests were used. p: p value for comparing between the studied groups, p1: Groups A & B; p2: Groups A & C; p3: Groups A & D; p4: Groups A & E; p5: Groups B & C; p6: Groups B & D; p7: Groups B & E; p8: Groups C & D; p9: Groups C & E; p10: Groups D & E. *: p value statistically significant, **: p value statically highly significant. T2DM: Type 2 diabetes mellitus; HTN: Hypertension; CKD: Chronic kidney disease; BUN: Blood urea nitrogen; eGFR: Estimated glomerular filtration rate.

59% were male, with a mean age of 58.1 years. In the present study, based on the CKD stages, 68% were in stage 5, 24% were in stage 4 CKD, and the rest of the participants were in lower CKD stages (Table 1). As per Aneez et al. [4], 34% of the study participants are in stage 4 CKD. The present study results are in alignment with the study by Swartling et al. [11], which showed that men have a high rate of mortality, an increased risk of CKD progression, and a rapid decline in eGFR.

In the present study, Table 2 shows the comparison of biomarkers across the groups—stages 3a, 3b, 4, and 5—classified based on eGFR by the CKD-EPI equation. Since the study participants were classified based on eGFR, statistically high significance was expected across the stages of CKD with regard to BUN, creatinine, and eGFR (Table 2). According to Suriyong et al. [12], the major predisposing factors in Asia include an increasing elderly population, low literacy rate, increased preva-

**Table 4. Distribution of CBC markers according to the duration of T2DM and HTN among CKD patients**

| CBC markers | T2DM/ HTN | Duration (years) | | | | | p |
|---|---|---|---|---|---|---|---|
| | | Group A (0–5) | Group B (6–10) | Group C (11–15) | Group D (16–20) | Group E (>20) | |
| Hb (g/dL) | T2DM | 8.80 (2.26) | 8.72 (1.97) | 8.46 (1.81) | 8.86 (1.58) | 8.89 (1.59) | 0.835 |
| | HTN | 8.62 (2.10) | 8.73 (2.12) | 8.92 (1.76) | 9.54 (2.04) | 8.67 (0.90) | 0.329 |
| WBC-total (cells/µL) | T2DM | 8652.55 (3282.64) | 8528 (2966.71) | 10122.7 (4652.07) | 8369 (2439.86) | 10739.23 (4176.37) | 0.006** |
| | | Significance between groups: p10=0.031* | | | | | |
| | HTN | 8851.73 (3591.32) | 8920.18 (3032) | 9198.05 (4118.09) | 8137.5 (2402.60) | 10006 (4626.30) | 0.649 |
| ANC (cells/µL) | T2DM | 6405.42 (3035.86) | 6372.55 (2723.74) | 7840.32 (4547.17) | 5901.9 (2170.40) | 8324.38 (4068.10) | 0.004** |
| | | Significance between groups: p1=0.001**, p10=0.014* | | | | | |
| | HTN | 6656.54 (3403.12) | 6537.78 (2692.88) | 7011.33 (4020.15) | 5845.33 (2158.78) | 7845.9 (4467.49) | 0.499 |
| AMC (cells/ µL) | T2DM | 446.01 (203.02) | 457.51 (345.33) | 465.43 (210.21) | 490.8 (200.57) | 548.61 (246.22) | 0.577 |
| | HTN | 455.36 (265.74) | 450.32 (204.82) | 443.83 (193.61) | 503.25 (222.83) | 539.6 (300.98) | 0.701 |
| ALC (cells/ µL) | T2DM | 1395.98 (739.61) | 1360.27 (658.93) | 1418.25 (626.97) | 1549.23 (626.97) | 1499.76 (744.80) | 0.741 |
| | HTN | 1365.50 (700.37) | 1479.25 (754.70) | 1428.27 (599.14) | 1403.83 (596.07) | 1332.2 (528.55) | 0.713 |
| PLT ($10^5$ cells/ µL) | T2DM | 2.27 (0.98) | 2.409 (1.07) | 2.63 (1.18) | 2.22 (1.107) | 2.52 (0.84) | 0.15 |
| | HTN | 2.31 (1.01) | 2.58 (1.14) | 2.28 (1.12) | 2.14 (0.64) | 2.13 (0.59) | 0.133 |

Expressed as mean and SD, ANOVA and post-hoc tests were used. p: p value for comparing between the studied groups, p1: groups A & B, p2: groups A & C, p3: groups A & D, p4: groups A & E, p5: groups B & C, p6: groups B & D, p7: groups B & E, p8: groups C & D, p9: groups C & E, p10: groups D & E. *: p value statistically significant; **: p value statically highly significant. T2DM: Type 2 diabetes mellitus; HTN: Hypertension; CKD: Chronic kidney disease, CBC: Complete blood count; Hb: Hemoglobin; WBC: White blood cell; ANC: Absolute neutrophil count; AMC: Absolute monocyte count; ALC: Absolute lymphocyte count; PLT: platele; SD: Standard deviation.

lence of comorbidities, and inappropriate use of nonsteroidal anti-inflammatory drugs. In the present study, when compared among the stages of CKD, hemoglobin (Hb) showed statistically significant differences between the stages of CKD, with a consistent decrease in Hb levels as CKD advanced. As kidney disease advances, there is decreased erythropoietin synthesis leading to decreased Hb levels. Additionally, other factors contribute, such as repeated infections, decreased food intake due to loss of appetite, and blood loss during hemodialysis (Table 2). A similar finding was obtained by Kutuby et al. [13] and Akinsola et al. [14] in their studies. In the present study, Hb concentration was 8.45 g/dL in CKD stage 5. Anemia is one of the risk factors for cardiovascular diseases in patients with CKD. According to a study by Pan et al. [15], CKD patients with Hb less than 8.6 g/dL show an increased risk of CKD progression. Demir et al. [16] have demonstrated that CKD with decreasing Hb along with increasing serum creatinine is a risk factor for the occurrence of coronary artery disease.

The kidney is highly vulnerable to inflammatory damage because it receives around 25% of blood circulation, lacks antioxidant activity, has altered gut microbiota along with the loss of the intestinal barrier, and altered intrarenal perfusion distribution [3]. Inflammatory markers such as CRP, pentraxin 3 (PTX3), serum component of amyloid A (SAA), and procalcitonin (PCT) are found to be useful in assessing cardiac complications in CKD patients with eGFR close to normal [17]. According to Alves et al. [18], markers of subclinical CKD include TNF-α, monocyte chemoattractant protein-1 (MCP-1), and E-selectin. However, these inflammatory markers are not easily accessible in countries with a low economic background. Also, since CKD is an asymptomatic disease, frequent screening of individuals, especially those with comorbidities who have the potential to develop CKD, is not feasible. Hence, relying on the composite CBC markers, which are derived from the routine hemogram, will be of use to clinicians in the regular management of CKD.

In the present study, analysis of CBC across the stages of CKD showed that individual WBC counts did not show any statistical significance. However, the inflammatory indices such as NLR, MLR, and PLR were significantly increased across the stages of CKD, with the highest levels in stage 5 compared to other stages of CKD. During inflammation, neutrophils, monocytes, and platelets increase, and they contribute to inflammation by free radical production and thrombosis. Lymphocytes play a protective role in cellular function, but their counts decrease, impairing their function. This is reflected by the alterations in the ratios, indicating disease activity and risk for increased mortality and morbidity in CKD due to cardiac and non-cardiac events. High NLR indicates increased inflammation and worse prognosis in CKD (Table 2). A similar finding was obtained by Yoshitomi et al. [6]. Zhang et al. [7] concluded that MLR is an early indicator of CKD, especially in individuals with GFR closer to normal. NLR and PLR show positive correlations with the urine protein-creatinine ratio (UPCR) and serum creatinine only in the advanced CKD stages [19]. According to Okyay et al. [19],

**Table 5. Distribution of derived inflammatory indices according to the duration of T2DM and HTN among CKD patients**

| Derived inflammatory indices | T2DM/ HTN | Duration (years) | | | | | p |
|---|---|---|---|---|---|---|---|
| | | Group A (0–5) | Group B (6–10) | Group C (11–15) | Group D (16–20) | Group E (>20) | |
| NLR | T2DM | 5.73 (4.48) | 6.15 (5.85) | 7.19 (6.31) | 7.90 (6.07) | 8.82 (5.99) | 0.048* |
| | HTN | 4.95 (2.86) | 5.70 (4.80) | 6.64 (3.43) | 6.73 (2.17) | 7.18 (5.94) | 0.029* |
| MLR | T2DM | 0.35 (0.19) | 0.36 (0.17) | 0.41 (0.28) | 0.44 (0.24) | 0.50 (0.33) | 0.022* |
| | | Significance between groups: p4=0.039*, p7=0.049* | | | | | |
| | HTN | 0.38 (0.21) | 0.39 (0.21) | 0.41(0.18) | 0.46 (0.20) | 0.55 (0.25) | 0.022* |
| | | Significance between groups: p7=0.011* | | | | | |
| PLR | T2DM | 186.12 (100.93) | 189.99 (98.78) | 214.73 (134.15) | 234.35 (87.74) | 264.02 (202.47) | 0.035* |
| | HTN | 191.98 (102.67) | 198.42 (118.05) | 249.18 (146.74) | 245.74 (77.79) | 272.99 (230.08) | 0.045* |
| SII | T2DM | 116.15 (91.42) | 123.33 (91.03) | 131.08 (97.29) | 138.42 (60.03) | 192.00 (133.84) | 0.038* |
| | | Significance between groups: p4=0.01*, p7=0.027* | | | | | |
| | HTN | 109.57 (70.31) | 125.89 (89.99) | 130.44 (60.53) | 147.25 (55.52) | 193.11 (137.55) | 0.013* |
| | | Significance between groups: p4=0.007**, p7=0.048* | | | | | |
| SIRI | T2DM | 216.96 (163.96) | 215.15 (162.22) | 265.37 (218.58) | 294.85 (169.49) | 356.74 (261.46) | 0.008** |
| | | Significance between groups: p1=0.01*, p4=0.013* | | | | | |
| | HTN | 214.20 (150.69) | 219.26 (165.43) | 245.79 (146.39) | 312.97 (173.30) | 346.97 (176.13) | 0.025* |

Expressed as mean and SD, ANOVA and post-hoc tests were used. p: p value for comparing between the studied groups; p1: Groups A & B; p2: Groups A & C; p3: Groups A & D; p4: Groups A & E; p5: Groups B & C; p6: Groups B & D; p7: Groups B & E; p8: Groups C & D; p9: Groups C & E; p10: Groups D & E. *: p value statistically significant; **: p value statically highly significant. T2DM: Type 2 diabetes mellitus; HTN: Hypertension; CKD: Chronic kidney disease; NLR: Neutrophil to lymphocyte ratio; MLR: Monocyte to lymphocyte ratio; PLR: Platelet to lymphocyte ratio; SII: Systemic immune inflammation index; SIRI: Systemic inflammation response index.

CKD patients on dialysis have high NLR, IL-6, and high-sensitivity CRP (hs-CRP) [20]. According to Chen et al. [21], CKD patients with high NLR show an increased risk of poor renal outcomes. Macrophages within adipocytes also produce various pro-inflammatory cytokines [22]. Uduagbamen et al. [23] and Yuan et al. [24] suggest that NLR is higher in CKD patients compared to healthy controls; moreover, NLR is used in the risk assessment of CKD stage 4 patients with replacement therapies.

In the present study, inflammatory indices such as SII and SIRI were significantly increased in stage 5 compared to the other stages of CKD. Both SII and SIRI involve neutrophil and lymphocyte counts along with either platelet or monocyte counts. Hence, these composite indices could be better indicators of inflammation than NLR, PLR, and MLR (Table 2). In the study by Ustundag et al. [9], SII and PLR values are elevated in patients with low-grade inflammation, as indicated by a mild increase in CRP. High PLR predicts the onset of cardiovascular complications. The systemic inflammation response index (SIRI) has a high independent positive predictive value for those individuals with high-risk scores for cardiac diseases [10]. SII and SIRI are associated with CKD prevalence, especially in the US population [25]. There is a strong linkage between SII and CKD in older patients with HTN or T2DM [26]. Screening for CKD is mandatory for older individuals with hypertension or diabetes mellitus [27]. SII levels have a strong association with diabetic nephropathy (DN). According to Li et al. [28], decreased eGFR and urinary albumin excretion are associated with SIRI. SIRI is used to evaluate the risk of mortality in patients with CKD who are subjected to maintenance peritoneal dialysis (PD) [29].

In the present study, the study participants were grouped into five based on the duration of T2DM and HTN. There was no significant alteration in Hb and BUN across the groups with regard to the duration of T2DM or HTN. There was a statistically significant increase in serum creatinine levels across the groups based on the duration of T2DM. However, there was no statistically significant difference with regard to HTN. eGFR was statistically significant across the groups with the duration of either T2DM or HTN. Irrespective of the presence of hypertension or diabetes, the magnitude of kidney damage was almost similar (Table 3). T2DM and HTN are the primary risk factors for CKD and its complications [30]. According to Kaneyama et al. [31], HTN has a higher influence than T2DM on the progression of CKD in Japanese individuals. As per the present study, the minimum duration of hypertension or T2DM was five years. According to Gembillo et al. [32], diabetics show an average time for the onset of CKD of seven to ten years. The risk of progression of CKD to end-stage renal disease (ESRD) and cardiac complications is much higher in T2DM. According to Wang et al. [33], in China, the prevalence of CKD is 10.8%; among them, around 40% and 60% have T2DM and HTN, respectively.

**Table 6. Shows the correlation among the variables in CKD patients**

| | BUN | Creat | Hb | WBC total | eGFR | ANC | AMC | PLT | ALC | NLR | MLR | PLR | SII |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WBC-total | | | | | | | | | | | | | |
| r | 0.131 | -0.025 | 0.018 | | | | | | | | | | |
| p | 0.007 | 0.617 | 0.719 | | | | | | | | | | |
| eGFR | | | | | | | | | | | | | |
| r | -0.486 | -0.631 | 0.249 | -0.035 | | | | | | | | | |
| p | <0.001 | <0.001 | <0.001 | 0.473 | | | | | | | | | |
| ANC | | | | | | | | | | | | | |
| r | 0.165 | -0.023 | -0.053 | 0.964 | -0.066 | | | | | | | | |
| p | 0.001 | 0.638 | 0.280 | <0.001 | 0.178 | | | | | | | | |
| AMC | | | | | | | | | | | | | |
| r | 0.019 | 0.044 | 0.064 | 0.431 | -0.040 | 0.355 | | | | | | | |
| p | 0.701 | 0.365 | 0.191 | <0.001 | 0.411 | <0.001 | | | | | | | |
| PLT | | | | | | | | | | | | | |
| r | 0.029 | -0.093 | 0.087 | 0.333 | 0.119 | 0.271 | 0.154 | | | | | | |
| p | 0.553 | 0.058 | 0.076 | <0.001 | 0.015 | <0.001 | 0.002 | | | | | | |
| ALC | | | | | | | | | | | | | |
| r | -0.136 | -0.088 | 0.288 | 0.250 | 0.157 | 0.010 | 0.177 | 0.303 | | | | | |
| p | 0.005 | 0.074 | <0.001 | <0.001 | 0.001 | 0.842 | <0.001 | <0.001 | | | | | |
| NLR | | | | | | | | | | | | | |
| r | 0.169 | 0.008 | -0.223 | 0.393 | -0.098 | 0.556 | 0.041 | -0.027 | -0.568 | | | | |
| p | 0.001 | 0.878 | <0.001 | <0.001 | 0.046 | <0.001 | 0.396 | 0.581 | <0.001 | | | | |
| MLR | | | | | | | | | | | | | |
| r | 0.100 | 0.069 | -0.182 | 0.162 | -0.111 | 0.258 | 0.527 | -0.039 | -0.490 | 0.646 | | | |
| p | 0.052 | 0.185 | <0.001 | 0.001 | 0.028 | <0.001 | <0.001 | 0.526 | <0.001 | <0.001 | | | |
| PLR | | | | | | | | | | | | | |
| r | 0.170 | 0.026 | -0.220 | 0.046 | -0.062 | 0.188 | -0.039 | 0.372 | -0.544 | 0.659 | 0.551 | | |
| p | <0.001 | 0.595 | <0.001 | 0.347 | 0.207 | <0.001 | 0.428 | <0.001 | <0.001 | <0.001 | <0.001 | | |
| SII | | | | | | | | | | | | | |
| r | 0.224 | 0.008 | -0.210 | 0.498 | -0.069 | 0.622 | 0.113 | 0.423 | -0.398 | 0.794 | 0.531 | 0.805 | |
| p | <0.001 | 0.863 | <0.001 | <0.001 | 0.161 | <0.001 | 0.021 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | |
| SIRI | | | | | | | | | | | | | |
| r | 0.125 | 0.034 | -0.165 | 0.488 | -0.099 | 0.572 | 0.565 | 0.061 | -0.362 | 0.735 | 0.903 | 0.481 | 0.668 |
| p | 0.011 | 0.489 | 0.001 | <0.001 | 0.044 | <0.001 | <0.001 | 0.214 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

BUN: Blood urea nitrogen; Hb: Hemoglobin; WBC: White blood cell; eGFR: Estimated glomerular filtration rate; ANC: Absolute neutrophil count; AMC: Absolute monocyte count; PLT: Platelet; ALC: Absolute lymphocyte count; NLR: Neutrophil to lymphocyte ratio; MLR: Monocyte to lymphocyte ratio; PLR: Platelet to lymphocyte ratio; SII: Systemic immune inflammation index.

In the present study, there was a statistically significant difference in total WBC count and absolute neutrophil count (ANC) according to the duration of T2DM. There was a significant increase in counts when T2DM existed for more than 20 years. There was a statistically significant difference in patients with T2DM with regard to ANC. ANC seemed to be a better marker than total WBC count since there were statistically significant changes in the early stages of CKD (Table 4). There were statistically significant differences in all the composite markers (NLR, MLR, PLR, SII, and SIRI) in CKD patients with either T2DM or HTN (Table 5). Similar findings were reported by other studies [34, 35]. There is an association between MLR and the risk of all-cause death in patients with DN [27]. Turkmen et al. [36] showed that PLR is superior to NLR in assessing inflammation in ESRD patients. T2DM patients with DN have high SII levels [37]. SII and CKD are high in individuals with HTN or T2DM [26]. Low eGFR and albuminuria are positively correlated with SIRI [29].

In the present study, Table 6 shows the correlation among the biochemical variables. With advancing renal disease, hemoglobin and lymphocyte counts decreased. Due to the increase in neutrophils, monocytes, and platelets and the decrease in lymphocytes, the composite indices also showed a similar pattern. All the composite markers showed positive correlations among themselves. However, as individual WBC counts, this type of association was not seen. In T2DM, neu-

trophils have a high predilection to move to the glomerular basement membrane and initiate a sequence of inflammatory reactions, causing further damage to the kidney (Table 6). Neutrophil esterase (NE) is toxic to glomerular cells, thus damaging renal cells [38]. Elderly CKD patients may not present with leukocytosis, unlike younger individuals [39]. The findings of the present study were similar to other studies [40–42]. According to Xiong et al. [43], NLR, MLR, and PLR have the capacity to be strong predictors of 30-day mortality in ESRD patients requiring renal replacement therapy. Thus, the derived composite indices are valid, and appropriate markers can assess the extent of systemic inflammation, especially in resource-limited settings.

### Limitations

The data regarding urine albumin excretion, ESR, and peripheral smears were not obtained. Comparison with well-known inflammatory markers such as IL-6 and hs-CRP could not be done. The drug histories of the participants were not complete enough to analyze the effect of confounders. Also, the confounding effects of diabetes mellitus, hypertension, and obesity were not assessed. A cohort study would have helped in assessing the markers associated with the progression of CKD. Since this was a retrospective study, data on CKD in the earlier stages could not be obtained.

## Conclusion

The study included CKD patients from stage 3a to stage 5, with most of them being male and more than 50 years old. NLR, MLR, PLR, SII, and SIRI are significantly elevated in CKD stage 5 compared to other CKD stages. All the composite indices showed a correlation with renal parameters, hemoglobin, and inflammatory markers. Hence, the inflammatory markers are potential markers for the diagnosis and prognosis of the various stages of CKD. Thus, they can be used to monitor the progression of CKD, especially in individuals with metabolic diseases.

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## References

1. Dorgelo A, Oostrom TAJ. An integrated approach towards a public health perspective on chronic kidney disease. Nat Rev Nephrol 2022;18(3):131–2. [CrossRef]
2. Ene-Iordache B, Perico N, Bikbov B, Carminati S, Remuzzi A, Perna A, et al. Chronic kidney disease and cardiovascular risk in six regions of the world (ISN-KDDC): A cross-sectional study. Lancet Glob Health 2016;4(5):e307–19. [CrossRef]
3. Mihai S, Codrici E, Popescu ID, Enciu AM, Albulescu L, Necula LG, et al. Inflammation-related mechanisms in chronic kidney disease prediction, progression, and outcome. J Immunol Res 2018;2018:2180373. [CrossRef]
4. Aneez FA, Shariffdeen N, Haleem FA, Thangarajah BR, Rasaratnam K. Correlation between neutrophil to lymphocyte ratio and platelet to lymphocyte ratio with proteinuria in different stages of chronic kidney disease. Egypt J Intern Med 2024;36(1):6. [CrossRef]
5. MacIsaac RJ, Ekinci EI, Premaratne E, Lu ZX, Seah JM, Li Y, et al. The Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) equation does not improve the underestimation of Glomerular Filtration Rate (GFR) in people with diabetes and preserved renal function. BMC Nephrol 2015;16:198.
6. Yoshitomi R, Nakayama M, Sakoh T, Fukui A, Katafuchi E, Seki M, et al. High neutrophil/lymphocyte ratio is associated with poor renal outcomes in Japanese patients with chronic kidney disease. Ren Fail 2019;41(1):238–43. [CrossRef]
7. Zhang JM, An J. Cytokines, inflammation, and pain. Int Anesthesiol Clin 2007;45(2):27–37. [CrossRef]
8. Gong S, Wang C, Xiong J, Zhao J, Yang K. Activated platelets, the booster of chronic kidney disease and cardiovascular complications. Kidney Dis (Basel) 2022;8(4):297–307.
9. Ustundag Y, Huysal K, Gecgel SK, Unal D. Relationship between C-reactive protein, systemic immune-inflammation index, and routine hemogram-related inflammatory markers in low-grade inflammation. Int J Med Biochem 2018;1:24–28.
10. Deniz L, Yuksel Y, Arslan HF, Aral H, Bulut U. Evaluation of systemic inflammation markers in predicting cardiac risk in patients with acute chest pain. Int J Med Biochem 2023;6(2):75–83.
11. Swartling O, Rydell H, Stendahl M, Segelmark M, Trolle Lagerros Y, Evans M. CKD progression and mortality among men and women: A nationwide study in Sweden. Am J Kidney Dis 2021;78(2):190–9.e1. [CrossRef]
12. Suriyong P, Ruengorn C, Shayakul C, Anantachoti P, Kanjanarat P. Prevalence of chronic kidney disease stages 3-5 in low- and middle-income countries in Asia: A systematic review and meta-analysis. PLoS One 2022;17(2):e0264393. [CrossRef]
13. Kutuby F, Wang S, Desai C, Lerma EV. Anemia of chronic kidney disease. Dis Mon 2015;61(10):421–4. [CrossRef]
14. Akinsola A, Durosinmi MO, Akinola NO. The haematological profile of Nigerians with chronic renal failure. Afr J Med Med Sci 2000;29(1):13–6.
15. Pan W, Han Y, Hu H, He Y. Association between hemoglobin and chronic kidney disease progression: A secondary analysis of a prospective cohort study in Japanese patients. BMC Nephrol 2022;23(1):295. [CrossRef]

16. Demir M, Kahraman F, Sen T, Astarcioglu MA. The relationship of the hemoglobin to serum creatinine ratio with long-term mortality in patients with acute coronary syndrome: A retrospective study. Medicine (Baltimore) 2023;102(41):e35636.
17. Lavín-Gómez BA, Palomar-Fontanet R, Gago-Fraile M, Quintanar-Lartundo JA, Gómez-Palomo E, González-Lamuño D, et al. Inflammation markers, chronic kidney disease, and renal replacement therapy. Adv Perit Dial 2011;27:33–7.
18. Alves HF, Dos Santo BD, Vallap JG, Da Silva JG, Gonçalves LD, Kimura LH, et al. Inflammatory markers and chronic kidney disease. J Hypertens 2021;39(Suppl 1):e110. [CrossRef]
19. Okyay GU, Inal S, Oneç K, Er RE, Paşaoğlu O, Paşaoğlu H, et al. Neutrophil to lymphocyte ratio in evaluation of inflammation in patients with chronic kidney disease. Ren Fail 2013;35(1):29–36.
20. Liu X, Li X, Chen Y, Liu X, Liu Y, Wei H, et al. Systemic immune-inflammation Index is associated with chronic kidney disease in the U.S. population: Insights from NHANES 2007-2018. Front Immunol 2024;15:1331610. [CrossRef]
21. Chen Y, Nie Y, Wu J, Li C, Zheng L, Zhu B, et al. Association between systemic inflammatory indicators with the survival of chronic kidney disease: A prospective study based on NHANES. Front Immunol 2024;15:1365591. [CrossRef]
22. Tang L, Deng Y, Lai J, Guo X, Liu P, Li S, et al. Predictive effect of system inflammation response index for progression of chronic kidney disease in non-dialyzing patient. J Inflamm Res 2023;16:5273–85. [CrossRef]
23. Uduagbamen PK, Oyelese AT, AdebolaYusuf AO, Thompson MU, Alalade BA, Ehioghae O. Neutrophil lymphocyte ratio as an inflammatory marker in chronic kidney disease: Determinants and correlates. Open J Nephrol 2022;12(1):23–35.
24. Yuan Q, Wang J, Peng Z, Zhou Q, Xiao X, Xie Y, et al; C-STRIDE study group. Neutrophil-to-lymphocyte ratio and incident end-stage renal disease in Chinese patients with chronic kidney disease: Results from the Chinese Cohort Study of Chronic Kidney Disease (C-STRIDE). J Transl Med 2019;17(1):86.
25. Huang P, Mai Y, Zhao J, Yi Y, Wen Y. Association of systemic immune-inflammation index and systemic inflammation response index with chronic kidney disease: observational study of 40,937 adults. Inflamm Res 2024;73(4):655–67.
26. Li L, Chen K, Wen C, Ma X, Huang L. Association between systemic immune-inflammation index and chronic kidney disease: A population-based study. PLoS One 2024;19(2):e0292646.
27. Qiu C, Liu S, Li X, Li W, Hu G, Liu F. Prognostic value of monocyte-to-lymphocyte ratio for 90-day all-cause mortality in type 2 diabetes mellitus patients with chronic kidney disease. Sci Rep 2023;13(1):13136. [CrossRef]
28. Li X, Cui L, Xu H. Association between systemic inflammation response index and chronic kidney disease: A population-based study. Front Endocrinol (Lausanne) 2024;15:1329256.
29. Li J, Li Y, Zou Y, Chen Y, He L, Wang Y, et al. Use of the systemic inflammation response index (SIRI) as a novel prognostic marker for patients on peritoneal dialysis. Ren Fail 2022;44(1):1227–35. [CrossRef]
30. Erfanpoor S, Etemad K, Kazempour S, Hadaegh F, Hasani J, Azizi F, et al. Diabetes, hypertension, and incidence of chronic kidney disease: Is there any multiplicative or additive interaction? Int J Endocrinol Metab 2020;19(1):e101061.
31. Kaneyama A, Hirata A, Hirata T, Imai Y, Kuwabara K, Funamoto M, et al. Impact of hypertension and diabetes on the onset of chronic kidney disease in a general Japanese population. Hypertens Res 2023;46(2):311–20. [CrossRef]
32. Gembillo G, Ingrasciotta Y, Crisafulli S, Luxi N, Siligato R, Santoro D, et al. Kidney disease in diabetic patients: From pathophysiology to pharmacological aspects with a focus on therapeutic inertia. Int J Mol Sci 2021;22(9):4824. [CrossRef]
33. Wang M, Li J, Li Y, Yao S, Zhao M, Wang C, et al. The effects of hypertension and diabetes on new-onset chronic kidney disease: A prospective cohort study. J Clin Hypertens (Greenwich) 2020;22(1):39–46. [CrossRef]
34. Ciray H, Aksoy AH, Ulu N, Cizmecioglu A, Gaipov A, Solak Y. Nephropathy, but not angiographically proven retinopathy, is associated with neutrophil to lymphocyte ratio in patients with type 2 diabetes. Exp Clin Endocrinol Diabetes 2015;123(5):267–71.
35. Huang W, Huang J, Liu Q, Lin F, He Z, Zeng Z, et al. Neutrophil-lymphocyte ratio is a reliable predictive marker for early-stage diabetic nephropathy. Clin Endocrinol (Oxf) 2015;82(2):229–33. [CrossRef]
36. Turkmen K, Erdur FM, Ozcicek F, Ozcicek A, Akbas EM, Ozbicer A, et al. Platelet-to-lymphocyte ratio better predicts inflammation than neutrophil-to-lymphocyte ratio in end-stage renal disease patients. Hemodial Int 2013;17(3):391–6.
37. Guo W, Song Y, Sun Y, Du H, Cai Y, You Q, et al. Systemic immune-inflammation index is associated with diabetic kidney disease in Type 2 diabetes mellitus patients: Evidence from NHANES 2011-2018. Front Endocrinol (Lausanne) 2022;13:1071465. [CrossRef]
38. Zhang R, Chen J, Xiong Y, Wang L, Huang X, Sun T, et al. Increased neutrophil count Is associated with the development of chronic kidney disease in patients with diabetes. J Diabetes 2022;14(7):442–54. [CrossRef]
39. Arai Y, Kanda E, Limori S, Naito S, Noda Y, Sasaki S, et al. Low white blood cell count is independently associated with chronic kidney disease progression in the elderly: The CKD-ROUTE study. Clin Exp Nephrol 2018;22(2):291–8. [CrossRef]
40. Kahraman C, Kahraman NK, Aras B, Coşgun S, Gülcan E. The relationship between neutrophil-to-lymphocyte ratio and albuminuria in type 2 diabetic patients: A pilot study. Arch Med Sci 2016;12(3):571–5. [CrossRef]
41. Zhang J, Lu X, Wang S, Li H. High neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio are associated with poor survival in patients with hemodialysis. Biomed Res Int 2021;2021:9958081. [CrossRef]
42. Kawalec A, Stojanowski J, Mazurkiewicz P, Choma A, Gaik M, Pluta M, et al. Systemic immune inflammation index as a key predictor of dialysis in pediatric chronic kidney disease with the use of random forest classifier. J Clin Med 2023;12(21):6911.
43. Xiong Y, Zhong Q, Zhang Y, Qin F, Yuan J. The Association between the platelet to white blood cell ratio and chronic kidney disease in an aging population: A four-year follow-up study. J Clin Med 2023;12(22):7073. [CrossRef]

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Review

# Pituitary Adenylate Cyclase-Activating Peptide-38 in migraine: A systematic review and meta-analysis

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## Abstract

Migraine is a complex neurological disorder characterized by recurrent headaches accompanied by sensory disturbances. It involves a combination of genetic, environmental, and neurovascular factors. The objective of this systematic review and meta-analysis was to investigate the correlation between migraine and specific mutant genes by examining the association of Pituitary Adenylate Cyclase-Activating Peptide-38 genes with migraine. A comprehensive search was conducted in major scientific databases, such as PubMed, Scopus, and Embase, to identify relevant studies published up until September 2023. The inclusion criteria encompassed studies that examined the genes (PACAP-38) and various aspects of migraine. Two independent reviewers performed data extraction and quality assessment to ensure the accuracy and reliability of the collected information. Seven studies, comprising 737 patients, were included in the final analysis. The random effects model yielded a standardized mean difference (SMD) of 0.55 (95% CI:-0.15 to 1.25, t=1.45, p=0.19). Heterogeneity among the studies was substantial, with $I^2$ indicating 93% variability (95% CI: 84.6% to 96.5%). The heterogeneity was statistically significant (Q= 87.2, df=6, p<0.001). The prediction interval ranged from -1.40 to 2.51. This systematic review and meta-analysis establish a strong link between PACAP-38 and susceptibility to migraine. These findings highlight the significance of genetic factors in migraine development, emphasizing the need for further investigation to elucidate underlying mechanisms and explore the clinical implications of these genetic associations.

**Keywords:** Meta-analysis, migraine, mutant gene, review

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Migraine is a complex neurological disorder that impacts a significant portion of the global population [1]. It is a condition marked by repeated occurrences of headaches ranging from moderate to severe intensity. These headache episodes frequently co-occur with other symptoms, such as nausea, vomiting, heightened sensitivity to light and sound, and, in certain cases, visual disturbances called auras preceding the headache itself [2]. While the precise underlying reason for migraine is still unclear, it is believed to arise from an interplay of genetic influences, environmental triggers, and neurovascular mechanisms in the brain. It is important that the occurrence rates and degree of effects caused by migraine demonstrate variation across different geographic regions and nations [3]. Italy, for instance, has been identified as having the highest rate of migraine-related disability, followed closely by Thailand, Norway, Spain, Brazil, and Ethiopia [4, 5]. These variations in prevalence rates highlight the influence of geographic and cultural factors on the occurrence and management of migraine. In Asia, the estimated average prevalence of migraine is 12.7%, emphasizing the significant impact of the condition on the continent [6]. The World Health Organization (WHO) recognizes migraine as the sixth most debilitating disorder

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worldwide, causing significant disability and a reduced quality of life for affected individuals [7]. The consequences of migraine reach further than just the individual level, as it places a substantial strain on healthcare infrastructures and economic productivity due to the utilization of medical resources and missed workdays or inability to work effectively [8].

Genetic factors play a significant role in the development and susceptibility to migraine. Various studies have identified several mutant genes that may contribute to the pathophysiology of the condition [9]. PACAP-38, a neuropeptide involved in various physiological processes, has been suggested to play a role in the regulation of neuroinflammation and pain modulation in migraine [10]. Understanding the genetic basis of migraine can provide valuable insights into its underlying mechanisms and potential targets for therapeutic interventions [11, 12]. By undertaking a systematic review and meta-analysis that synthesizes studies examining the connection between these mutated genes and migraine, we can assess the collective strength of the evidence and determine the degree to which these genetic variants contribute to the development of the condition. This comprehensive analysis can help identify genetic markers or pathways that may be useful in diagnosing, managing, and developing targeted treatments for individuals with migraine. The findings from this systematic review and meta-analysis have the potential to contribute to the field of migraine research by shedding light on the genetic mechanisms involved in the development and manifestation of the condition.

## Methods

### Search strategy

Multiple electronic databases, including PubMed, Embase, Scopus, and Web of Science, were systematically searched to identify relevant studies published up until the literature search cutoff date of November 1, 2023. The search terms were carefully selected to capture articles related to migraine and specific mutant genes (PACAP-38). The search was limited to human studies and articles published in English.

### Study selection

Two reviewers independently screened the titles and abstracts of the retrieved articles based on predefined inclusion and exclusion criteria. Full-text articles were obtained for potentially relevant studies and further assessed for eligibility. Inclusion criteria included studies investigating the association between the PACAP-38 gene and migraine susceptibility using cross-sectional, cohort, or case-control study designs. Animal studies, case reports, reviews, and conference abstracts were excluded.

### Data extraction

Data from eligible studies were independently extracted by two reviewers using a standardized form. The extracted information included study characteristics, participant demographics, genotype frequencies, and relevant outcomes. Any discrepancies or disagreements were resolved through discussion and consensus.

### Risk of bias assessment

Two separate authors conducted a risk of bias assessment according to the ROBINS-I tool guidelines, ensuring independent evaluation. This evaluation covered six areas: choice of comparison groups, confounding bias, determination of exposure, assessment of outcomes, handling missing data, and presentation of findings. Bias risk was categorized as low, moderate, serious, or critical based on domain assessment [13].

### Data synthesis and statistical analysis

We utilized either random-effects or fixed-effects models based on the heterogeneity observed among the studies included in our analysis. Effect sizes (Mean Standardized Difference, SMD) and their corresponding 95% confidence intervals were calculated. The $I^2$ statistic was used to assess heterogeneity between studies. We conducted subgroup and sensitivity analyses to investigate potential sources of heterogeneity and assess the robustness of our results. The analysis was carried out using R statistical software version 4.0.2.

## Results

### Study characteristics

Following a systematic search, we initially identified 172 studies. Through screening of titles, abstracts, and full texts, seven case-control studies were deemed eligible and met the inclusion criteria (Fig. 1) [14–20]. In total, 737 patients were included in the qualitative analysis, consisting of 427 individuals with migraine and 310 control participants. The included studies spanned diverse geographic regions and age groups to investigate the role of PACAP levels in migraine pathophysiology (Table 1). Across the studies, participants' ages varied widely, ranging from pediatric (8 years) to middle-aged and older adults (42 years). The research was conducted in countries including the United Kingdom [18], Hungary [15], Iran [19], China [14, 17], and Spain [16, 20], reflecting a global perspective on migraine research. The minimum sample size was 9, and the maximum sample size was 106 among the experimental group. Various study designs, including experimental, exploratory, and case-control approaches, were employed, each providing unique insights into PACAP's involvement in migraine. All studies included in our analysis measured PACAP levels utilizing enzyme-linked immunosorbent assay (ELISA) methodology.

### Meta-analysis

The random-effects model showed an SMD of 0.55 (95% CI:-0.15 to 1.5), indicative of a potential positive effect, though lacking statistical significance (p=0.12). Interestingly, the inclusion of PACAP-38 mutation status did not substantially alter the observed effect size. Nevertheless, considerable heterogeneity persisted among studies, with an $I^2$ value of 93%, suggesting notable variability in effect sizes even after accounting for PACAP-38 mutations. The wide prediction interval, spanning from -1.40 to 2.51, underscores the uncertainty surrounding the true effect size (Fig. 2). We attempted a sensitivity and specificity analysis to overcome the heterogeneity.

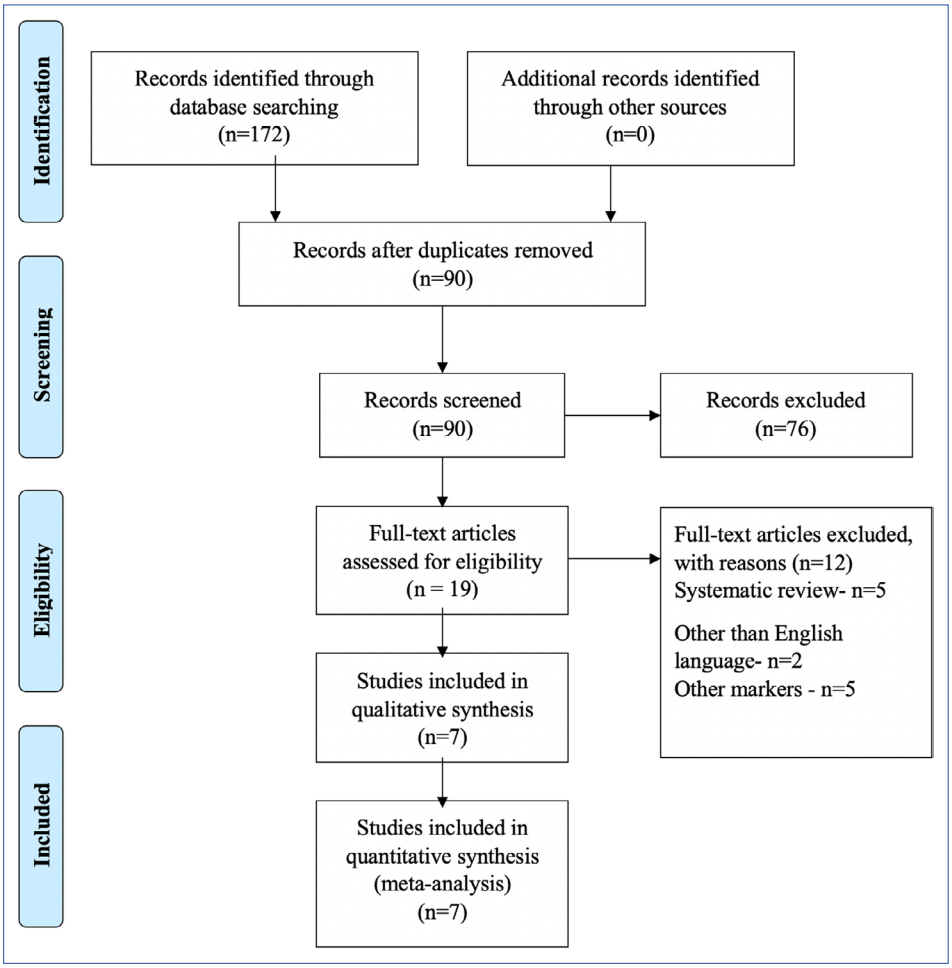


**Figure 1.** A PRISMA diagram illustrating the search strategy.

The updated meta-analysis (Fig. 3), conducted after removing Liu et al. [14] and Perez-Pereda et al. [20], includes 5 studies with a total of 386 observations (250 experimental, 136 control). The random-effects model reveals a standardized mean difference (SMD) of 0.07 (95% CI: [-0.0006, 0.15]), suggesting a small positive effect that is borderline significant (p = 0.05). Notably, the analysis demonstrates very low heterogeneity among the included studies, with $I^2$=0.0% [0.0%; 79.2%], $Tau^2$=0.0003, and a Q statistic of 0.27 (p=0.99). The prediction interval [-0.27, 0.42] indicates the range within which true effect sizes in similar studies are likely to fall.

### Risk of bias

Within the included studies (Table 2), two were identified to possess an overall moderate risk of bias, whereas the remaining studies were classified as having a low risk. Specifically, two studies exhibited moderate risks of bias related to confounding and selection of participants [14, 19]. None of the studies were identified as having serious or critical risks of bias.

## Discussion

The present meta-analysis synthesized findings from seven studies investigating the impact of PACAP levels in migraine pathophysiology. Our analysis indicated an SMD of 0.55, implying a potential positive effect of PACAP on migraine. However, it is important to note that this effect did not reach statistical significance (p=0.12). Notably, the inclusion of PACAP-38 mutation status did not significantly alter the observed effect size, indicating that other factors may contribute to the variability in PACAP levels observed across studies. Despite the intriguing trend toward a positive effect, considerable heterogeneity was evident among the included studies, with an $I^2$ value of 93%. The significant heterogeneity observed suggests notable variability in effect sizes, likely stemming from differences in study populations, methodologies, and clinical characteristics among migraine patients. The wide prediction interval, spanning from -1.40 to 2.51, underscores the uncertainty surrounding the true effect size of PACAP on migraine. This uncertainty may be attributed to the limited number of studies included in the analysis, as well as the complex and multifactorial nature of migraine pathophysiology.

PACAP is believed to have multiple roles in the development of migraines, including the activation of TVS and intracranial vasodilation [17]. Previous studies propose that reduced interictal PACAP-38 concentrations in individuals with migraines may originate from various factors, such as suboptimal brain energy

**Table 1. Characteristics of the included studies**

| Author | Country | Age | Type of the participants | Study design | Sample size | Marker | Result |
|---|---|---|---|---|---|---|---|
| Zagami et al. [18] 2014 | United Kingdom | 37±8 years | Migraine with or without aura | Experimental study | T=15 | PACAP | PACAP levels are elevated migraineurs during spontaneous migraines |
| Tuka et al. [15] 2016 | Hungary | 40.1±12 | Migraine | Exploratory study | T=18 EHC=9 C=9 | PACAP | PACAP-38 levels were found higher during CH attacks compared to the inter-bout phase |
| Han et al. [17] 2015 | China | 40.86±11.97 | Migraine & Tension-type headache | Case control | M=133 TTH=106 C=50 | PACAP | Migraine patients had lower interictal plasma PACAP levels than patients with TTH and healthy controls |
| Cernuda-Morollό et al. [16] 2016 | Spain | 42.8±13.4 | Women with Chronic Migraine | Case control study | T=86 M=35 C=32 | PACAP and vasoactive intestinal peptide (VIP) | No difference found in PACAP levels |
| Pérez-Pereda et al. [20] 2020 | Spain | 41±10 years | Chronic migraine patients | Case control study | CM=101 EM=98 C=97 | PACAP-38, Calcitonin gene-related peptide (CGRP), | PACAP serum levels were higher in CM than in EM or HC |
| Liu et al. [14] 2022 | China | 4–18 years | Children with Migraine | Case control study | T=143 M=76 C=77 | PACAP-38 and calcitonin gene-related peptide (CGRP), | PACAP-38 and CGRP levels in migraine patients during the ictal and interictal periods were higher than those in controls |
| Togha et al. [19] 2021 | Iran | 39 years | Migraine patients | Case control | T=89 CM=36 EM=23 C=30 | TRPV1, PACAP, and VIP | Serum level TRPV1, PACAP, and VIP were higher among the migraine patients |

PACAP: Pituitary Adenylate Cyclase-Activating Peptide; CM: Chronic migraine; EM: Episodic migraine.

levels, mitochondrial abnormalities, imbalances in neuronal $Mg^{2+}$, and the degradation of PACAP-releasing circuits [17]. Administering PACAP might result in an expansion of the superficial temporal artery diameter and a reduction in mean blood flow velocity in the middle cerebral artery [21]. Numerous studies have examined the relationship between plasma PACAP levels and various phases of migraines, yielding mixed results. Specifically, two studies have focused on interictal peripheral levels of PACAP in migraine patients, revealing decreased levels in individuals experiencing migraines [14, 15]. Another study observed decreased interictal serum PACAP levels in patients with EM, but no significant difference was detected between CM patients and controls [22]. The findings from Han et al.'s [17] study indicate a noteworthy decrease in PACAP levels in plasma among individuals with both episodic migraine (EM) and chronic migraine (CM) when compared to those in the healthy control group. Some evidence suggests that sumatriptan treatment may cause decreased PACAP levels [18]. Additionally, interictal PACAP levels have been observed to show a negative correlation with the duration of migraine disease [15, 17]. In contrast to our findings, elevated serum PACAP levels in patients with CM were identified by Pérez-Pereda et al. [20], distinction that more effectively differentiated them from cases of EM and control subjects.

Overall, while our analysis suggests a potential association between PACAP levels and migraine, the findings should be interpreted with caution due to the high heterogeneity and wide prediction interval. Future research should aim to elucidate the underlying mechanisms driving the observed variability in PACAP levels and explore potential therapeutic implications for targeting the PACAP pathway in migraine management. Furthermore, larger-scale studies employing standardized methodologies are necessary to provide deeper insights into the role of PACAP in migraine and its potential as a therapeutic target.

## Limitations

A limitation of our meta-analysis is the intrinsic heterogeneity among the studies included, potentially contributing to the observed variability in effect sizes. The diverse study populations, methodologies, and clinical characteristics of migraine patients across different geographic regions and age groups could have influenced

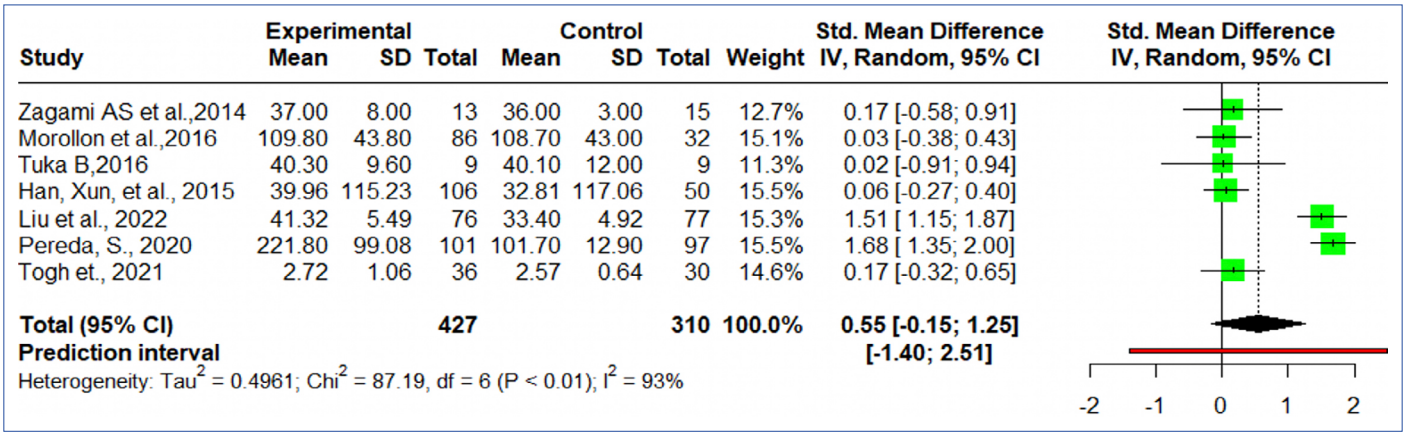


**Figure 2.** Forest plot for the PACAP-38 mutation gene in Migraine.

SD: Standard deviation; CI: Confidence interval; $I^2$: I-squared (measure of heterogeneity); PACAP-38: Pituitary Adenylate Cyclase-Activating Peptide-38.

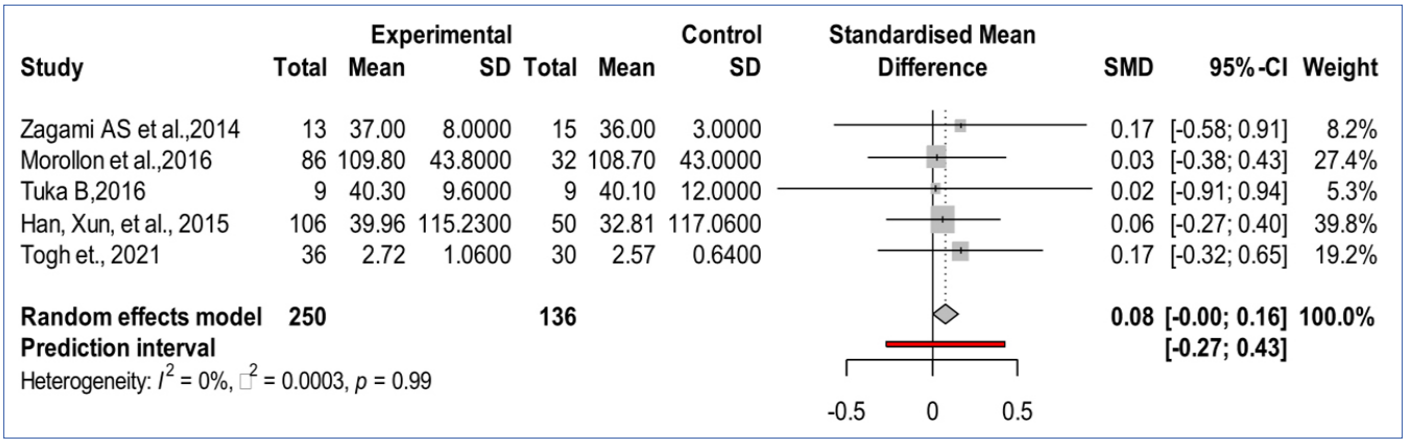


**Figure 3.** Forest plot after sensitivity analysis of the included studies.

SD: Standard deviation; SMD: Standardized mean difference; CI: Confidence interval; $I^2$: I-squared (measure of heterogeneity).

the results. Additionally, the limited number of studies available for inclusion may have restricted the generalizability of our findings and increased the risk of publication bias.

### Strengths

Despite these limitations, our meta-analysis offers several strengths. By synthesizing data from multiple studies, we provided a comprehensive overview of the current literature regarding PACAP levels in migraine pathophysiology. The inclusion of studies from diverse geographic regions and age groups enhances the external validity of our findings, providing insights into PACAP's role in migraine across different populations. Additionally, our analysis utilized rigorous statistical methods, including a random-effects model, to account for heterogeneity among studies and provide robust estimates of effect sizes. Overall, our study contributes to the growing body of evidence on PACAP and migraine, highlighting the need for further research in this area.

**Table 2. Risk of bias**

| Author | Selection of comparison groups | Bias due to confounding | Ascertainment of exposure | Measurement of outcome | Missing data | Reporting of results | Overall risk of bias |
|---|---|---|---|---|---|---|---|
| Zagami et al. [18] 2014 | Low | Low | Low | Low | Low | Low | Low |
| Tuka et al. [15] 2016 | Low | Low | Low | Low | Low | Low | Low |
| Han et al. [17] 2015 | Low | Low | Low | Low | Low | Low | Low |
| Cernuda-Morollón et al. [16] 2016 | Low | Low | Low | Low | Low | Low | Low |
| Pérez-Pereda et al. [20] 2020 | Low | Low | Low | Low | Low | Low | Low |
| Liu et al. [14] 2022 | Moderate | Low | Low | Low | Low | Low | Moderate |
| Togha et al. [19] 2021 | Low | Moderate | Low | Low | Low | Low | Moderate |

## Conclusion

In conclusion, the results of this systematic review and meta-analysis suggest a potential involvement of PACAP-38 genes in migraine development. However, it is imperative to conduct further research to validate these findings and comprehensively grasp the intricate interplay between genetics and migraine. Future research should focus on investigating the potential mechanisms underlying the association between PACAP-38 genes and migraine, as well as examining other genetic and environmental factors that may contribute to the development of this disorder. Moreover, longitudinal studies are necessary to determine the temporal relationship between PACAP-38 genes and the onset of migraine.

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## References

1. Ashina M, Katsarava Z, Do TP, Buse DC, Pozo-Rosich P, Özge A, et al. Migraine: Epidemiology and systems of care. Lancet 2021;397(10283):1485–95. [CrossRef]
2. GBD 2016 Headache Collaborators. Global, regional, and national burden of migraine and tension-type headache, 1990-2016: A systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol 2018;17(11):954–76. [CrossRef]
3. Steiner TJ, Stovner LJ. Global epidemiology of migraine and its implications for public health and health policy. Nat Rev Neurol 2023;19(2):109–17. [CrossRef]
4. Safiri S, Pourfathi H, Eagan A, Mansournia MA, Khodayari MT, Sullman MJM, et al. Global, regional, and national burden of migraine in 204 countries and territories, 1990 to 2019. Pain 2022;163(2):e293–309. [CrossRef]
5. Li XY, Yang CH, Lv JJ, Liu H, Zhang LY, Yin MY, et al. Global, regional, and national epidemiology of migraine and tension-type headache in youths and young adults aged 15-39 years from 1990 to 2019: Findings from the global burden of disease study 2019. J Headache Pain 2023;4(1):126. [CrossRef]
6. Stovner LJ, Hagen K, Linde M, Steiner TJ. The global prevalence of headache: An update, with analysis of the influences of methodological factors on prevalence estimates. J Headache Pain 2022;23(1):34. [CrossRef]
7. Leonardi M, Steiner TJ, Scher AT, Lipton RB. The global burden of migraine: Measuring disability in headache disorders with WHO's Classification of Functioning, Disability and Health (ICF). J Headache Pain 2005;6(6):429–40. [CrossRef]
8. Amiri P, Kazeminasab S, Nejadghaderi SA, Mohammadinasab R, Pourfathi H, Araj-Khodaei M, et al. Migraine: A review on ıts history, global epidemiology, risk factors, and comorbidities. Front Neurol 2022;12:800605. [CrossRef]
9. Waschek JA, Baca SM, Akerman S. PACAP and migraine headache: Immunomodulation of neural circuits in autonomic ganglia and brain parenchyma. J Headache Pain 2018;19(1):23.
10. Thapa S, Shah S, Bhattarai A, Yadav R, Yu F, Chand S, et al. The role of biomarkers pituitary adenylate cyclase-Activating Polypeptide (PACAP) and Vasoactive Intestinal Peptide (VIP) in chronic and episodic migraines: A meta-analysis. Acta Neurol Scand 2024;2024:2954374. [CrossRef]
11. Kesavan P, Satheesh AP, Rasheed AHRS, Veerappan U, Kannaian S, Veerabathiran R. Association analysis of serotonin transporter gene polymorphism among the South-Indian migraineurs. Curr J Neurol 2023;22(1):44–9. [CrossRef]
12. Kesavan P, Satheesh AP, Husain RSRA, Veerappan U, Kannaian S, Ahmed SS, et al. Genetic predisposition of TNFα gene polymorphism in South-Indian Migraineurs and meta-analysis. Front Biosci (Elite Ed) 2021;13(2):226–36. [CrossRef]
13. Sterne JA, Hernán MA, Reeves BC, Savović J, Berkman ND, Viswanathan M, et al. ROBINS-I: A tool for assessing risk of bias in non-randomised studies of interventions. BMJ 2016;355:i4919.
14. Liu J, Wang G, Dan Y, Liu X. CGRP and PACAP-38 play an important role in diagnosing pediatric migraine. J Headache Pain 2022;23(1):68. [CrossRef]
15. Tuka B, Szabó N, Tóth E, Kincses ZT, Párdutz Á, Szok D, et al. Release of PACAP-38 in episodic cluster headache patients - An exploratory study. J Headache Pain 2016;17(1):69.
16. Cernuda-Morollón E, Riesco N, Martínez-Camblor P, Serrano-Pertierra E, García-Cabo C, Pascual J. No change in interictal PACAP levels in peripheral blood in women with chronic migraine. Headache 2016;56(9):1448–54. [CrossRef]
17. Han X, Dong Z, Hou L, Wan D, Chen M, Tang W, et al. Interictal plasma pituitary adenylate cyclase-activating polypeptide levels are decreased in migraineurs but remain unchanged in patients with tension-type headache. Clin Chim Acta 2015;450:151–4.
18. Zagami AS, Edvinsson L, Goadsby PJ. Pituitary adenylate cyclase activating polypeptide and migraine. Ann Clin Transl Neurol 2014;1(12):1036–40. [CrossRef]
19. Togha M, Ghorbani Z, Ramazi S, Zavvari F, Karimzadeh F. Evaluation of serum levels of transient receptor potential cation channel subfamily V member 1, vasoactive ıntestinal polypeptide, and pituitary adenylate cyclase-activating polypeptide in chronic and episodic migraine: The possible role in migraine transformation. Front Neurol 2021;12:770980.
20. Pérez-Pereda S, Toriello-Suárez M, Ocejo-Vinyals G, Guiral-Foz S, Castillo-Obeso J, Montes-Gómez S, et al. Serum CGRP, VIP, and PACAP usefulness in migraine: A case-control study in chronic migraine patients in real clinical practice. Mol Biol Rep 2020;47(9):7125–38. [CrossRef]
21. Schytz HW, Birk S, Wienecke T, Kruuse C, Olesen J, Ashina M. PACAP38 induces migraine-like attacks in patients with migraine without aura. Brain 2009;132(Pt 1):16–25. [CrossRef]
22. Schytz HW, Olesen J, Ashina M. The PACAP receptor: A novel target for migraine treatment. Neurotherapeutics 2010;7(2):191–6.

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Review

# Advancing green laboratory practices: A review of sustainability in healthcare

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**Abstract**

As awareness of the environmental impact of healthcare facilities, including laboratories, grows, there is a pressing need to adopt sustainable practices within laboratory medicine. Sustainability is therefore a key goal in the quickly evolving healthcare landscape. This review explores forward steps in green medical laboratory practices and offers perspectives on sustainability in healthcare, specifically within the context of laboratory medicine. The review assesses the environmental footprint of laboratory operations, considering factors such as energy consumption, resource utilization, chemical usage, and waste generation. Technological advancements and educational initiatives aimed at fostering a culture of sustainability are also discussed as essential components of the transition toward greener medical laboratory practices. This review underscores the importance of integrating sustainability principles into laboratory medicine to mitigate environmental impact while ensuring the delivery of high-quality healthcare services for present and future generations.

**Keywords:** Environmental impact, green lab, sustainability

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In the rapidly changing field of healthcare, sustainability is a key goal. Attention to sustainability is crucial for governments, the general population, and the healthcare system [1]. In 1987, the United Nations released the Brundtland Report, which defined sustainable development as "development that meets the needs of the present without compromising the ability of future generations to meet their own needs" [2, 3]. Although healthcare facilities work to maintain and enhance public health, their effects on the environment can have a detrimental effect on the welfare of people and other living things [4]. Sustainability in healthcare has evolved from being primarily environmental to being a comprehensive concept that takes socio-ecological and socio-technical system balance into account [5].

Ironically, the healthcare sector is one of the biggest emitters of greenhouse gases despite its commitment to protecting and enhancing human health. It is critical that healthcare practices embrace sustainability and actively work towards achieving carbon net zero in the face of this worsening global catastrophe.

## Green Laboratory Design and Infrastructure

Medical laboratories are essential for diagnostics but pose significant environmental challenges due to their energy-intensive operations. Traditional lab designs prioritize functionality over sustainability, leading to high energy consumption, inefficient space use, and negative environmental impacts. Com-

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pared to an office block, a typical laboratory needs three to six times as much energy per unit surface area [6]. This high energy demand increases operating costs and contributes to environmental degradation, including greenhouse gas emissions and waste generation. Poor lab layouts also result in underutilized space and poor indoor air quality, posing health risks. In addition to energy consumption, labs are significant water users and waste producers. Sustainable water conservation measures include acquiring instruments with reduced water usage, installing automatic shut-off valves on sinks, and using adjustable water settings. Prioritizing eco-friendly manufacturers with ISO certification can further enhance sustainability. As healthcare institutions strive to meet rising service demands while reducing their ecological footprint, adopting sustainable design principles in labs is imperative. Sustainable labs offer numerous benefits, including reduced energy consumption and operating costs through optimized space utilization and energy-efficient technologies. Enhanced ventilation and filtration systems improve indoor air quality, creating a healthier work environment. Additionally, sustainable design future-proofs lab infrastructure against evolving regulatory requirements and market dynamics, enhancing organizational resilience.

## Passive Energy Systems

Leveraging natural light, passive solar heating, and natural ventilation mitigates energy usage while enhancing occupant comfort, forming a cornerstone of sustainable laboratory design. Natural light reduces the need for artificial lighting by incorporating large windows, skylights, and light wells, creating a more pleasant and productive environment. Passive solar heating maximizes solar heat gain in winter and minimizes it in summer through strategic window placement, shading devices, and thermal mass, reducing reliance on traditional heating systems. Natural ventilation, using operable windows, louvers, and vents, improves air quality and reduces the need for mechanical systems. These passive energy systems not only cut energy consumption but also enhance the overall experience for staff and visitors [7].

The U.S. Environmental Protection Agency (EPA) has criteria for sustainable laboratory design focusing on reducing emissions, improving technology efficiency, and promoting resource management. Adhering to these standards helps laboratories reduce environmental impact while enhancing operational efficiency and cost-effectiveness [8].

## Emission Reduction

Sustainable laboratory design and operation aim to minimize emissions of pollutants and greenhouse gases. This can be achieved through the use of energy-efficient equipment, such as low-emission fume hoods and energy-efficient lighting systems. Additionally, laboratories can implement waste reduction and recycling programs to minimize the generation of hazardous waste and reduce emissions associated with waste disposal [9].

## Technology Efficiency

The EPA encourages laboratories to adopt energy-efficient technologies that reduce energy consumption and improve overall efficiency. This includes the use of energy-efficient HVAC (Heating, Ventilation, and Air Conditioning) systems, high-efficiency lighting, and energy-efficient laboratory equipment. Laboratories can also implement energy management systems to monitor and optimize energy use, further reducing energy consumption and costs [10].

## Prudent Resource Management

Sustainable laboratory design and operation also involve prudent resource management, including the efficient use of water, materials, and chemicals. Laboratories can implement water-saving measures, such as low-flow faucets and water-efficient equipment, to reduce water consumption. Additionally, laboratories can use environmentally friendly materials and chemicals, such as non-toxic cleaning products and recycled materials, to minimize their environmental impact [11].

Load minimization is a critical aspect of sustainable laboratory design and operation. It involves aggregating areas with similar energy demands and minimizing loads to reduce energy consumption and improve air quality within the laboratory. By optimizing the distribution of energy loads and reducing the overall energy demand, laboratories can achieve significant energy savings and create a healthier indoor environment for occupants. One of the key strategies for load minimization is to aggregate areas with similar energy demands. This involves grouping laboratory spaces based on their energy requirements, such as temperature and humidity control, lighting, and equipment usage. By clustering areas with similar energy needs, laboratories can optimize the distribution of energy loads and reduce the overall energy demand. Laboratories can also implement energy management systems to monitor and optimize energy use, further reducing energy consumption and costs [12].

## HVAC Systems Optimization

Employing efficient HVAC systems, variable air volume (VAV) ventilation, and low-flow hoods significantly reduces energy consumption and enhances air quality management. Selecting energy-efficient HVAC systems minimizes energy use while providing optimal comfort, leading to substantial savings. VAV systems adjust airflow based on demand, reducing energy consumption and improving indoor air quality. Additionally, low-flow hoods provide necessary ventilation with minimal air conditioning, further reducing energy use and enhancing the working environment [13].

## Renewable Energy Integration

Harnessing wind turbines, solar panels, and biomass systems as renewable energy sources is crucial for sustainable laboratory operations. Integrating renewable energy reduces reliance on fossil fuels and lowers carbon footprints. Solar panels,

commonly installed on roofs or walls, convert sunlight into electricity for laboratory equipment, lighting, and HVAC systems. Wind turbines, placed on or near laboratory buildings, capture wind energy for similar uses. Biomass systems generate heat and electricity from organic materials like wood chips and agricultural residues, further reducing dependence on grid electricity and fossil fuels [14].

## Cost Analysis

Utilizing building simulation technology enables precise cost analysis, ensuring alignment with energy efficiency and financial goals. This technology accurately models the energy performance of laboratory buildings, allowing identification of potential improvements. Detailed cost analysis helps determine the most cost-effective energy efficiency measures, maximizing energy savings. Additionally, building simulation technology predicts long-term energy savings, assessing the financial viability and payback period of these measures. This ensures laboratories achieve a positive return on investment while meeting their energy efficiency objectives.

## Design Flexibility

Flexible infrastructure enables seamless integration of emerging technologies and facilitates future upgrades, making it a key aspect of green labs. This design flexibility allows laboratories to adapt to changing needs and incorporate new technologies, ensuring continued efficiency and sustainability. It helps labs stay at the forefront of innovation by easily integrating new technologies and future-proofing their facilities. Additionally, flexible infrastructure simplifies future upgrades, allowing labs to meet new requirements without costly and disruptive renovations, maintaining efficiency and sustainability over time [15].

## Comprehensive Commissioning

Third-party commissioning validates laboratory systems, enhancing quality assurance and performance optimization, a critical aspect of green labs. Comprehensive commissioning identifies and addresses deficiencies or inefficiencies through thorough testing and verification. This ensures systems function as intended and meet performance objectives. Additionally, it identifies opportunities for energy savings, recommending measures to enhance efficiency, reduce energy consumption, and lower costs [16].

## Rethinking Renovation

Repurposing existing facilities with sustainable systems or renovating buildings to align with eco-friendly practices offers a pragmatic approach to minimizing environmental impact. This is crucial for green labs, allowing them to reduce their carbon footprint and leverage existing infrastructure. Renovating with sustainable features like energy-efficient lighting, HVAC systems, and water-saving fixtures creates more sustainable and comfortable working environments, benefiting both the environment and laboratory staff [11].

## Green Procurement Practices

### Procuring green lab concept

Implementing green revolution measures in a clinical laboratory involves adopting environmentally sustainable practices to minimize resource consumption, reduce waste, and promote overall eco-friendliness.

### Chemical Management

Chemicals play an integral role in our society, contributing to advancements in human health and the extension of life expectancy, particularly within the realms of healthcare and clinical laboratories. Despite their crucial contributions, these substances also pose potential risks to both human health and the environment due to their inherent hazardous nature. While chemicals have undeniably enhanced various aspects of our lives, there is a need for careful consideration and management to mitigate any adverse effects they may pose to health and the environment [17]. The risks associated with chemicals encompass their entire lifecycle, spanning production, transport, use, and disposal. Ensuring the proper and sustainable management of chemicals is of utmost importance. Hazardous chemicals, identified as significant contributors to various health conditions in the European Union (EU), have been linked to ailments such as cancer, neurodevelopmental disorders, reproductive issues, metabolic disorders, cardiovascular diseases, and respiratory disorders. Addressing these risks requires comprehensive measures at every stage of the chemical lifecycle to safeguard both human health and the environment [18]. Typically, population subgroups facing greater vulnerability, such as children from lower socioeconomic backgrounds, are more prone to developing diseases linked to pollution [19]. Moreover, exposure to chemicals, even at low levels, can contribute to enduring health effects. This includes issues like decreased fertility, lower birth weights, and neuropsychiatric conditions in children. Approximately 10–15% of all births exhibit neurobehavioral development disorders, with attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorder presenting a widespread distribution. Addressing these challenges is crucial for promoting the long-term health and well-being of vulnerable populations, particularly children. By embracing green chemistry, there is a concerted effort to evaluate the overall life cycle impact of a particular chemical. This approach aims not only to enhance the efficiency of chemical processes but also to prioritize sustainability, safety, and environmental responsibility throughout the entire life cycle of chemical products [20].

To eliminate or reduce hazardous chemicals effectively, the following actions, as suggested in reference, can be implemented [21]:

1. Eliminate Hazardous Chemicals Whenever Possible:
    - Replace mercury thermometers with safer alternatives.
    - Cease the use of ethidium bromide for gels.
    - Explore and adopt solventless chemical reactions when feasible.

- Utilize computer simulations as substitutes for experimental procedures.

2. Reduce Quantities of Harmful Chemicals, Reagents, and Precursors:
   - Implement more efficient chemical reactions to minimize the overall quantity of hazardous substances.
   - Embrace green chemistry principles to substitute harmful chemicals with less toxic alternatives.
3. Procurement:
   - Prioritize the procurement of chemicals with lower toxicity and environmental impact.
   - Collaborate with suppliers who adhere to sustainable and environmentally friendly practices.
   - Consider life cycle assessments when selecting chemicals to understand their overall impact.

By actively implementing these measures, laboratories can contribute to the reduction of hazardous chemicals, promoting safety, sustainability, and environmental responsibility in their practices.

## Equipment Selection

The selection of equipment for a laboratory should prioritize environmentally friendly options to promote sustainability. Laboratory instruments such as fume cupboards, -80°C freezers, autoclaves, and incubators frequently operate around the clock and, on average, consume energy equivalent to or greater than that of a typical single-family house. Efficient freezer management plays a crucial role in reducing energy consumption. This involves regular defrosting, discarding unnecessary samples, and frequent cleaning of filters and condensers to facilitate the dissipation of generated heat. Minimizing the duration of freezer door openings also contributes significantly to energy conservation [22]. A significant measure to reduce energy consumption involves adjusting the freezer temperature from -80°C to -70°C [23]. This modification can result in a decrease in energy usage by 20%–34% while simultaneously improving temperature consistency (±1°C at -70°C compared to ±4.5°C at -80°C). Powering off instruments when not in use can also yield a substantial impact. In a clinical laboratory, for instance, this practice contributes to energy conservation. Finally, it is crucial for laboratories to embrace sustainable procurement practices. This involves establishing an efficient ordering process, implementing proper inventory management to reduce unnecessary transport and CO2e emissions, and actively seeking out more sustainable products.

## Education and Training for Sustainable Practices

### A circular economy – minimal usage of Earth's resources

Global healthcare waste creation is increasing at an accelerated pace of 2%–3% as healthcare facilities expand to meet demand worldwide [24]. The amount of trash generated by the global healthcare industry is quickly becoming an environmental problem. To reduce this impact, appropriate treatment plans and focused management must be implemented prior to the waste becoming waste. Healthcare settings must implement secure systems that collect, transport, separate, and handle waste before disposing of it. As practitioners of healthcare science, we may take steps to lessen clinical laboratory practice's carbon impact and ultimately assist the healthcare industry in meeting its carbon-zero goals.

According to a 2015 evaluation done by the World Health Organization (WHO), very few nations were found to have adequate waste disposal systems. Outdated technologies, lack of worker skills, and restricted budget allocation were the causes of this shortfall. A clinical laboratory must collaborate and work with all other links in the supply chain, including other departments, organizations, suppliers, and regional education providers, for reuse, repair, and reconditioning to be successful. Reselling resources can result in financial gains, sharing resources lessens the demand for new manufacturing, and repurposing equipment in educational settings not only promotes the circular economy model but also offers practical training possibilities for the workforce of the future.

## Advocating Practice: The Carbon Footprint of Travel

When analyzing the travel connected to healthcare that occurs in laboratories, we must take into account travel by the staff, suppliers, patients, and samples. Administrators in laboratories should think about advocating for a decrease in carbon-intensive forms of transportation and implementing practices that can lessen the carbon footprint of delivering samples and resources. Frequent variations in the need for tests can frequently result in urgent "kit" orders, where providers receive several requests for a single supply of goods—often carried out using carbon-intensive methods—from suppliers. Carbon emissions can be reduced by promoting the sharing of kits and consumables among groups in times of need, thereby doing away with the requirement for single-item, long-distance deliveries.

## Sample Collection and Processing

The laboratory's carbon footprint will be impacted by the quantity of samples collected, the mode of transportation, the delivery route, and the frequency of collection. It is simple to understand how the laboratory contributes to the plastic waste of healthcare when you take into account the creation, usage, and disposal of sample transport bags. The majority of healthcare laboratories make an unquantifiable contribution. Although the switch to single-use plastics is a major factor in the creation of plastic trash, we can take action to change this behavior. One proposal is to replace single-use sample bags with sample transport boxes. Not only may samples be carried in boxes safely and securely, but moving away from bags also increases sample loss prevention and confidentiality. With just

a little initial work, a straightforward modification like a reusable transport box can cut down on the usage of single-use plastics, speed up the process of unbagging samples, and shorten turnaround times [25].

## Sustainability in Clinical Laboratories

Phlebotomy practitioners may encounter many challenges, such as mislabeling, sample rejection, and retests. However, we must not overlook the necessity of teaching medical professionals how to reduce the number of unnecessary tests they order. Overall, meaningful carbon savings and environmental advantages can be achieved by lowering the quantity of samples that are carried, processed, and disposed of.

## Avoiding Damages

In order to address the climate and public health crises, it is imperative that we, as ethical healthcare practitioners, collaborate, teach, and exchange sustainable methods. Individually, we must integrate the sustainability agenda into our practice both now and in the future and make sure that sustainability practice education permeates every aspect of our work, including professional qualifications, professional development materials, and inductions. Due to its straightforward premise that encourages product reuse, repair, and reconditioning, the circular economy is a workable alternative. However, we must manage the sustainable healthcare supply chain before a circular model can be implemented. Information gathering, supply analysis, dialogue and cooperation with suppliers, evaluation of service providers, and investment from both internal and external sources, as well as the involvement of end users, are all necessary to get there [26]. Numerous studies [27] have demonstrated that pre-analytical errors can lead to an unwarranted carbon footprint; hence, the demand for higher education is pressing.

## Point-of-Care Testing and Its Pertinence

Point-of-care testing (POCT) is considered to have a significantly lesser impact than laboratory analysis, frequently due to the fact that fewer staff, sample, and patient transportation requirements apply. Additionally, the method improved the environment since the POCT test would eliminate the requirement for laboratory analysis. However, it is pertinent that essential clinical patient needs and quality control procedures are taken into account while implementing POCT. Sustainability in Quality Improvement (SusQI) is one such project that serves as a solid paradigm for Education for Sustainable Development (ESD) in healthcare professional development. The healthcare version of ESD, known as SusQI, was created by The Centre for Sustainable Healthcare (CSH) [28] and considers four principles of sustainable healthcare [29]: Prevention, patient self-care, lean delivery, and low-carbon alternatives. Healthcare professionals who promote sustainability across many professional groups and disciplines can receive virtual training and help from CSH to integrate these ideas into ESD. Therefore, the foundation for professional sustainability education delivery in higher education and the healthcare industry can be formed by the principles of ESD and SusQI.

Since 2015, the advancement of ESD spearheaded by UNESCO has evolved, and it is now being incorporated into high-quality frameworks for continuing professional development in both global healthcare and education [30]. ESD's overarching goal is to transform society by equipping learners with essential information, skills, values, and attitudes. Healthcare practitioners can learn about ESD abilities by applying the following principles: Thinking (future, systems, and creatively), learning (collaborative and participatory), and critical reflection. Future thinking is the process of analyzing social, environmental, and economic goals as well as the effects of inactivity through peer debate. Systems thinking involves applying case studies that are connected to students' current work to help them comprehend carbon measurement, circular economy modeling, and the teamwork needed to bring about change. Using team-based learning techniques, reflective practice, didactic instruction, and critical analysis of research with supporting data, critical reflection is accomplished. Peer and experiential learning that takes action research in the workplace into account is known as participatory learning. Using creative thinking and project-based learning to promote change, problem-based learning is connected to local Quality Improvement standards. Collaborative learning includes interprofessional collaboration, local and guest expert contributions, and collaborative learning methodologies. As a healthcare laboratory expert begins to think about sustainability, knowledge is necessary for an accepted impact measurement. The laboratory professional must gain knowledge of social impact measurement, circular economy modeling, and carbon measurement. To obtain maximum good social results, the proposed modification concept must take into account both the positive and negative social repercussions of the current system. Single-use plastic products are extensively used from a quality and safety as well as health standpoint. In addition, there is no avoiding the sector's high energy usage. Through ESD, students should be able to make decisions and take actions that will help the economy and the environment. Education providers acknowledge the importance of ESD in providing high-quality instruction. Therefore, it is essential to instill ESD as a core skill in both present and upcoming graduates as well as practicing professionals. The "triple bottom line" strategy is incorporated into ESD initiatives, which enables the student to use social, economic, and environmental factors to strike a balance for sustainable development [31].

## Utilization of SusQI for Laboratory Professional ESD

Any healthcare professional group can readily implement SusQI as a method [32], and any part of the patient pathway,

including laboratory diagnoses, can incorporate social and environmental predicaments. Sustainable and green professional practice can be integrated into the laboratory by applying SusQI principles to healthcare laboratory scenarios and promoting problem-solving on how these can be addressed. Interprofessional collaboration also has the added benefit of increasing the profession's visibility.

## Conclusion

In conclusion, the imperative for sustainable practices in healthcare, particularly in laboratory medicine, cannot be overstated. The traditional approach to laboratory design and operation is inefficient and environmentally detrimental. However, by embracing green practices and implementing sustainable design principles, laboratories can significantly reduce their ecological footprint while enhancing operational efficiency and cost-effectiveness.

Key strategies such as emission reduction, technology efficiency, prudent resource management, passive energy systems, HVAC systems optimization, renewable energy integration, cost analysis, design flexibility, comprehensive commissioning, and rethinking renovation all contribute to the overarching goal of creating a more sustainable healthcare infrastructure.

The transition to sustainable laboratory practices is not only a moral imperative to protect our planet for future generations but also a strategic necessity to ensure the long-term viability of healthcare systems worldwide. By prioritizing sustainability, laboratories can contribute to mitigating climate change and foster healthier and more productive work environments for laboratory staff and patients alike.

Advancing green medical laboratory practices is not merely an option but a responsibility that healthcare institutions must embrace to safeguard public health, protect the environment, and pave the way for a more sustainable future.

In essence, the integration of sustainable practices within clinical laboratories is paramount for mitigating environmental impact, promoting resource conservation, and ensuring the long-term viability of healthcare systems. Through collaboration and innovation, laboratories can lead the way towards a healthier and more environmentally conscious future.

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## References

1. Molero A, Calabrò M, Vignes M, Gouget B, Gruson D. Sustainability in healthcare: Perspectives and reflections regarding laboratory medicine. Ann Lab Med 2021;41:139–44. [CrossRef]
2. Biason KM, Dahl P. Strategic steps to sustainability in healthcare - Sustainable Operations. Available at: https://healthcarefacilitiestoday.com/posts/Strategic-steps-to-sustainability-in-healthcare--13629. Accessed Aug 20, 2024.
3. Marimuthu M, Paulose H. Emergence of sustainability-based approaches in healthcare: Expanding research and practice. Procedia Soc Behav Sci 2016;224:554–61. [CrossRef]
4. Buffoli M, Capolongo S, Bottero M, Cavagliato E, Speranza S, Volpatti L. Sustainable healthcare: How to assess and improve healthcare structures' sustainability. Ann Ig 2013;25:411–8.
5. Faggini M, Cosimato S, Nota FD, Nota G. Pursuing sustainability for healthcare through digital platforms. Sustain Times 2019;11:1–15. [CrossRef]
6. Lopez JB, Jackson D, Gammie A, Badrick T. Reducing the environmental impact of clinical laboratories. Clin Biochem Rev 2017;38:3–11.
7. Gammie AJ, Lopez JB, Scott S. Imperative: Reducing the environmental impact of clinical laboratories. Clin Chem Lab Med 2023;61:634–8. [CrossRef]
8. National Research Council. Laboratory Design, Construction, and Renovation: Participants, Process, and Product. Washington DC: Natl Academies Press; 2000.
9. Lopez JB, Badrick T; ad hoc IFCC Panel on the Environmental Responsibility of Clinical Laboratories; APFCB Laboratory Management Committee. Proposals for the mitigation of the environmental impact of clinical laboratories. Clin Chem Lab Med 2012;50:1559–64. [CrossRef]
10. Rampi V, Bisazza O. The EU green deal: The challenge of greening medical technologies. Clin Chem Lab Med 2023;61:651–3. [CrossRef]
11. National Research Council (US) Committee on Prudent Practices in the Laboratory. Prudent Practices in the Laboratory: Handling and Management of Chemical Hazards: Updated Version. Washington DC: Natl Academies Press; 2011.
12. McAlister S, Barratt AL, Bell KJ, McGain F. The carbon footprint of pathology testing. Med J Aust 2020;212:377–82. [CrossRef]
13. Meagan Fitzpatrick. UV Lights in Biosafety Cabinets. Office of Environmental Health and Safety. Available at: https://ehs.princeton.edu/laboratory-research/biological-safety/biological-safety-cabinets/uv-lights-biosafety-cabinets. Accessed Aug 20, 2024.
14. National Renewable Energy Laboratory (NREL). Laboratories for the 21st Century: An Introduction to Low-Energy Design. 2008. Available at: https://www.nrel.gov/docs/fy08osti/29413.pdf. Accessed Aug 20, 2024.
15. Hackman S, Durham J. Emerging practices in clinical laboratory design: It's time to rethink the clinical laboratory from the ground up. Today's Clinical Lab. Available at: https://www.clinicallab.com/emerging-practices-in-clinical-laboratory-design-341. Accessed Aug 20, 2024.

16. Ross J, Penesis J, Badrick T. Improving laboratory economic and environmental performance by the implementation of an environmental management system. Accred Qual Assur 2019;24:319–27. [CrossRef]
17. Ozben T, Fragão-Marques M. Chemical strategies for sustainable medical laboratories. Clin Chem Lab Med 2022;61:642–50. [CrossRef]
18. Giovanni C, Marques FL, Günther WM. Laboratory chemical waste: Hazard classification by GHS and transport risk. Rev Saude Publica 2021;55:102. [CrossRef]
19. Carpintero A. Guidelines for National Waste Management Strategies: Moving from Challenges to Opportunities. United Nations Environment Programme (UNEP), United Nations Institute for Training and Research (UNITAR), Inter-Organisation for the Sound Management of Chemicals (IOMC): Osaka, Japan; 2013.
20. Goldenman G. Study for the strategy for a non-toxic environment of the 7th Environment Action. Available at: https://cswab.org/wp-content/uploads/2018/03/European-Strategy-for-Persistent-Chemicals-2017-.pdf. Accessed Aug 20, 2024.
21. Scott S, Ozben T, Rampi V, Gruson D, Gammie A, Lopez J, et al. EFLM Guidelines for green and sustainable medical laboratories. Available at: https://www.eflm.eu/upload/docs/EFLM-GREEN-LAB-BOOKLET.pdf. Accessed Aug 20, 2024.
22. Hafer M. Quantity and electricity consumption of plug load equipment on a university campus. Energy Efficiency 2017;10:1013–39. [CrossRef]
23. Pali-Schöll I, Hermuth-Kleinschmidt K, Dramburg S, Agache I, Mayerhofer H, Jensen-Jarolim E, et al. An EAACI review: Go green in health care and research. Practical suggestions for sustainability in clinical practice, laboratories, and scientific meetings. Allergy 2023;78:2606–22. [CrossRef]
24. Ranjbari M, Shams Esfandabadi Z, Shevchenko T, Chassagnon-Haned N, Peng W, Tabatabaei M, et al. Mapping healthcare waste management research: Past evolution, current challenges, and future perspectives towards a circular economy transition. J Hazard Mater 2022;422:126724. [CrossRef]
25. Sheri Scott. Laboratory Sustainability: The Need for Green. Available at: https://thepathologist.com/inside-the-lab/lab-sustainability-the-need-for-green. Accessed 20 Aug, 2024.
26. Daú G, Scavarda A, Scavarda LF, Portugal VJT. The Healthcare Sustainable Supply Chain 4.0: The circular economy transition conceptual framework with the corporate social responsibility mirror. Sustainability 2019;11:3259. [CrossRef]
27. Alcantara JC, Alharbi B, Almotairi Y, Alam MJ, Muddathir ARM, Alshaghdali K. Analysis of preanalytical errors in a clinical chemistry laboratory: A 2-year study. Medicine Baltimore 2022;101(27):e29853. [CrossRef]
28. Marsden O, Clery P, D'Arch Smith S, Leedham-Green K. Sustainability in quality improvement (SusQI): Challenges and strategies for translating undergraduate learning into clinical practice. BMC Med Educ 2021;21:1. [CrossRef]
29. Stancliffe R, Bansal A, Sowman G, Mortimer F. Towards net zero healthcare. BMJ 2022;379:e066699. [CrossRef]
30. Beaudoin F, Brundiers K. AASHE. A guide for applied sustainability learning projects: Advancing sustainability outcomes on campus and in the community. Available at: https://static.sustainability.asu.edu/docs/reports/a-guide-for-applied-sustainability-learning-projects-2017.pdf. Accessed Aug 20, 2024.
31. Scott S. Embedding education into clinical laboratory professional training to foster sustainable development and greener practice. Clin Chem Lab Med 2022;61:638–41. [CrossRef]
32. Spooner R, Stanford V, Parslow-Williams S, Mortimer F, Leedham-Green K. Concrete ways we can make a difference: A multi-centre, multi-professional evaluation of sustainability in quality improvement education. Med Teach 2022;44:1116–24. [CrossRef]

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Case Report

# Multiple myeloma in a 27-year-old: A rare presentation with cutaneous involvement and literature insights

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## Abstract

Multiple myeloma (MM) is a hematological malignancy marked by the abnormal proliferation of plasma cells that invade the bone marrow and, in most cases, secrete a monoclonal protein. While typically diagnosed in older individuals with an average age of 70, MM is rarely observed in young adults. Here, we present a unique case of MM in a 27-year-old man with nodular lesions on the trunk, histologically confirmed as cutaneous MM. Laboratory investigations revealed acute renal impairment, hypercalcemia, and the presence of IgG-Lambda paraprotein. The myelogram confirmed plasmacytosis in more than 12% of the bone marrow mononuclear cells. This case highlights the relevance of considering MM in the differential diagnosis of young patients with unusual cutaneous manifestations.

**Keywords:** Multiple myeloma, paraproteins, renal insufficiency, skin diseases

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MM is a malignant plasma cell condition representing around 1% of all cancer cases and approximately 10% of all hematologic malignancies [1]. The clinical manifestations of the disease are caused by the malignant cells, monoclonal protein, or cytokines produced by the malignant cells. The acronym "CRAB" briefly summarizes the principal symptoms and complications: "C" denotes hypercalcemia, "R" renal failure, "A" anemia, and "B" is a bone lesion [2]. Skin infiltration is rarely observed. The median age at which MM is diagnosed is approximately 70 years old, and less than 2% of patients are diagnosed before the age of 40 [3]. The present case, which we describe, is a rare case of MM in a 27-year-old young man with skin involvement.

## Case Report

A 27-year-old male reported acute and intense left lower limb pain that lasted three weeks, as well as low back pain, epistaxis, asthenia, loss of appetite, and weight loss. A physical examination of his trunk revealed multiple asymptomatic nodular lesions.

## Results

During the initial appointment, routine laboratory tests revealed acute renal impairment (creatinine: 4.96 mg/dL, urea: 178 mg/dL), hypercalcemia (11.60 mg/dL), moderate normochromic normocytic anemia (10.3 g/dL), and elevated C-reactive protein (7.4 mg/dL). Urinalysis showed hematuria (12,000/mL) and leukocyturia (3,000/mL). The left knee X-ray revealed the existence of numerous osteolytic lesions on the lower extremity of the femur. Subsequently, the patient underwent hemodialysis to address the acute renal failure, antibiotic therapy was initiated to manage any underlying infection, and a complete investigation for MM was then performed. Serum protein

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electrophoresis found a monoclonal peak in the gamma globulin fraction (Fig. 1). The serum immunofixation assay identified the monoclonality of the IgG-Lambda isotype. Urine immunofixation showed a monoclonal IgG-Lambda band and an excess of monoclonal free lambda light chains. Serum free light chain (sFLC) assay revealed increased excretion of monoclonal lambda light chains >1026.98 mg/dL (normal: 122–437 mg/dL) and a Kappa/lambda ratio <0.05 (normal: 1.30–2.61). Serum β2 microglobulin levels were high at 10,520 ng/mL (normal: 970–2640 ng/mL) with normal lactate dehydrogenase (LDH) levels. Table 1 summarizes laboratory test results obtained at the initial assessment. The myelogram revealed plasmacytosis in more than 12% of the bone marrow mononuclear cells. A whole-body low-dose CT scan was performed, which revealed multiple osteolytic lesions of the axial and peripheral skeleton. A cutaneous nodule biopsy revealed an infiltrate of clonal plasma cells expressing CD138, CD56, lambda light chain, and Ki67 (95%) in immunohistochemistry, indicating cutaneous involvement of the patient's MM. The bone marrow biopsy results are shown in Figure 2. Once the patient's renal function improved, he was scheduled to see an oncologist.

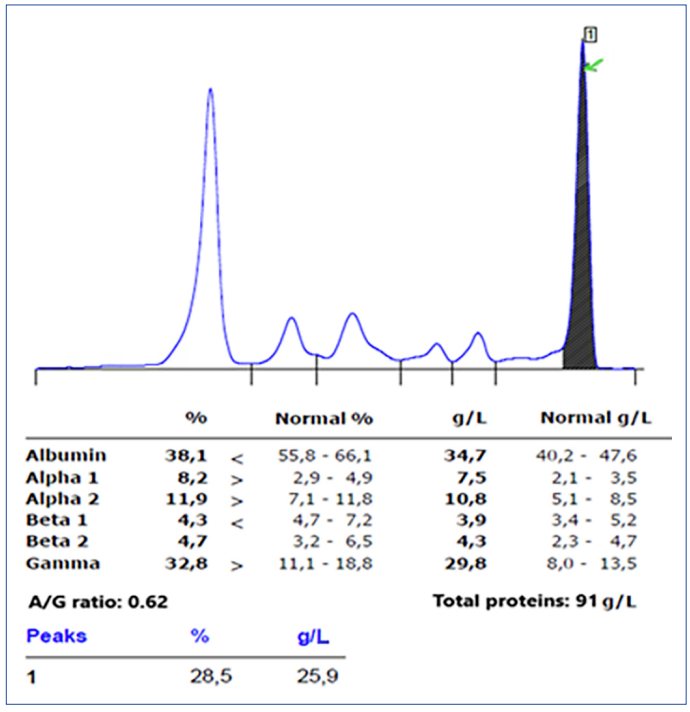


**Figure 1.** Gamma globulin peak on serum protein electrophoresis. A/G ratio: Albumin/globulin ratio.

## Discussion

MM is a clonal B-cell malignancy that affects terminally differentiated plasma cells and is the second most prevalent hematological malignancy after non-Hodgkin lymphoma [4]. M protein, which can be present as intact immunoglobulin or light chain alone, is a key disease characteristic. MM is most common among the elderly, with the peak incidence occurring in the seventh decade of life [5]. A small percentage of MM are identified before age 40 (around 2%) or younger than 30 (0.3%) [6]. Due to their uncommon occurrence, the clinicopathological characteristics and prognosis of early-onset MM patients remain unclear. The limited evidence available in this field primarily consists of individual case reports or small series [6, 7].

While most studies suggest that the clinical and laboratory features of younger individuals with MM resemble those reported in the general MM patient population, some investigations have reported a higher prevalence of osteolytic lesions and light chain myeloma in younger patients than their older counterparts [8].

For most MM patients, plasma cell growth is primarily confined to the bone marrow. However, some MM patients develop extramedullary myeloma, characterized by the growth of clonal plasma cells outside the bone marrow (skin, muscle, pleura, lymph nodes, liver, and the central nervous system). Cutaneous involvement is a rare occurrence in MM, with an incidence of 1.14%, and often indicates a high tumor load, poor prognosis, and late stages of the disease [9]. This extramedullary spread in MM is caused by the downregulation of chemokine receptors and adhesion molecules, allowing plasma cells to evade the bone marrow microenvironment [10]. This is associated with high-risk genetics, accelerated proliferation, apoptosis escaping, and treatment resistance [11]. Histology is crucial in distinguishing between cutaneous MM,

**Table 1. Summary of laboratory test results**

| Laboratory test | Result | Reference value |
|---|---|---|
| Creatinine (mg/dL) | 4.96 | 0.72–1.25 |
| Urea (mg/dL) | 178 | 15–45 |
| Calcium (mg/dL) | 11.6 | 8.4–10.2 |
| Hemoglobin (g/dL) | 10.3 | 13–18 |
| MCV (fL) | 81 | 80–98 |
| MCH (pg) | 28.9 | 27–32 |
| Hematocrit (%) | 28.9 | 40–54 |
| C-reactive protein (mg/dL) | 7.4 | <0.5 |
| sFLC | | |
| Kappa (mg/dL) | 52.4 | 122–437 |
| Lambda (mg/dL) | >1027 | 62–231 |
| Kappa/Lambda ratio | <0.05 | 1.30–2.61 |
| Serum β2 microglobulin (ng/mL) | 10520 | 970–2640 |
| Urine β2 microglobulin (ng/mL) | 390 | <320 |
| LDH (U/L) | 174 | 125–243 |
| Albumin (mg/dL) | 3500 | 3500–5000 |
| Total proteins (mg/dL) | 9100 | 6000–7800 |
| Uric acid (mg/dL) | 10.2 | 3.5–7.2 |
| IgG (mg/dL) | 5308 | 540–1822 |
| IgA (mg/dL) | 78 | 63–484 |
| IgM (mg/dL) | 43 | 22–240 |

MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; sFLC: Serum free light chains; LDH: Lactate dehydrogenase; IgG: Immunoglobulin G; IgA: Immunoglobulin A; IgM: Immunoglobulin M.

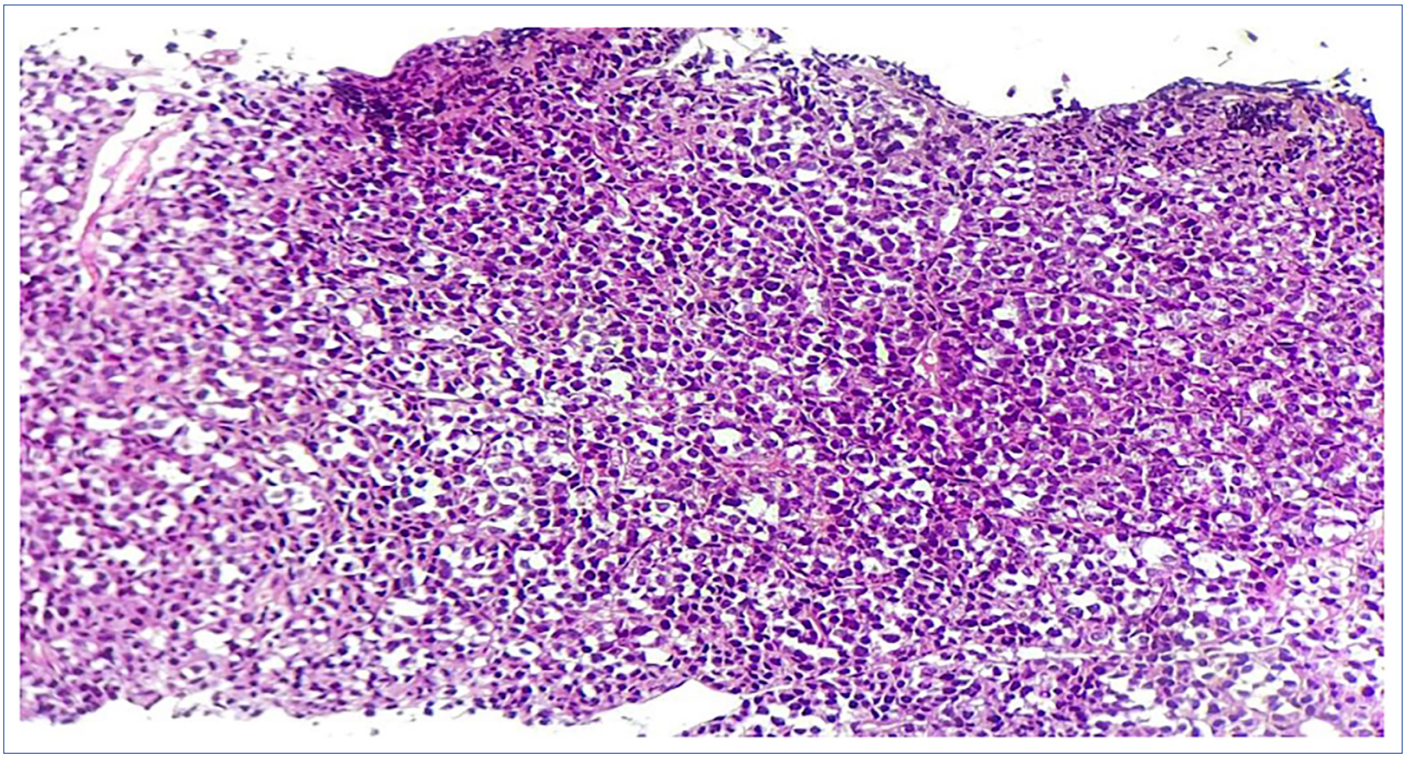

**Figure 2.** Bone marrow biopsy showing a diffuse proliferation of atypical plasma cells (HES, ×100).
HES: Hematoxylin-eosin-saffron stain.

skin infections, and paraneoplastic conditions, including skin light chain amyloidosis (AL) and scleromyxedema.

The survival rates of MM patients have lately improved considerably as a result of novel therapy techniques that include proteasome inhibitors, immunomodulatory treatments, monoclonal antibodies, and stem cell transplantation. However, MM is still considered an incurable disease, with remissions and relapses marking its clinical course. This inability to successfully cure MM suggests the presence of atypical drug-resistant MM cells and significant intra-tumor heterogeneity [12]. Despite continued progress in treating MM, there is still no clear, recognized therapy protocol for young MM patients. Moreover, identifying optimal treatment alternatives becomes critical for these populations to improve long-term outcomes while minimizing the burden of treatment-related toxicities.

Yet, active therapy for young adults with MM should be started as rapidly as possible, according to the criteria specified by the International Myeloma Working Group (IMWG). The treatment approach for cutaneous manifestations focuses on targeting the underlying MM, local radiation therapy, and surgical excision of the skin lesions if needed [13]. Moreover, while allogeneic transplantation remains an option for young patients with high risk, it should still be regarded as experimental and considered only if a suitable donor is available. New approaches include bone-targeting drugs, monoclonal antibodies (Isatuximab, Elotuzumab, anti-BCMA), vaccinations, cellular therapy, and other targeted drugs, which may enlarge the therapeutic armamentarium for managing early-onset MM [14, 15].

## Conclusion

MM in young adults is uncommon, although it does exist. When young adults present to healthcare professionals, they may exhibit symptoms of MM similar to those seen in older patients or more rare manifestations, such as skin symptoms, as observed in our patient. Even so, MM must be considered early in the differential diagnosis.

Despite the recent introduction of several novel therapies, there is still a lack of curative therapy alternatives for young MM patients. A better understanding of disease behavior in this specific group of patients requires prospective multicentric studies, potentially recognizing this group as a distinct biological and clinical entity.

**Informed Consent:** Written informed consent was obtained from the patient for the publication of the case report and the accompanying images.

**Authorship Contributions:** Concept – O.G., N.M., N.D.; Design – O.G., A.H.; Supervision – D.E., E.H.S., A.B., M.C.; Funding – O.G.; Materials – O.G.; Data collection &/or processing – O.G.; Analysis and/or interpretation – O.G.; Literature search – O.G.; Writing – O.G.; Critical review – O.G.

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## References


1. Rajkumar SV. Multiple myeloma: 2022 update on diagnosis, risk stratification, and management. Am J Hematol 2022;97(8):1086–107. [CrossRef]
2. Rodriguez-Otero P, Paiva B, San-Miguel JF. Roadmap to cure multiple myeloma. Cancer Treat Rev 2021;100:102284. [CrossRef]
3. Caulier A, Roussel M, Morel P, Lombion N, Branco B, Galtier J, et al. Epidemiological landscape of young patients with multiple myeloma diagnosed before 40 years of age: The French experience. Blood 2021;138(25):2686–95. [CrossRef]
4. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66(1):7–30. [CrossRef]
5. Pál I, Illés Á, Váróczy L. Multiple myeloma of the young - A single center experience highlights future directions. Pathol Oncol Res 2020;26(1):419–24. [CrossRef]
6. Jurczyszyn A, Davila J, Kortüm KM, Jayabalan DS, Vij R, Fiala M, et al. Multiple myeloma in patients up to 30 years of age: A multicenter retrospective study of 52 cases. Leuk Lymphoma 2019;60(2):471–6. [CrossRef]
7. de Freitas Ferreira HH, Silva ASJ, Júnior LSDS, de Medeiros Oliveira GH, Araujo M das GP, Soares V lima, et al. Multiple myeloma course with renal insufficiency in young patient: Case report. Blood 2020;136(Suppl 1):26. [CrossRef]
8. Veerendra A, Anand N, Saxena S, Nandennavar M, Karpurmath SV. Clinical profiles and outcomes of young versus elderly patients with multiple myeloma: A retrospective observational study from a tertiary cancer center in South India. Cancer Res Stat Treat 2023;6(3):392–9. [CrossRef]
9. Forsyth C, Lai S, Jagger J. Cutaneous multiple myeloma. Br J Haematol 2020;190(2):134.
10. Bansal R, Rakshit S, Kumar S. Extramedullary disease in multiple myeloma. Blood Cancer J 2021;11(9):161. [CrossRef]
11. Liu Y, Jelloul F, Zhang Y, Bhavsar T, Ho C, Rao M, et al. Genetic Basis of extramedullary plasmablastic transformation of multiple myeloma. Am J Surg Pathol 2020;44(6):838–48. [CrossRef]
12. Tiedemann RE, Tagoug I, Erdmann N, Mahdipour-Shirayeh A, Chan Chung K. Multiple myeloma b cells and pre-plasma cells are important reservoirs for myeloma relapse following plasma cell-directed therapy and prevent cure with standard therapies. Blood 2023;142:642. [CrossRef]
13. Santos G, Sousa L, Fernandes T, João A. Case for diagnosis. An Bras Dermatol 2014;89(1):173–4.
14. Shah N, Chari A, Scott E, Mezzi K, Usmani SZ. B-cell maturation antigen (BCMA) in multiple myeloma: rationale for targeting and current therapeutic approaches. Leukemia 2020;34(4):985–1005. [CrossRef]
15. Gandolfi S, Prada CP, Richardson PG. How I treat the young patient with multiple myeloma. Blood 2018;132(11):1114–24. [CrossRef]

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Case Report

# Naphthalene toxicity in a patient with G6PD deficiency

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## Abstract

Naphthalene, an aromatic hydrocarbon prevalent in mothballs and deodorizers, poses significant health risks upon exposure, primarily through ingestion or dermal absorption. Herein, we report the case of a previously healthy 28-year-old male who presented with hemolysis, methemoglobinemia, and acute renal failure. The patient had a history of ingestion of mothballs, mistaking them for candy, prompting consideration of naphthalene intoxication as the clinical diagnosis, which was subsequently confirmed by laboratory findings. Given concurrent hepatic dysfunction and a diagnosis of glucose-6-phosphate dehydrogenase (G6PD) deficiency, N-acetyl cysteine was administered instead of methylene blue. The patient's condition improved after he was managed with aggressive fluid resuscitation, noninvasive ventilation, blood transfusions, and hemodialysis. Naphthalene ingestion can result in hemolysis, methemoglobinemia, and acute kidney injury, with heightened susceptibility observed in patients with G6PD deficiency.

**Keywords:** G6PD deficiency, intravascular hemolysis, methemoglobinemia, mothball, naphthalene

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Naphthalene, with the chemical formula C10H8, is a polycyclic aromatic hydrocarbon used in the formulation of moth repellents and toilet deodorizers. Absorption can occur through ingestion, dermal contact, or inhalation. Prolonged or excessive exposure to naphthalene commonly induces symptoms including headache, confusion, excitement, malaise, profuse sweating, nausea, vomiting, and abdominal pain [1]. Studies indicate that the lethal dose of acute naphthalene toxicity ranges from 5 to 15 grams for adults and 2 to 3 grams for children [2]. Ingestion of naphthalene leads to methemoglobinemia, characterized by a leftward shift of the oxygen-hemoglobin dissociation curve, resulting in decreased release of oxygen to the tissues. Naphthalene, being a potent oxidizing agent, initiates the generation of free oxygen radicals and triggers the depletion of glutathione, leading to intravascular hemolysis. This cascade of events further manifests as anemia, hematuria, leukocytosis with neutrophil predominance, jaundice, and dysfunction of the hepatic and renal systems [3].

## Case Report

A 28-year-old male presented to the emergency department reporting ingestion of naphthalene balls (2 tablets of naphthalene balls—6 grams each) three days before admission. The patient subsequently experienced progressive dyspnea and yellowish discoloration of the skin and sclera over the following two days, which was accompanied by a 1-day history of fever. Initially managed at another facility, the patient underwent nasogastric lavage and received supplemental oxygen via a face mask because of low oxygen saturation. The following day, the patient was transferred to noninvasive ventilation because of persistent hypoxemia despite oxygen therapy. Jaundice developed two days before presentation, accompanied by one episode of dark-colored urine the day before. On examination, the patient had tachypnea (a respiratory rate of 26/26/min), a pulse rate of 122/min, normal blood pressure (110/70 mm Hg), and a temperature of 100.2°F. On admission, his oxygen saturation in room air was 64%, prompting the initiation of noninvasive ventilation

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with 100% oxygen. Despite this intervention, his saturation remained between 84% and 88%. The patient remained alert and oriented; however, he had evidence of jaundice and cyanosis of the lips and bilateral palms. Systemic examination revealed no other abnormalities. A urinary catheter was inserted, revealing dark-colored urine resembling cola (Fig. 1), while blood samples exhibited a dark brown to blackish hue (Fig. 2).

## Results

Arterial blood gas (ABG) performed while the patient was on NIV showed a pH of 7.37, $pCO_2$ of 28 mm Hg, $pO_2$ of 145 mm Hg, and $HCO_3$ of 16 mmol/L, while peripheral oxygen saturation ($SpO_2$) ranged from 84% to 88%. The investigations of the patient are summarized in Table 1. The direct and indirect Coombs tests were negative, but the patient's urine tested positive for hemoglobin, with no red blood cells observed in urine cytology. The chest X-ray was normal. Methemoglobin was detected in his blood via spectrophotometry, with a recorded value of 15.3%. Methemoglobin levels on the day of admission or serial monitoring of methemoglobin could not be performed because it was not available at our center. The management of naphthalene toxicity typically involves the administration of N-acetyl cysteine. However, in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, the administration of N-acetyl cysteine can intensify the condition. Hence, the patient's G6PD levels were examined, revealing a value of 2.2 units/gram of hemoglobin (normal range: 5.5 to 20.5 units/gram of hemoglobin) via the kinetic method. Management: The patient was started on intravenous fluids, injections of N-acetyl cysteine (1800 mg in 3 divided doses), and vitamin C tablets (1000 mg/day). However, because of low G6PD levels, injections of methylene blue could not be administered to the patient. The patient received 6 units of packed red blood cells and underwent 9 sessions of hemodialysis because of elevated creatinine levels and reduced urine output. NIV was gradually discontinued, and on the fifth day of hospitalization, the patient transitioned to oxygen via a face mask. Oxygen supplementation was discontinued on the eighth day after the patient's saturation level became normal. Urine color normalized by the ninth day (Fig. 3), with gradual improvement in urine output, and he was discharged on the thirteenth day. Follow-up at two months revealed normal hemoglobin, liver function, and renal profile.

## Discussion

The prevalence of naphthalene mothball usage is rampant. A common method for identifying the composition of a mothball is to submerge it in water, where a naphthalene-based product will float. In addition to its role as a moth repellent, naphthalene is used as a toilet bowl deodorizer and soil fumigant, as well as a component in numerous other industrial products [2].

Naphthalene poisoning causes methemoglobinemia. Normally, red blood cells (RBC) contain iron in its reduced form ($Fe^{2+}$), which can combine with oxygen by sharing an electron, thus forming oxyhemoglobin. When oxyhemoglobin releases oxygen into the tissue, the iron molecule is restored to its original


**Figure 1.** Urobag of the patient.

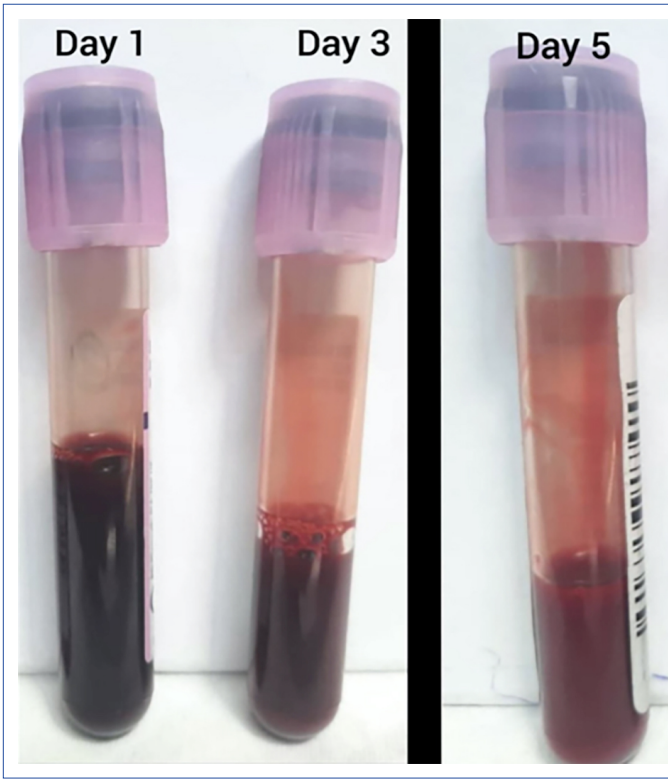
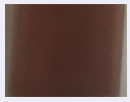
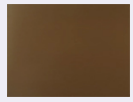
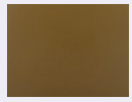
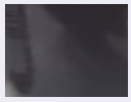
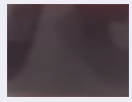
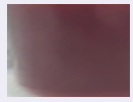



**Figure 2.** Colour of the patient's blood on day 1,3 and 5.

**Table 1. Summary of patient investigations**

| Day of admission | Day 1 14/2/22 | Day 2 15/2/22 | Day 3 16/2/22 | Day 4 17/2/22 | Day 5 18/2/22 | Day 7 20/2/22 | Day 8 21/2/22 | Day 9 22/2/22 |
|---|---|---|---|---|---|---|---|---|
| Haemoglobin in (gm/dl) | 4.4 | 5.5 | | 9.7 | 11 | | | 9.4 |
| Retics corrected | 5.2 | | | | | | | |
| Leucocyte count in cell/cu | 32400 | 32400 | | 23100 | 19800 | | | 12700 |
| Platelets /cmm | 320000 | 255000 | | 227000 | | | | 374000 |
| Creatinine in (mg/dl) | 3.48 | 7.02 | 7.65 | 7.3 | 6.5 | | 7.9 | |
| Blood Urea in mg/dl | 139 | 240 | 205 | 160 | 143 | | 192 | |
| Urine colour |  |  | |  | |  | |  |
| LDH (U/L) | 4028 | | 3692 | | | | | |
| T. bilirubin in (mg/dl) | 6.1 | | | | 6.1 | | | |
| D. bilirubin in (mg/dl) | 0.44 | | | | 1.7 | | | |
| AST in U/L | 282 | | | | 48 | | | |
| ALT in U/L | 42 | | | | 183 | | | |
| Blood colour |  | | |  | |  | |  |

LDH: Lactate dehydrogenase; AST: AST: Aspartate aminotransferase; ALT: Alanine aminotransferase

ferrous state. Hemoglobin can accept and transport oxygen only when the iron atom is in the ferrous state. Naphthalene causes the oxidation of hemoglobin to methemoglobin, i.e., oxidized iron is converted to the ferric state ($Fe^{3+}$), which lacks the electron needed to form a bond with oxygen and hence makes it incapable of transporting oxygen, resulting in hypoxia. Therefore, the measurement of oxygen saturation using a pulse oximeter is unreliable, as observed in this case, where the pulse oximeter saturation was less than 88% and the partial pressure of oxygen in arterial blood gas was 145 mm Hg. Co-oximetry is the gold standard for these patients [4]. Usually, methemoglobin levels are less than 1%, and levels greater than 20% cause anxiety, headaches, and dizziness. Methemoglobin ranges between 30% and 50% can cause fatigue, confusion, and tachypnea. At levels >50%, arrhythmias, acidosis, seizures, and coma can occur [1].

Naphthalene causes the depletion of glutathione, which is required for Nicotinamide Adenine Dinucleotide Phosphate (NADPH) production, resulting in the accumulation of methemoglobin as NADPH is responsible for approximately 5% reduction of methemoglobin. Also, depletion of glutathione decreases the tolerance of erythrocytes to oxidative stress, resulting in extensive hemolysis. Studies have also demonstrated naphthalene's role in the enhanced production of free

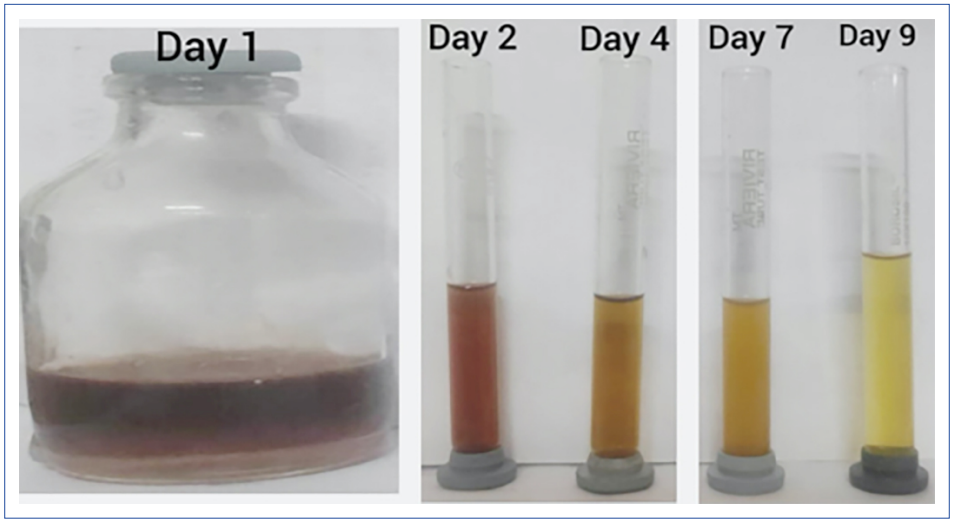



**Figure 3.** Colour of the urine of the patient on days 1, 2, 4, 7 and 9.

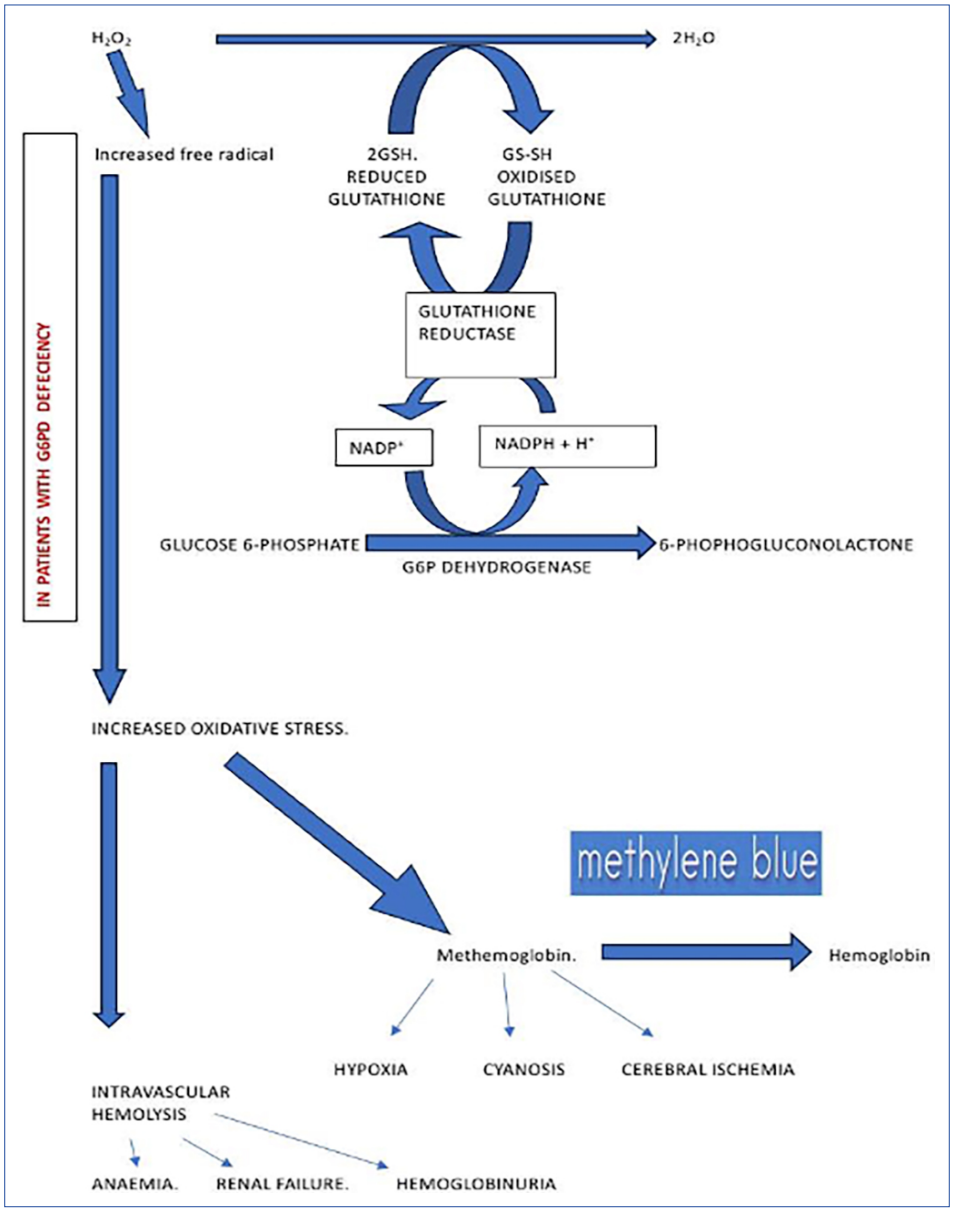



**Figure 4.** Flow chart explaining the pathophysiology of naphthalene toxicity.

oxygen radicals, which in turn increases methemoglobin levels and hemolysis [4]. NADPH production requires G6PD; thus, G6PD deficiency also augments methemoglobinemia and hemolysis [1], which is probably the cause of the disease severity in this case. Our patient had methemoglobinemia and G6PD deficiency, which was confirmed by elevated methemoglobin and low G6PD levels. His investigations also revealed elevated bilirubin levels, elevated LDH, and severe anemia with hemoglobinuria secondary to hemolysis. Hemoglobinuria and the direct renal toxicity of naphthalene resulted in acute kidney injury, with anuria requiring multiple hemodialysis sessions. Despite the potential risk, a heightened suspicion or a strong family history of G6PD deficiency should not entirely discourage the use of methylene blue; instead, its administration should be approached cautiously. Given the diverse manifestations and varying degrees of severity associated with G6PD enzyme deficiency, responses to different oxidative stressors can be unpredictable, making clinical outcomes challenging to anticipate. Therefore, the use of methylene blue should be considered judiciously, particularly when the potential benefits outweigh the perceived risks. If therapeutic doses of methylene blue fail to improve methemoglobin levels, further administration should be avoided to mitigate the elevated risk of hemolysis. Alternative treatment modalities should be explored, and personalized interventions can be tailored on the basis of individual patient presentations (Fig. 4).

Free radical scavengers such as ascorbic acid [5, 6], N-acetyl cysteine (a reducing agent) [7], and exchange transfusion have been found to be useful in treatment. Methylene blue augments methemoglobin's reduction to hemoglobin. A dose of 1 mg/kg body weight in adults is administered as an intravenous slow infusion in a 1% sterile aqueous solution. However, methylene blue was

not used in this case because it may induce hemolysis in cases of G6PD deficiency and may result in paradoxical methemoglobinemia. Hemolysis and hemolysis-induced acute kidney injury are managed by PRBC transfusion and hemodialysis [8].

## Conclusion

Naphthalene exposure typically presents with mild symptoms; however, patients with G6PD deficiency may experience severe toxicity. Potential complications, such as methemoglobinemia and hemolytic anemia, should be anticipated and managed promptly. While there is limited evidence to direct the treatment of complex naphthalene toxicity, understanding its underlying mechanisms can facilitate appropriate management and supportive measures, leading to improved patient outcomes.

**Informed Consent:** Written informed consent was obtained from the patient for the publication of the case report and the accompanying images.

**Authorship Contributions:** Concept – E.S., A.A.A., A.T., A.M.A.; Design – E.S., A.A.A., A.T., A.M.A.; Supervision – E.S., A.A.A., A.T., A.M.A.; Funding – E.S., A.A.A., A.T., A.M.A.; Materials – E.S., A.A.A., A.T., A.M.A.; Data collection &/or processing – E.S., A.A.A., A.T., A.M.A.; Analysis and/or interpretation – E.S., A.A.A., A.T., A.M.A.; Literature search – E.S., A.A.A., A.T., A.M.A.; Writing – E.S., A.A.A., A.T., A.M.A.; Critical review – E.S., A.A.A., A.T., A.M.A.

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## References

1. Lyle ANJ, Spurr R, Kirkey D, Albert CM, Billimoria Z, Perez J, et al. Case report of congenital methemoglobinemia: An uncommon cause of neonatal cyanosis. Matern Health Neonatol Perinatol 2022;8(1):7. [CrossRef]
2. Uthuman AAA, Jayasinghe CS, Fernando AHN. Acute intravascular hemolysis due to naphthalene toxicity: A case report. J Med Case Rep 2019;13(1):91. [CrossRef]
3. Kundra TS, Bhutatani V, Gupta R, Kaur P. Naphthalene poisoning following ingestion of mothballs: A case report. J Clin Diagn Res 2015;9(8):UD01–2. [CrossRef]
4. Iolascon A, Bianchi P, Andolfo I, Russo R, Barcellini W, Fermo E, et al; SWG of red cell and iron of EHA and EuroBloodNet. Recommendations for diagnosis and treatment of methemoglobinemia. Am J Hematol 2021;96(12):1666–78. [CrossRef]
5. Reeves DJ, Saum LM, Birhiray R. I.V. ascorbic acid for treatment of apparent rasburicase-induced methemoglobinemia in a patient with acute kidney injury and assumed glucose-6-phosphate dehydrogenase deficiency. Am J Health Syst Pharm 2016;73(9):e238–42. [CrossRef]
6. Tahura S. Naphthalene induced acute hemolysis in a G6PD deficient Bangladeshi boy - A case report. Moj Toxicol 2016;2(2):48–9. [CrossRef]
7. Chughlay MF, Kramer N, Spearman CW, Werfalli M, Cohen K. N-acetylcysteine for non-paracetamol drug-induced liver injury: A systematic review. Br J Clin Pharmacol 2016;81(6):1021–9. [CrossRef]
8. Volney G, Tatusov M, Yen AC, Karamyan N. Naphthalene toxicity: Methemoglobinemia and acute intravascular hemolysis. Cureus 2018;10(8):e3147. [CrossRef]

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Letter to the Editor

# Distribution of thalassemia trait in Balikesir Province according to trait type and age group

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## Dear Editor,

Previously, an article entitled "Distribution of Thalassemia Trait in Balikesir Province According to Trait Type and Age Group" by Uğur Erçin was published International Journal Of Medical Biochemistry [1]. First of all, I congratulate the researchers of this article who have conducted detailed and difficult research. This valuable research can be completed with some other theoretical and practical experiments by the same group or other researchers.

First, in more comprehensive investigations, indices such as the Mentzer index [2] and the England & Frazer formula [3] can be used. The Mentzer index is obtained by dividing the mean corpuscular volume (MCV) by the number of red blood cells (RBC) per milliliter, and the England & Frazer formula is obtained by MCV – RBC -5× Hemoglobin (Hb) - 3.4. These indicators are also obtained in the laboratory by a simple blood count. These indices can distinguish iron deficiency anemia from thalassemia trait to a favorable extent. With these indices, we can determine to what extent the results of examining globin chains are consistent with the results of these indices. We can also find out what percentage of the patients identified have indices similar to those of iron deficiency anemia.

Second, after identifying patients with beta-thalassemia trait, PCR techniques can be used to find the common genetic mutations that cause this disease. The benefit of this research is that in the case of marriages between persons with this disease in trait form, we can more easily find the specific mutations that caused this disease in the person volunteering for marriage and prevent the birth of an infant suffering from this disease.

In the first case mentioned above, there is no need for advanced equipment, and only by means of special formulas for each index do we reach the index number of the desired index in each patient. In the second case, there is a need for a thermocycler device and specific primers for each mutation, which, according to the progress of universities, are available in almost all universities of medical sciences.

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## References

1. Ugur Ercin. Distribution of thalassemia trait in Balikesir Province according to trait type and age group. Int J Med Biochem 2021;4(3)166–71. [CrossRef]
2. Mentzer WC Jr. Differentiation of iron deficiency from thalassaemia trait. Lancet 1973;1(7808):882. [CrossRef]
3. England JM, Fraser PM. Differentiation of iron deficiency from thalassaemia trait by routine blood-count. Lancet 1973;1(7801):449–52. [CrossRef]

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