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Research Article

The relationship between serum vitamin D levels and hematological inflammatory indices in patients with heart failure

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Abstract

Objectives: Recent studies have suggested that chronic systemic inflammation increases the risk of development and progression of heart failure (HF). Vitamin D may contribute to the pathogenesis of HF by modulating inflammatory pathways. Changes in brain natriuretic peptide (BNP) levels are critical for the diagnosis and assessment of HF severity. We aimed to investigate the association between serum vitamin D levels, BNP, and novel hematological systemic inflammation indices in chronic heart failure (CHF) patients.

Methods: In this retrospective study, we report data from 187 participants admitted to the outpatient clinic, with 85 CHF and 102 without CHF (control group). Vitamin D, BNP, and complete blood cell samples were analyzed. Novel hematological systemic inflammation indices—the systemic immune-inflammation index (SII; neutrophil × platelet / lymphocyte), the systemic inflammation response index (SIRI; neutrophil count × monocyte/lymphocyte count), the pan-immune-inflammation value (PIV; neutrophil count × platelet count × monocyte count)/lymphocyte count, monocyte-to-lymphocyte ratio (MLR), and platelet-to-lymphocyte ratio (PLR)—were calculated.

Results: Binomial logistic regression showed that only MLR was significantly associated with CHF ($P < 0.001$). A weak, negative, and statistically significant correlation was found between BNP and vitamin D ($r=-0.185$, $p=0.011$) levels. There was a weak negative correlation between vitamin D and PLR ($r=-0.196$, $p=0.007$), PIV ($r=-0.145$, $p=0.048$), and SIRI ($r=-0.156$, $p=0.033$).

Conclusion: An independent association between systemic hematological inflammatory indicators and vitamin D with the severity of CHF expressed by elevated BNP levels was revealed.

Keywords: BNP, heart failure, lymphocyte, neutrophil, vitamin D

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Heart failure (HF) is a clinical syndrome that occurs as a result of structural and/or functional abnormalities in the heart. It can lead to the impairment of ventricular filling and/or ejection. Multiple processes, including those involving myocardial damage and ventricular remodeling, are involved in the pathogenesis of HF [1].

Inflammation is one of the major pathophysiological contributors to both acute and chronic heart failure (CHF) [1]. The activation of neurohormonal systems and hemodynamic overload may trigger myocardial inflammatory responses [1, 2]. Consequently, inflammatory activation plays an important role in CHF disease progression through my-

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ocardial contractile dysfunction, hypertrophy, apoptosis, and remodeling [3, 4].

Measurements of blood cell count levels associated with maintaining a subclinical inflammatory environment are inexpensive markers for detecting inflammatory activity. Chronic sterile inflammation plays a critical role in cardiac hypertrophy and cardiac failure [5]. Neutrophilia, lymphocytosis, and thrombocytopenia are common findings in patients with acute or chronic HF, regardless of etiology [6–8]. Circulating inflammatory cells correlate with the severity of CHF [9, 10].

Changes in natriuretic peptide concentrations, either brain natriuretic peptide (BNP) or N-terminal pro-B-type natriuretic peptide (NTproBNP), are critical for the diagnosis of HF, accurate assessment of prognosis, and assessment of HF severity [11, 12]. BNP secretion is mainly dependent on ventricular volume expansion and pressure overload. BNP levels provide objective inclusion criteria for clinical trials [12]. Natriuretic peptides are also elevated in inflammation as a protective mechanism to maintain inflammatory homeostasis [13].

In clinical practice, novel systemic inflammatory hematological indices that reflect a balance between the host's inflammatory and immune responses are used for predicting outcomes in patients with acute HF [14, 15]. Recent research has raised the possibility that inflammatory markers may be complementary biomarkers of CHF [1, 16], which can be simply calculated with the data obtained from the complete blood count. The systemic immune-inflammation index (SII; neutrophil × platelet / lymphocyte), the systemic inflammation response index (SIRI; neutrophil count × monocyte / lymphocyte count), the pan-immune-inflammation value (PIV; neutrophil count × platelet count × monocyte count) / lymphocyte count, and platelet-to-lymphocyte ratio (PLR) based on lymphocyte, neutrophil, and platelet counts, may more comprehensively represent the inflammatory state [14–18].

Vitamin D has effects on the cardiovascular system as well as its well-known effects on bone health and metabolism [19]. Additionally, recent data have revealed the effects of vitamin D on innate and adaptive immunity [19, 20].

An association between an increased risk of developing HF and vitamin D deficiency has been observed in patients [21–23]. It has also been suggested that vitamin D may contribute to the pathogenesis of HF by modulating inflammatory pathways [24].

We aimed to investigate the associations between serum vitamin D levels, novel hematological systemic inflammation, and B-type natriuretic peptide (BNP) levels in CHF patients.

Materials and Methods

Our study protocol was approved by the institutional ethics committee (2011-KAEK-25 2022/11-19).

In this retrospective study, we reviewed the medical records of all patients with confirmed CHF diagnoses who were admitted to the outpatient department of Bursa Yuksek Ihtisas Education and Research Hospital between September 1, 2021, and March 30, 2022. Among the 11,000 patients who had blood BNP levels measured, outpatients with simultaneous blood collections for BNP, complete blood cell count, and vitamin D were included. Those with BNP levels between 100 to 500 pg/mL and those with incomplete medical records were excluded.

We included a total of 187 participants in the study, all at least 18 years of age. These participants were divided into two groups according to their BNP levels. The BNP concentrations in patients with and without CHF were between 518–8374 pg/ml and <100 pg/ml, respectively. The comparison group was composed of patients admitted to the outpatient clinic for control purposes.

The patients' demographic characteristics, medical histories, and blood test results were collected from the hospital's medical records. We excluded patients with acute coronary syndrome, active cancer, end-stage kidney disease, total white blood counts exceeding 1.0×10^4 cells/μL, active viral or bacterial infections, or any history of hematological diseases.

The complete blood cell samples were analyzed using an automated hematology analysis system (Mindray BC-6000 hematology analyzer; Mindray, Shenzhen, China). The BNP concentrations were measured using an Abbott Architect Analyzer (Abbott, Chicago, Illinois).

SII was calculated as follows: neutrophil × platelet / lymphocyte [17]. SIRI was defined as neutrophil count × monocyte / lymphocyte count [14]. The pan-immune-inflammation value (PIV) included all the immune-inflammatory cells in the peripheral blood count: (neutrophil count × platelet count × monocyte count) / lymphocyte count [18]. Monocyte-to-lymphocyte ratio (MLR), neutrophil to lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and red cell distribution width to platelet ratio (RDW to PLT) were calculated from whole blood counts [10,14–18]. Due to the retrospective nature of the study, we could not include other inflammatory markers such as C-reactive protein, fibrinogen, interleukins, and others.

Statistical analysis

The statistical software package SPSS 21.0 (SPSS, Chicago, IL) was used to conduct the statistical analysis. The characteristics of the study sample were reported using descriptive statistics. The Kolmogorov-Smirnov test was used to test for normal distribution. Groups were compared using Student's t-test or Mann-Whitney U test for continuous variables and chi-squared test for categorical variables. Pearson correlation analysis was used to verify the correlation between selected variables. Correlation coefficients (r) were deemed statistically significant when the p-value was <0.05. Binomial logistic regression analysis was performed to quantify the correlation between variables and CHF.

Results

The study included 187 patients admitted to the outpatient department, of which 85 had CHF and 102 did not. The de-

mographic, clinical, and laboratory parameters of the two groups are presented in Table 1. The median age of the study sample was 67 years (IQR: 19), and 48% of the patients were male (97 females, 90 males). Vitamin D levels were lower in CHF patients (p<0.001), and the inflammatory indices NLR, MLR, PLR, SII, SIRI, PIV, and RDW to PLT were significantly higher in CHF patients than in the control group (p<0.001). Binomial logistic regression analysis showed that only MLR was significantly associated with CHF (p<0.001), but other hematological indices were statistically insignificant (p>0.005), and vitamin D is not an independent risk factor for CHF. Data from the two groups were pooled to analyze the relationships between vitamin D and BNP using other hematological parameters (Table 2). The correlation between BNP and MLR (r=0.485, p<0.001), SIRI (r=0.504, p<0.001), and PIV (r=0.423, p<0.001) was higher than the correlation with other inflammatory indexes. There was a very weak negative correlation between vitamin D and PLR (r=–0.196, p=0.007), PIV (r=–0.145, p=0.048), and SIRI (r=–0.156, p=0.033).

Table 1: Demographic and laboratory parameters in patients with and without CHF

Parameter	CHF group (n=85) median (IQR)	Control group (n=102) median (IQR)	p
Age (years)	69 (23)	64 (19)	0.083
Sex			
Female	39	58	0.144
Male	46	44	
BNP (pg/mL)	1288 (1711)	29 (20)	<0.001
Vitamin D (ng/mL)	10.8 (18)	17.3 (11)	<0.001
Total WBC (×10^9/L)	7.7 (2.8)	7.0 (2.9)	0.068
Neu (×10^9/L)	5.5 (1.7)	4.3 (2.1)	<0.001
Lym (×10^9/L)	1.1 (0.9)	2.0 (0.7)	<0.001
Mono (×10^9/L)	0.49 (0.30)	0.46 (0.25)	<0.001
PLT (×10^9/L)	237 (119)	253 (90)	0.024
NLR	4.76 (3.54)	1.94 (0.95)	<0.001
MLR	0.41 (0.40)	0.21 (0.19)	<0.001
SII	1078 (1109)	491 (284)	<0.001
SIRI	2.05 (2.01)	0.92 (0.68)	<0.001
PIV	531 (594)	214 (226)	<0.001
RDW to PLT	0.067 (0.032)	0.055 (0.022)	<0.001

CHF: Chronic heart failure; IQR: Interquartile range; BNP: Brain natriuretic peptide; WBC: White blood cell; Neu: Neutrophil; Lym: Lymphocyte; Mono: Monocyte; PLT: Platelet; NLR: Neutrophil to lymphocyte ratio; MLR: Monocyte to lymphocyte ratio; SII: Systemic immune-inflammation index; SIRI: Systemic inflammatory response index; PIV: Pan immune inflammation value; RDW to PLT: Red cell distribution width to platelet.

Table 2. Correlation of BNP and vitamin D and inflammatory indices

	BNP		Vitamin D	
	rho	p	rho	p
Vitamin D	–0.185	0.011*		
Neu	0.409	<0.001**	–0.81	0.269
Lym	–0.405	<0.001**	156	0.33
NLR	0.368	<0.001	–0.176	0.16
MLR	0.485	<0.001**	–0.176	0.16
PLR	0.361	<0.001**	–0.196	0.007*
SII	0.324	<0.001**	–0.145	0.048*
SIRI	0.504	<0.001**	–0.156	0.033*
PIV	0.423	<0.001**	–0.145	0.047*
RDW to PLT	0.053	0.472	–0.030	0.688

*: Correlation is significant at the 0.05 level; **: Correlation is significant at the 0.01 level. PLR: Platelet to lymphocyte ratio.

Discussion

The present study had four main findings. First, we found lower serum vitamin D levels in CHF patients compared to control subjects. This finding is confirmed by a number of previous studies suggesting that insufficient vitamin D status may increase the risk of HF [21–26]. We also found a weak negative correlation between BNP levels and vitamin D levels [25–27]. These patients are at risk of having low serum vitamin D levels due to their limited exercise capacity and inadequate exposure to sunlight. Additionally, we observed a positive relationship between BNP concentration and hematologic inflammatory indices in patients with CHF, a finding supported by previous reports [1–3, 14, 28].

The most important finding of our study was that complex hematological indices better elucidate the relationship between vitamin D and inflammation in patients with CHF than single-cell counts. Blood cell count differences are either a cause or a consequence of CHF. Neutrophils act on cardiac hypertrophy by regulating hypoxic stress in the myocardium [29]. A higher monocyte count is associated with a greater degree of ventricular remodeling [30, 31]. Lymphocyte apoptosis is stimulated in CHF [32]. The present results are consistent with previous studies demonstrating that MLR is an independent risk factor for CHF and predicts hospitalization [33, 34]. PLT count levels could also be related to maintaining a low inflammatory environment and could change due to several factors [35]. Pulmonary congestion in HF may inhibit platelet hematopoiesis in the lungs [36]. Although all these blood cells are involved in the same general process, none are specific to CHF. Hematological indices involve ratios of different but complementary immunological pathways, which is why they can adequately reflect a patient's inflammatory state.

Recent data also show a correlation between decreased vitamin D and other inflammatory markers, such as C-reactive protein and fibrinogen [37]. However, they usually reflect acute inflammation, whereas complex hematological markers could better reflect the long-term inflammatory state of CHF. These markers could be auxiliary biomarkers of severity and may represent a treatment target [8, 38].

A study by Tabatabaeizadeh et al.[39] demonstrates that high-dose supplementation of vitamin D leads to reductions in NLR levels. However, anti-inflammatory clinical trials have shown limited success in patients with CHF [40]. The complexity of inflammatory responses may partially explain these unsatisfactory results.

Limitations

The main limitation of our study is that it was conducted retrospectively at a single center. Second, the study was based on data from patients' medical histories, and other inflammatory markers could not be included. Furthermore, the effects of comorbidities, rheumatological diseases, smoking status, the effects of the drugs patients used, and vitamin D ingestion were not taken into account. The number of patients evaluated is very low, and causal relationships cannot be established from this observational study. Well-designed, larger prospective multicenter studies with more comprehensive data are needed to confirm the findings of this study.

Conclusion

An independent association between systemic hematological inflammatory indicators and vitamin D with the severity of CHF expressed by elevated BNP levels was revealed. These results may contribute to a better understanding of how patients' inflammatory responses could be related to vitamin D levels. They could also lead to further studies that will elucidate this relationship.

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

Ethics Committee Approval: The study was approved by The University of Health Sciences Bursa Yuksek Ihtisas Training and Research Hospital Clinical Research Ethics Committee (No: 2011-KAEK 25 2022/11-19, Date: 30/11/2022).

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Research Article

Effect of dapagliflozin on oxidative stress in heart embryonic H9c2 cardiomyocytes

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Abstract

Objectives: Dapagliflozin is a drug used to treat type 2 diabetes and is also used in certain heart failure and chronic kidney disease conditions. In this study, we investigated the effects of dapagliflozin (DAPA) on malondialdehyde (MDA), lipid hydroperoxide (LOOH), superoxide dismutase (SOD), total thiol (T-SH), and total antioxidant capacity (TAC) as oxidative stress parameters in heart embryonic H9c2 cardiomyocytes.

Methods: H9c2 cardiomyocyte cells were treated with methotrexate (MTX) (10-0.160 µM) and DAPA (10-0.150 µM). The cell viability and oxidative stress parameters were measured.

Results: MDA and LOOH levels were significantly lower in the control ($p<0.001$ for both) and DAPA groups ($p<0.001$; $p<0.05$, respectively) compared to the MTX groups, while SOD ($p<0.001$ for both), T-SH ($p<0.001$; $p<0.01$, respectively), and TAC ($p<0.01$; $p<0.05$, respectively) were significantly higher in the control and DAPA groups compared to the MTX groups. There was no significant difference between the control and DAPA groups in other parameters except for MDA. However, MDA levels were significantly higher in the DAPA group ($p<0.05$) compared to the control group. The decrease in MDA levels was significantly correlated with the increase in SOD activity (r: -0.814; p: 0.014) in the DAPA treatment group.

Conclusion: Cell viability increased, and the levels of MDA and LOOH decreased, while the SOD, T-SH, and TAC levels increased in H9c2 cardiomyocytes induced by oxidative stress. The findings obtained in this study suggest that DAPA may have beneficial effects in cardiomyopathy caused by oxidative stress.

Keywords: Dapagliflozin, H9c2 cardiomyocyte cells, malondialdehyde, methotrexate, oxidative stress, superoxide dismutase

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Dapagliflozin (DAPA), a sodium-glucose cotransporter 2 (SGLT-2) inhibitor, has been shown to have favorable effects on cardiac events such as cardiovascular death or hospitalization for heart failure (HF) in patients with type 2 diabetes mellitus (T2DM), along with a reduced risk of cardiovascular events [1, 2]. However, the cardiovascular safety of DAPA was studied in comparison to placebo and other drugs across a total of 21 studies, including 2 involving a large number of patients with a history of cardiovascular disease (CVD) [3]. The primary endpoints were the timing of cardiovascular death, hospitalization for myocardial infarction, stroke, or unstable angina, with secondary endpoints including unplanned coronary revascularization and hospitalization for HF in addition to the primary endpoints. When all the data were combined, it was concluded that cardiovascular risk would not increase with the use of DAPA [3]. DAPA may also attenuate cardiotoxicity by reducing oxidative stress, mitochondrial dysfunction, fibrosis, hypertrophy, and inflammation through PI3K/AKT/Nrf2 signaling [4, 5].

The mechanism of methotrexate (MTX)-induced cardiotoxicity is attributed to free radical formation, stimulation of lipid

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peroxidation, and subsequent alteration of cellular membrane integrity. This hypothesis is supported by the reported cytoprotective effect of antioxidants against MTX toxicity [6, 7].

Oxidative stress is a condition in which reactive oxygen-containing compounds are present in higher numbers than under normal conditions within a cell or group of cells [8]. Oxidative stress is a contributing factor in chronic CVD [8, 9] and is particularly significant in cardiovascular aging [10]. In recent years, many studies have focused on the role of various drugs in eliminating oxidative stress and reducing the damage caused by MTX [4, 5, 11, 12].

However, the exact mechanisms by which these favorable effects occur on MTX-induced cardiomyopathy are not fully understood. In our study, we aimed to determine the effects of DAPA treatment on MTX-induced oxidative stress by measuring (MDA), (LOOH), (SOD), (T-SH), and (TAC) as oxidative stress levels before and after DAPA treatment in H9c2 cardiomyocyte cells.

Materials and Methods

All chemicals used in the study were provided by Sigma-Aldrich, Istanbul, Türkiye.

Cell culture and treatment

H9c2 (2–1) cardiomyocyte cells were purchased from The American Type Culture Collection (ATCC). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin) at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were subcultured with 0.25% trypsin-EDTA. The trypsin-EDTA-cell suspension was centrifuged at 120xg for 5 minutes. After centrifugation, the supernatant was discarded, fresh medium was added to the cell pellet, and cells were seeded into 96-well plates at 1×10^4/well. The effects on cell viability were analyzed by applying MTX and DAPA (Forziga®AstraZeneca, Türkiye) at different concentrations and times.

Cell viability assay

Cell viability was determined using the MTT reduction assay. Briefly, H9c2 cells were incubated with DMEM containing 10% FBS overnight in 96 well plates at a density of 1×10^4 cells/well. After reaching 80% confluence, the cells were washed twice with D-PBS and incubated with medium containing various concentrations of MTX (10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156 µM) and DAPA (100, 50, 25, 12.5, 6.25, 3.125, and 1.562 µM) for 24, 48, and 72 hours at 37˚C in a humidified atmosphere containing 5% CO_2. The medium was removed, and 100 µL DMEM and 20 µL MTT (5 mg/mL) was added to each well for 3 hours. The formazan crystals formed in intact cells were dissolved in 100 µL DMSO. Absorbance was recorded at a wavelength of 490 nm and at a reference wavelength of 570 nm using a microplate reader (Multiskan GO-Thermo). Using optical density (OD) values, IC50 and EC50 values for MTX and DAPA were calculated as 2.2 µM and 10 µM for 48 hours respectively with the GraphPad Prism 9 program.

Experimental groups

Groups were formed to include control, 2.2 µM MTX, 10 µM DAPA, and 2.2 µM MTX+10 µM DAPA. Cells were seeded at $3–4\times10^5$ cells/well on 6-well plates for each group. 24 hours after cell seeding, the control group was replaced with normal medium, and the others with medium containing MTX and DAPA alone or in combination at the given concentrations. While the other groups were incubated for 48 hours, the MTX+DAPA group was treated with DAPA for 48 hours after 48 hours of MTX. At the end of the experiment, cell lysates were prepared for the measurement of oxidative stress parameters from all groups.

All measurements related to the cell culture model, cell viability assay, cell lysate preparation, and oxidative stress were done as clearly stated in our previous studies [11, 12]. Differently, the cells were incubated with MTX in complete culture medium for 48h prior to the addition of DAPA. H9c2 (2–1) cardiomyocyte cells were treated with DAPA (Forziga®AstraZeneca, Türkiye) (10–0.153 µM) for 24, 48, and 72h at 37˚C in a humidified atmosphere containing 5% CO_2. DAPA was dissolved in DMSO. 2.7 µM MTX and 2.5 µM DAPA were used for 48 hours. The cell viability was calculated by considering the control as 100%.

Cell lysate preparation

Using 1xRipa lysis buffer and a protease inhibitor cocktail set (Merck KGaA, Darmstadt, Germany), a cell lysate from all groups was created at the conclusion of the experiment. 300 µL of Ripa lysis buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) together with a protease inhibitor cocktail (1:200) were added after the cells had been washed twice with cold 1xPBS. The cells were lysed by pipetting on ice, and the cell suspension was incubated for 30 minutes at +4°C in a shaking water bath. It was then centrifuged at 14000×g for 30 minutes at +4°C. After centrifugation, the supernatants were transferred to fresh Eppendorf tubes, and the resulting cell lysates were stored in a deep freezer at –80°C until measurement.

Oxidative stress parameters

Each experimental group was repeated at least three times. Lipoperoxidation was ascertained by the formation of malondialdehyde (MDA), which was estimated using the modified thiobarbituric acid (TBA) method [13]. LOOH levels were determined spectrophotometrically according to the method of ferrous oxidation with xylenol orange version 2 (FOX2) [14]. Cu, Zn-SOD activity was determined using the method of Sun et al. [15] by inhibition of nitroblue tetrazolium (NBT) reduction, with xanthine/xanthine oxidase used as a superoxide

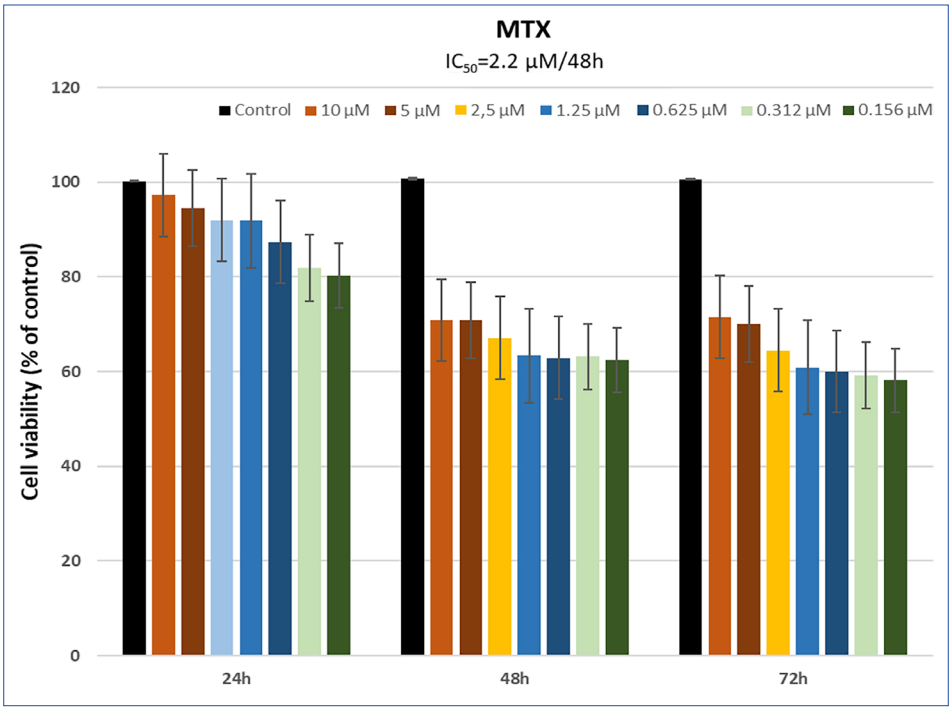

Figure 1. Time- and dose-dependent effects of MTX and DAPA in cell viability in H9c2 cells as % of control.

Data are presented as the mean±standard error of the mean (n=8). MTX: Methotrexate; DATA: Dapagliflozin.

generator. T-SH levels were determined using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) as introduced by Hu [16]. The non-enzymatic TAC levels were measured with the ferric reducing antioxidant power assay and were performed according to the protocol of Benzie and Strain [17].

Statistical methods

All statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS) v. 22.0 (IBM, Armonk, NY, USA) package program. The distribution of all analyzed parameters was confirmed using the Shapiro-Wilk test. All parameters were normally distributed and expressed as mean± standard deviation. One-way ANOVA and the Tukey test as *post-hoc* were used in the comparison of groups. Correlation analysis was performed using Spearman's correlation analysis. Correlation/scatter graphs were plotted with Jamovi 2.3.18. A p-value below 0.05 was considered significant.

Results

The results of an MTT assay demonstrated that the number of viable cells decreased in response to increased concentration and times (24, 48, and 72 hours) of MTX treatment (Fig. 1). Conversely, the number of viable cells increased in response to increased concentration and times of DAPA treatment (Figs. 2, 3).

MDA ($p<0.001$; $p<0.05$, respectively) and LOOH ($p<0.001$ for both) levels were significantly lower in the control and DAPA groups compared to the MTX groups, while SOD ($p<0.001$ for both), T-SH ($p<0.001$; $p<0.01$, respectively), and TAC ($p<0.01$; $p<0.05$, respectively) were significantly higher in the control and DAPA groups compared to the MTX groups. There was no significant difference between the control and DAPA groups in other parameters except for MDA. However, MDA levels were significantly higher in the DAPA group ($p<0.05$) compared to the control group (Table 1).

There was a negative correlation between MDA and SOD (r: –0.814; p: 0.014) in the DAPA treatment group (Fig. 4). No correlation was observed between other oxidative parameters.

Discussion

In the present study, we found that in MTX-induced H9c2 cells, MDA and LOOH levels significantly increased, while Cu/Zn-SOD, T-SH, and TAC levels significantly decreased. These results indicate that the activation of systemic reactive oxygen species (ROS) triggers a sequence of events leading to cardiomyopathy. To the best of our knowledge, this is the first study to show that DAPA administration was able to improve oxidative stress induced by MTX. DAPA achieved this goal by reducing oxidative stress, increasing antioxidant status, and preventing cellular injury.

Free radicals, which occur during normal metabolism or pathologically, cause many damages in cells and tissues. Since oxidative damage caused by ROS affects biomolecules such as proteins, lipids, and nucleic acids, tests for oxidative products of these biomolecules have been used for many

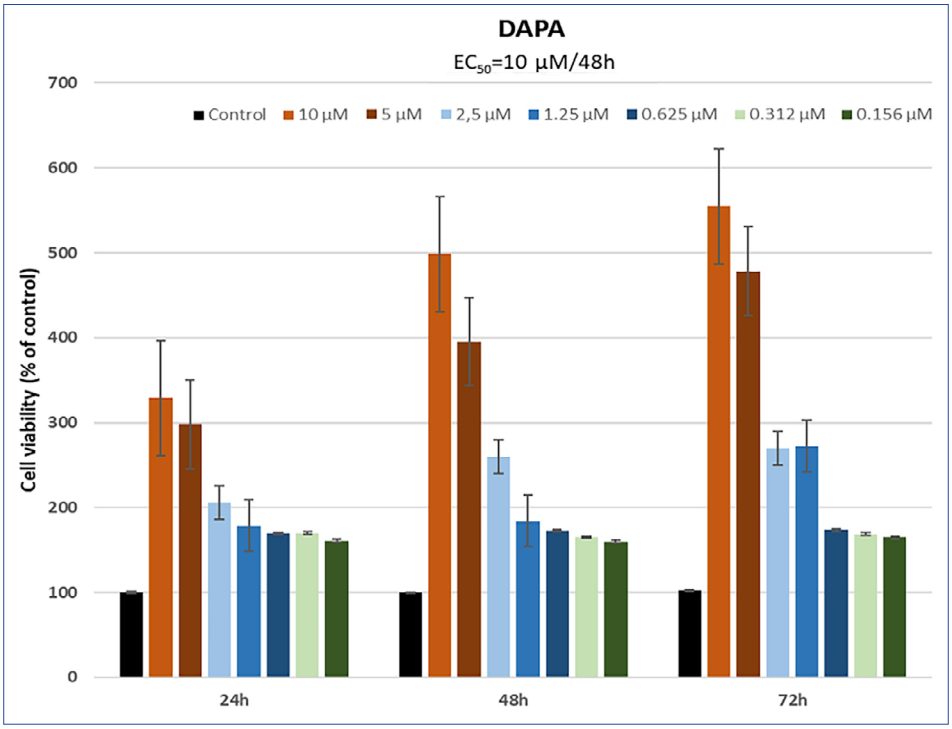

Figure 2. Protective effects of DAPA against MTX-induced decrease in cell viability in H9c2 cells as % of control.

Data are presented as the mean±standard error of the mean (n=8).

years in demonstrating oxidative stress [18]. Recent studies suggested that oxidative stress [19] may be held responsible for the development of coronary artery disease (CAD). The heart is one of the most vulnerable organs to oxidative stress due to its specific structure and function [20]. It is clear that the production of ROS in cardiac dysfunction is a major factor contributing to heart diseases including cardiomyocytes, endothelial cells, and neutrophils [21].

The relationship between oxidative stress and atherosclerosis has been investigated by various research groups both in humans and experimental animals [22]. In many studies where MDA was used as a marker, high levels of MDA were observed to play an important role in the development of atherosclerosis in rabbit aorta, and a positive correlation was found between atheromatous plaques and MDA levels [23]. On the other hand, blood samples were generally used in human studies and lipid peroxidation indicators were mostly analyzed. Many studies have shown that MDA, diene conjugates, or LOOHs increase in serum in atherosclerotic CVD [24–26]. In our previous studies [11, 12], MDA and LOOH levels were found to be increased in H9c2 rat cardiomyocyte cells by MTT assay. Su et al. [27] detected increased MDA activity and decreased SOD activity in H9c2 cells after H_2O_2 stimulation. Zilinyi et al. [28] showed that injection of 6×3 mg/kg doxorubicin (DOX) was associated with considerably elevated MDA levels compared to the control group. In line with the literature, our results also show increased oxidative stress evidenced by elevated MDA and LOOH levels in MTX-treated H9c2 rat cardiomyocyte cells.

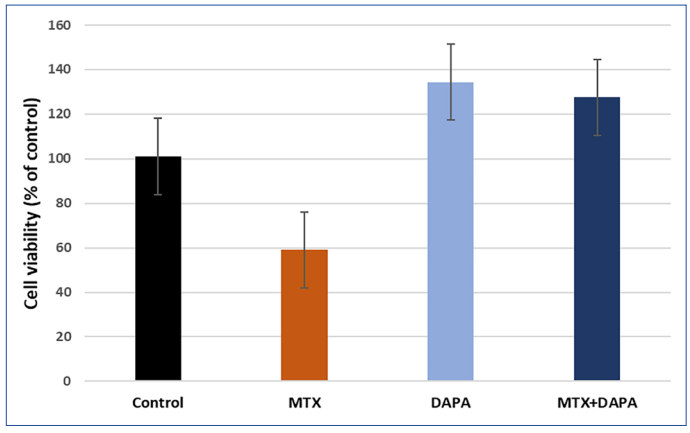

Figure 3. Protective effects of DAPA against MTX-induced decrease in cell viability in H9c2 cells as % of control.

Data are presented as the mean±standard error of the mean (n=8).

Studies have investigated the healing and antioxidant effects of DAPA on endothelial cells. DAPA has been reported to improve cell functions due to positive effects on oxidative stress [29, 30]. According to TAC and total oxidant status (TOS) analysis, DAPA increased TAC, but not TOS, in cultured human blood cells [31]. An animal study showed that DAPA administration attenuated macrophage polarization in infarcted rat hearts by regulating macrophage polarization via the STAT3-signaling pathway [32]. Solini et al. [33] reported significant reductions in blood pressure (BP) and oxidative stress due to the acute effects of DAPA on systemic and renal vascular function.

Table 1. Oxidative stress markers in H9c2 cardiomyocyte control cells, cells exposed to MTX without any treatment, and cells treated with DAPA

	Control (n=8)	MTX (n=8)	DAPA (n=8)
	Mean±SD	Mean±SD	Mean±SD
MDA (nmol/mL)	0.63± 0.06	0.78±0.07^{a***}	0.69±0.06^{a*,b*}
LOOH (nmol/mL)	13.67±1.81	21.36±2.31^{a***}	13.68±2.32^{b***}
SOD (U/mL)	3.94±0.39	3.06±0.28^{a***}	3.79±0.22^{b***}
T-SH (mM)	1.52±0.15	0.94±0.24^{a***}	1.38±0.26^{b**}
TAC (µg ascorbic acid equivalent/mL)	19.46±1.41	16.87±2.24^{a**}	19.16±2.35^{b*}

*: p<0.05; **: p<0.01; ***: p<0.001. ^{a}: versus control; ^{b}: versus MTX. MTX: Methotrexate; DAPA: Dapagliflozin; MDA: Malondialdehyde; LOOH: Lipid hydroperoxide; SOD: Superoxide dismutase; T-SH: Total thiol; TAC: Total antioxidant capacity.

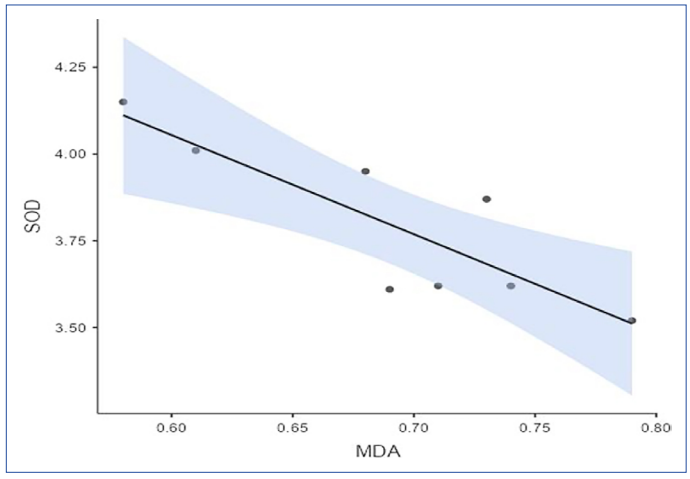

Figure 4. Correlation between MDA and SOD activity.
MDA: Malondialdehyde; SOD: Superoxide dismutase.

Shigiyama et al. [34] monitored T2DM patients using metformin by adding DAPA to their treatment and reported that urine 8-hydroxy-2'-deoxyguanosine, a biomarker of oxidative stress, was significantly lower and endothelial functions were better in the DAPA group than in the metformin group at 16 weeks. Buyukaydin et al. [35] demonstrated that patients using SGLT2 inhibitors (empagliflozin or dapagliflozin) had statistically higher total antioxidant status (TAS) levels in T2DM patients. While increased TAS may appear to be related to lower glucose values, there was no statistically significant difference in HbA1c between patients using SGLT2 inhibitors or not. DAPA has been reported to reduce mitochondrial ROS formation in aortic tissues and also to prevent atherosclerosis formation and suppress macrophage infiltration [36]. DAPA also suppressed high-glucose-induced oxidative stress in cultured mProx24 cells [37]. In addition to modulating inflammation, endothelial activation, and oxidative damage, DAPA regulated tubular ion channel expression and the non-classic renin-angiotensin-aldosterone system (RAAS) [38].

Limitations of the study

Our study has some limitations. The most significant shortcoming is the lack of transmission electron microscopic data. Another limitation is the absence of in vivo (animal and human studies) experiments in our study. Additionally, including another drug with a proven antioxidant effect that reduces oxidative stress could have enhanced the study.

In our study, MDA and LOOH values, which are lipid peroxidation products, were analyzed as markers of oxidative stress. SOD, T-SH, and TAC levels were analyzed as antioxidant indicators. MDA and LOOH levels were significantly lower in DAPA groups, while SOD, T-SH, and TAC were significantly higher in DAPA groups compared to the MTX groups. Additionally, there was a negative correlation between MDA and SOD activity in the DAPA treatment group. It was shown that oxidative stress markers increased in case of injury and then decreased significantly with DAPA treatment.

Conclusion

The results of the study demonstrate that DAPA reduces oxidative stress by decreasing the production of ROS and increasing antioxidant levels, in line with the literature. DAPA exhibited an antioxidant effect by reducing oxidative stress markers in heart embryonic H9c2 cardiomyocytes. However, the mechanism behind the positive effect of SGLT2 inhibitors (DAPA and EMPA) on cardiac function is not yet fully understood. Further studies are needed to explore how and why these changes occur in humans and through experimental research.

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

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Authorship Contributions: Concept – Z.D., H.U.; Design – Z.D., H.U.; Supervision – Z.D., H.U.; Funding – Z.D., H.U.; Materials – Z.D., H.U.; Data collection &/or processing – Z.D., H.U.; Analysis and/or interpretation – Z.D., H.U.; Literature search – Z.D., H.U.; Writing – Z.D., H.U.; Critical review – Z.D., H.U.

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Research Article

EZH2 gene and CA-IX prompt relation: An effective therapeutic target approach for melanoma progression

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Abstract

Objectives: Melanoma is a common type of skin cancer originating from melanocytes. Its standard treatments include surgery, chemotherapy, radiotherapy, and targeted therapy. However, due to limitations in drug treatments, new targets must be identified. One important enzyme in carcinogenesis, CA-IX, creates an acidic and hypoxic niche within tumor cells. Additionally, EZH2, a gene that encodes a histone-lysine N-methyltransferase, is involved in DNA methylation and plays a crucial role in cancer development by modulating epigenetic changes. In this study, our goal is to elucidate the effect of CA-IX inhibition on the EZH2 gene in melanoma.

Methods: We evaluated the effects of CA-IX inhibition with AZA on EZH2 gene expression levels in the A375 human melanoma cell line. Cell culture, ELISA, and qPCR experiments were conducted. The cytotoxic activities of AZA were assessed using the WST-1 assay. CA-IX protein levels were measured using a Human Carbonic Anhydrase IX ELISA Kit. qPCR was performed using the QuantiNova LNA Probe PCR assay.

Results: An IC_{50} value was observed at a concentration of 10.7 μM for AZA in the WST-1 assay. Decreased CA-IX protein levels were observed following AZA treatment ($p<0.0001$). Additionally, EZH2 mRNA levels were significantly reduced when CA-IX protein was inhibited by AZA ($p<0.05$).

Conclusion: Inhibition of CA-IX and the consequent changes in the acidity of the tumor microenvironment may modulate EZH2 levels. CA-IX could be a promising target for the epigenetic treatment of melanoma.

Keywords: Acetazolamide, carbonic anhydrase-IX, carbonic anhydrase inhibiton, enhancer of zeste homolog 2, melanoma

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Cancer is a disease caused by the accumulation of genetic and epigenetic alterations [1, 2]. In addition to genetic alterations, epigenetic regulations that occur during transcription are also important factors in carcinogenesis[3]. Melanoma is the most lethal and aggressive form of skin cancer and is one of the types with high malignancy [4, 5]. Although melanoma can be detected at an early stage, metastatic melanoma has a poor prognosis. As the drugs used in the treatment of melanoma are limited, new strategies to combat melanoma have been investigated [6].

The genes of the Polycomb group are a group of important epigenetic regulators that repress transcription [3]. One of the Polycomb genes, Enhancer of Zeste Homolog 2 (EZH2), encodes a histone-lysine N-methyltransferase that is involved in DNA methylation and also methylates the H3 lysine at position 27 [7]. EZH2 is the enzymatic catalytic subunit of Polycomb repressive complex 2 (PRC2). The catalysis of EZH2 is associated with the repression of gene expression and is considered a critical epigenetic event during tissue development. EZH2 acts as a regulator in the cell cycle, autophagy, and apoptosis processes and prevents cellular senescence by supporting DNA repair. EZH2 is involved in many biological processes, for example, in oncogenic metabolic pathways [3]. There are mutations and high expression of EZH2 in various cancers, suggesting that EZH2 plays a critical role in the development and progression of carcinogenesis [8]. EZH2 inhibitors

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have been investigated and developed as an important new therapeutic target. One of these inhibitors, tazemetostat, has been approved for the treatment of epithelioid sarcomas. The development of highly effective EZH2 inhibitors is of crucial importance for the treatment of cancer epigenetics [3]. EZH2 expression is also controlled by hypoxia via HIF response elements (HRE) in the EZH2 gene promoter. The expression of EZH2 increases, and thus the proliferation of cancer cells is promoted in a hypoxic environment [9, 10]. A prominent enzyme associated with hypoxia is carbonic anhydrase IX (CA-IX), a fundamental enzyme for all metabolic processes related to acid-base balance, and a transmembrane protein associated with carcinogenesis [11]. Since CA-IX is localized in the cell membrane, it increases extracellular acidosis and causes angiogenesis, epithelial-mesenchymal transformation, invasion, hypoxia, and signal transduction [12]. Because of all these functions, CA-IX inhibitors have become attractive for cancer treatment [13]. Acetazolamide (AZA), the most potent CA inhibitor, has been used clinically for many years and has also been used for CA-IX inhibition *in vivo* tumor models [14].

Due to its crucial role in carcinogenesis, gene expression, and developmental processes, CA-IX also activates the epigenetic program of the epithelial-mesenchymal transition in cancer cells, thus contributing to cancer cell survival [15, 16]. Since melanomas exhibit a higher expression level of CA-IX, epigenetic changes may play a crucial function in the development of melanomas. The role of EZH2 in this process, known to be associated with epigenetic regulation, is not yet clear. Therefore, in our study to clarify the relationship between EZH2 and the tumor-associated enzyme carbonic anhydrase CA-IX, we tested the hypothesis that inhibition of CA-IX is associated with EZH2 in melanoma.

Materials and Methods

Cell culture

The A375 human melanoma cell line was obtained from Hacettepe University Pharmacy Faculty Biochemistry Department, Ankara, Türkiye. All cells were cultured in DMEM (Capricorn, DMEM/HPA) including 10% Fetal Bovine Serum (FBS) (Capricorn, FBS-HI-11A), 1% Streptomycin/Penicillin (Capricorn, PS-B) at optimum conditions (37°C, 5% CO_2). Acetazolamide (AZA) was purchased from Sigma (BCBZ9159). It was prepared as a stock solution by dissolving it in DMSO at a concentration of 300 µM.

Cytotoxicity test

Using 96-well plates, all cells were cultured with DMEM supplemented with 10% FBS. The cells were treated with 0, 2.5, 5, 10, 25, 50, 100 µmol/L concentrations of AZA for 24 h and 48 h, in triplicate, in a humidified 5% CO_2 atmosphere. WST-1 reagent was added to the wells and incubated at 37°C for 2 h. The plates were then read at 450 nm on an Epoch™ Microplate Spectrophotometer (BioTek, USA). The IC_{50} value of AZA was determined as 10.7 µmol/L using a WST-1 assay (Cayman Chemical).

While calculating IC_{50} values:

% Viability = (Average absorbance of inhibitor × 100) / (Average absorbance of control)

% Inhibition = 100 – ((Average absorbance of inhibitor × 100) / (Average absorbance of control))

Then, the inhibitor concentration values were entered into the GraphPad Prism 9.1.0 program, converted into a graph, and the inhibitor dose (IC_{50}) value that inhibited 50% of the cells was calculated.

ELISA

CA-IX levels were measured using the Human Carbonic Anhydrase IX ELISA Kit BT Lab (Zhejiang, China). Firstly, the collected cells were diluted in DPBS to reach a concentration of one million/mL. The damaged cells were lysed with prepared freeze-thaw cycles. After centrifugation (4°C, 1500 g, 10 min), the supernatants were collected carefully. Briefly, 40 µL of supernatant, 10 µL of primary antibody, and 50 µL of Streptavidin-Horseradish Peroxidase were mixed to reach a final volume of 100 µL at 37°C for 60 min. After incubation, chromogen solutions A and B were added to the wells. Following a 10 min incubation at 37°C, a stop solution was used to end the reaction. All experiments were performed in triplicate (n=3) and read at 450 nm by an Epoch™ Microplate Spectrophotometer (BioTek, USA).

RNA isolation

The cells were trypsinized and washed with DPBS for RNA isolation. The miRNeasy Kit and QIACube Connect (QIAGEN) were used for total RNA isolation according to the manufacturer's instructions (QIAGEN). Total RNAs were quantified with the QIAxpert Instrument. cDNA synthesis was performed with the QuantiNova Kit.

qPCR

qPCR was performed using the QuantiNova LNA Probe PCR assay. PCRs were conducted at a final volume of 50 µL. All PCR reagents were purchased from QIAGEN. Each reaction mixture contained 10 µL of 2× QuantiNova Probe PCR Master Mix, 2 µL of 10x Probe PCR Assay, and 3 µL of RNase-free water. The reactions were run for 45 cycles on a Rotor Gene Q device (QIAGEN), with denaturation at 3 min at 25°C, annealing at 10 min at 45°C, and extension at 5 min at 85°C. Primer sequences' catalog numbers are UPFH0128337 for EZH2 and UPFH1132903 for CA9. The relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method. Relative mRNA amounts were calculated using the CT method. $\Delta CT = CT_{target} - CT_{GAPDH}$, $\Delta\Delta CT = CT_{treatment} - CT_{calibrator}$, where the calibrator was the no-treatment group. CT was then converted to fold change using the formula $2^{-\Delta\Delta CT}$ [17]. The GAPDH gene was selected as an internal control to normalize the relative expression levels of EZH2 and CA9. The comparative quantification of the individual target genes was based on the cycle threshold (CT), which was normalized to GAPDH using the ΔΔCT method. GAPDH is constitutively expressed at high levels in almost all tissues. For this reason, we use GAPDH

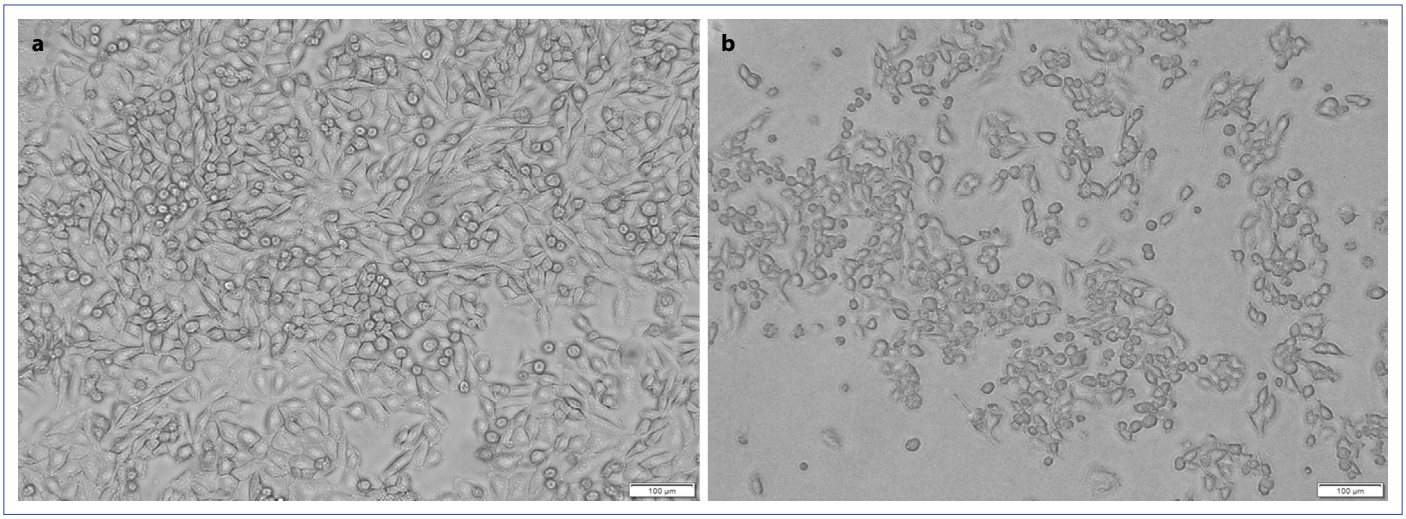

Figure 1. (a) Images of A375 melanoma cells without treatment. (b) 24 hours after AZA treatment.
AZA: Acetazolamide.

as the housekeeping gene for qPCR. In addition, according to the literature, the GAPDH gene was the most stable gene, so we used this gene as the housekeeping gene [18, 19].

Statistical analysis

Statistical analysis for ELISA was performed with Student's t-test in the GraphPad Prism 5.0 program. The relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method. When analyzing qPCR data, we first calculate Delta Ct (ΔCt), obtained by subtracting the reference gene Ct from the target gene Ct. Ct was then converted to fold change using the formula $2^{-\Delta\Delta CT}$. The qPCR results were analyzed with the GeneGlobe program. All significance levels were accepted as $p<0.05$.

Results

Acetazolamide reduces the viability of A375 melanoma cells

A375 melanoma cells were treated with AZA for 24h and 48h (Fig. 1). After treatment with 0, 2.5, 5, 10, 25, 50, 100 µM AZA concentrations for 24h, the viability of A375 melanoma cells were 100%, 100%, 70.2%, 50.3%, 29.6%, 28.6%, and 27.3%, respectively. Additionally, after 48h with the same AZA concentrations, the viabilities were 100%, 100%, 52.6%, 14.3%, 13.3%, 12.5%, and 12.1%, respectively. Based on these results, the dose of AZA for A375 melanoma cells was determined to be 10.7 µM at 24h.

CA-IX levels decreased in A375 melanoma cells treated with AZA

The CA-IX levels in A375 melanoma cells were 5.31 ng/mL in the AZA non-treated group, while it was 1.81 ng/mL in the AZA-treated group. CA-IX levels decreased in cells treated with AZA ($p<0.0001$). The decrease indicates that AZA can inhibit the CA-IX enzyme in melanoma cells. Moreover, AZA is not only a classical CA inhibitor but also effectively inhibits the CA-IX isoenzyme, which plays a role in tumorigenesis (Fig. 2).

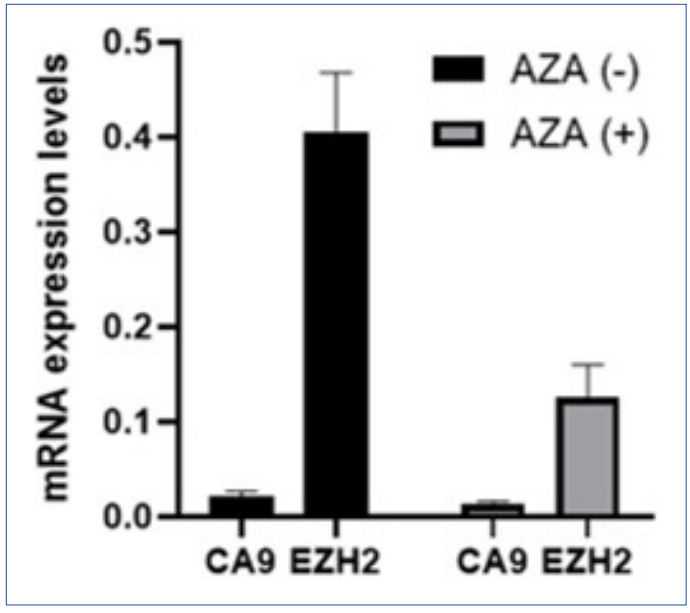

Figure 2. $2^{-\Delta\Delta Ct}$ values of GAPDH-normalized CA9 and EZH2 genes in A375 melanoma cells treated with 10.7 µmol/L AZA ($p=0.4136$, CA9; $p<0.05$, EZH2).
EZH2: Enhancer of Zeste Homolog 2.

EZH2 mRNA levels decreased in A375 melanoma cells treated with AZA

To assess the mRNA expression of EZH2 and CA9 in A375 melanoma cells, qPCR was performed. The results indicated that while the mRNA expression of CA9 decreased after AZA treatment, the change was not statistically significant ($p=0.4136$). However, EZH2 mRNA expression decreased significantly ($p<0.05$) (Fig. 3). AZA treatment resulted in a 1.67-fold decrease in CA9 and a 3.57-fold decrease in EZH2 gene expression (Fig. 4). The substantial reduction in EZH2 suggests that AZA could be used to inhibit this critical Polycomb protein.

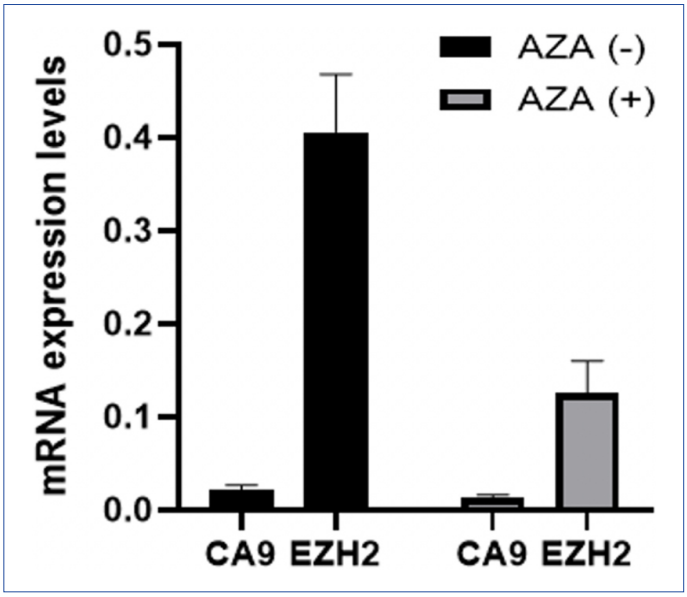

Figure 3. mRNA expression levels of CA9 and EZH2 genes in A375 melanoma cells treated with 10.7 µmol/L AZA (p=0.4136, CA9; p<0.05, EZH2).

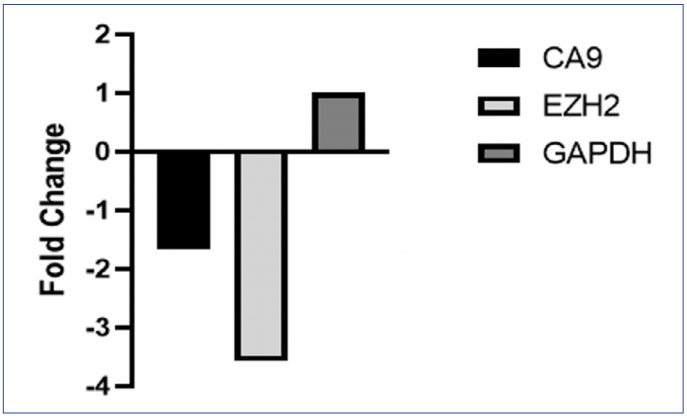

Figure 4. The fold increase rates of the mRNA expressions of CA9 and EZH2 genes in A375 melanoma cells treated with 10.7 µmol/L AZA after normalization with GAPDH compared to the control group (-1.67 fold change, CA9; -3.57 fold change, EZH2).

Discussion

CA-IX is a transmembrane isoform of CAs and regulates acidity in cancer cells [20, 21]. Additionally, acidosis is thought to be responsible for epigenetic changes in cancer [22]. In this study, we aimed to investigate the effect of CA-IX inhibition on the EZH2 polycomb protein, which is a key epigenetic modulator.

CA-IX is an important regulator of many processes involved in carcinogenesis and is highly expressed in melanomas [23]. Andreucci et al. [21] demonstrated that melanoma, breast, and colon cancer cells exposed to acid significantly increase CA-IX expression. Federici et al. [23] observed that CA-IX was expressed by malignant human melanoma cells and that specific CA-IX inhibitors suppressed the growth of melanoma cells. Chafe et al. [24] discovered that CA9 expression was associated with decreased immune activity in tumors from patients with metastatic melanoma and that the CA-IX inhibitor SLC-0111 may represent a significant therapeutic option. In summary, literature indicates that an aggressive melanoma phenotype with high CA-IX expression and the application of inhibitors to this protein should improve prognosis. In our study, we observed a notable decrease in CA-IX protein levels in melanoma cells post-AZA treatment. Given the role of CA-IX in melanoma, its inhibition may be a promising strategy [23, 25].

EZH2 is an epigenetic regulator that plays a crucial role in DNA methylation and melanoma development [26]. High expression of EZH2 has been shown to be associated with malignant forms of melanoma [27, 28]. Mahmoud et al. [29] highlighted a close association between EZH2 and metastatic melanoma, suggesting that EZH2 facilitates melanoma progression by activating oncogenic signaling pathways and deactivating tumor suppressive signaling pathways. Considering that epigenetic changes are known to be induced by acidosis, we postulate that CA-IX inhibition impacts EZH2, a key regulator of epigenetics. Huang et al. [16] observed increased expression of EZH2 and CA-IX under hypoxic conditions. Our study aimed to demonstrate the effect of CA-IX inhibition on EZH2 levels through qPCR experiments. Our results indicate that inhibiting CA-IX leads to a marked reduction in EZH2 levels. Our study suggests that CA-IX inhibition can modulate epigenetic alterations by lowering EZH2 levels, proposing an alternative method to modulate epigenetics by targeting CA-IX in melanoma (Fig. 1).

Conclusion

In summary, various innovative strategies for melanoma treatment have been explored over the years. One such strategy involves the pivotal modulator EZH2, which is essential for the survival of melanoma cells by regulating gene expression. Our preliminary data suggest that EZH2 gene levels decrease upon CA-IX inhibition, which is associated with poor prognosis in melanoma cells, making CA-IX inhibition a potentially valuable approach for advancing melanoma treatment strategies. Additionally, our research acts as a link between past and prospective studies on melanoma treatment approaches.

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

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Research Article

The association of serum proprotein convertase furin/PCSK3 concentrations with stable coronary artery disease and coroner artery lesion severity

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Abstract

Objectives: Furin (Proprotein Convertase Subtilisin/Kexin Type 3, PCSK3) is a proprotein convertase involved in the processing of precursor proteins. Furin substrates play significant roles in the initiation and progression of atherosclerosis, which is the primary cause of coronary artery disease (CAD). This study aimed to investigate the serum furin concentrations in stable CAD patients and their relationship with disease severity.

Methods: The study included 81 stable CAD patients and 50 subjects without coronary artery lesions. Coronary angiography was performed via the percutaneous femoral artery approach, and the severity of CAD was assessed using the Gensini score. Serum furin concentrations were measured using an enzyme-linked immunosorbent assay.

Results: Serum furin concentrations were significantly higher in CAD patients compared to CAD-negative subjects (p=0.0001). The serum furin levels of mild, moderate, and severe CAD patients were 1.53 ng/mL, 2.01 ng/mL, and 3.03 ng/mL, respectively, which were significantly different from CAD-negative subjects (p=0.018, p=0.002, and p=0.0001, respectively). Furin levels were found to be an independent predictor of CAD and exhibited potential diagnostic value for CAD and severe CAD.

Conclusion: The study concluded that serum furin concentrations could be considered a new risk factor for CAD, in addition to well-known biomarkers.

Keywords: Atherosclerosis, coronary arteries, dibasic processing enzyme, paired basic amino acid cleaving enzyme, proprotein convertases

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Cardiovascular diseases (CVDs) continue to be the most important health problem and are the leading cause of death globally [1]. CVDs are a group of disorders of the heart and blood vessels including coronary artery disease (CAD). The main cause of CAD is atherosclerosis, which is a complex pathophysiological process mainly driven by endothelial dysfunction, lipid accumulation, and inflammation [2]. While traditional risk factors including dyslipidemia, hypertension, diabetes mellitus contribute to the pathogenesis of CAD, other novel risk factors may be involved. The proprotein convertases

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(PCs) are serine proteases involved in the processing of precursor proteins; they often are responsible for the activation of their substrates but sometimes lead to inactivation [3, 4]. PCs are generally abbreviated as PCSK because of their similarity with bacterial subtilisin and yeast kexin proteases [5], and the genes encoding these enzymes are called *PCSKs* [6]. There are nine mammalian PC families: PC1/3, PC2, Furin, PC4, PC5/6, PACE4, PC7, SKI-1/S1P, and PCSK9 [4]. Furin (PCSK3) is the first endoprotease identified among the PCs and is expressed ubiquitously in all mammalian tissues and cell lines [7]. This PC is localized primarily in the trans-Golgi network (TGN) but can cycle between the TGN and the cell surface. Furin, a type-I membrane-bound protein, can be present in the extracellular milieu with ectodomain shedding from the membrane [8, 9]. There are many mammalian substrates of furin including cytokines, hormones, growth factors, receptors, and it also cleaves bacterial and viral substrates [5, 10]. Any dysfunction in the expression or activity of furin may be associated with a variety of disorders, such as atherosclerosis, cardiovascular diseases (CVDs), cancer, diabetes, infectious diseases, inflammation [5, 11–14]. In particular, the potential targets of furin are involved in the initiation and progression of atherosclerosis by regulating lipid and lipoprotein metabolism, inflammatory response, blood pressure, and formation of atherosclerotic lesions [11, 15]. In recent years, it has been shown that the expression of furin and PCSKs5-7 is associated with key molecular pathways and mechanistic networks for CVD [12]. Also, it has been observed that furin is among the proprotein convertases with the highest novel CVD therapeutic potential [12], and it would be important to determine the exact role of furin in CAD pathogenesis as a new target for the treatment of the disease.

To the best of our knowledge, studies focusing on the clinical use of furin as a biomarker for CAD are insufficient. We hypothesized that furin might be a circulating candidate biomarker for CAD and its level could be related to coronary artery lesion severity. The aim of the present study was to investigate the serum level of furin protease in subjects with CAD and to evaluate the relationship between its concentration and the severity of coronary artery disease determined by Gensini score.

Materials and Methods

Study population

The study population consisted of eighty-one (81) stable coronary artery disease patients who were diagnosed by coronary angiography [CAD (+)] and fifty (50) individuals who did not have a lesion in their coronary arteries [CAD (–)], who applied to the outpatient clinic of Karadeniz Technical University Faculty of Medicine, Department of Cardiology. A detailed anamnesis from all subjects was taken, and physical examinations were performed before coronary angiography. Individuals younger than 18 years of age, who had malignant neoplasm, severe renal/hepatic disease, systemic infection, recent history of surgery, or were pregnant were excluded from the study. An informed consent form was signed by the individuals participating in the study. Ethics committee approval was obtained for the research from the Karadeniz Technical University (KTU) Faculty of Medicine Scientific Researches Ethics Committee (Submission number: 2011/3, approval date: October 17, 2011) and was in accordance with the Declaration of Helsinki.

Gensini score

Coronary angiography was performed for all individuals via the percutaneous femoral artery approach following the standard procedure [16]. The Gensini score [17] was used to assess the severity of coronary artery disease. Gensini scores were determined using the following steps: 1. Definition of the concentric or eccentric luminal narrowing degrees; 1 point for ≤25% stenosis, 2 points for 26–50% stenosis, 4 points for 51–75% stenosis, 8 points for 76–90% stenosis, 16 points for 91–99% stenosis, and 32 points for 100% occlusion. 2. Definition of the coronary artery lesion site; 5 points for the left main coronary artery, 2.5 points for the proximal left anterior descending branch and left circumflex artery, 1.5 points for midsegment of the left anterior descending coronary artery, 1 point for the diagonal branch and obtuse marginal branches, and 0.5 points for the second diagonal and left circumflex artery posterolateral branch. 3. The summation of the individual coronary segment scores (the narrowing scores were multiplied by a coefficient defined for each main coronary artery and each segment). The CAD (+) patients were classified into three groups according to the tertile of their Gensini score: Mild (Gensini score 2–7.5 points), moderate (Gensini score 8–35.5 points), and severe (Gensini score 36–177 points).

Biochemical analyses

Blood samples were obtained from an antecubital vein of each subject into the serum separator tubes without anticoagulant. Serum samples were separated after centrifugation at 1800g for 10 min and stored at –80°C until analysis. Serum concentrations of glucose, triglyceride (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) were measured enzymatically using a Roche/cobas 6000 series clinical chemistry analyzer (Mannheim, Germany). Serum hs-CRP concentrations were assigned by a latex-enhanced immunonephelometric method on a Siemens Dade Behring BN II nephelometer (Marburg, Germany). The measurement of serum hs-TroponinT concentrations was determined by an Electrochemiluminescence (ECL) method on a Roche Elecsys 2010 (Mannheim, Germany). The original reagents of the analyzers were used to measure the concentrations of variables. The quantification of these variables was carried out in the Medical Biochemistry Laboratory of Farabi Hospital at Karadeniz Technical University Faculty of Medicine after daily quality control procedures were completed. Serum furin concentrations were determined by using an enzyme-linked immunosorbent assay kit (Human furin USCN Life Science Inc., Wuhan, PRC) and expressed as ng/mL. The minimum

Table 1. Demographic, anthropometric and biochemical variables in CAD (-) and CAD (+) subjects

Variables	CAD (-) n=50		CAD (+) n=81		p
	n	%	n	%	
Age, years	58±14		62±12		0.043*
Male,	16	32	65	80	0.0001**
Hypertension	24	48	41	51	0.771**
Diabetes mellitus	2	4	18	22	0.005**
Smoking	2	4	18	22	0.005**
Family history	21	42	36	44	0.878**
BMI, kg/m^2	28 (26–32)		29 (26–32)		0.636
Waist cimcumference, cm	100 (90–106)		102 (94–110)		0.293
SBP, mm/Hg	120 (110–136)		125 (118–133)		0.703
DBP, mm/Hg	78 (60–80)		78 (68–80)		0.710
Heart rate, beats/min	70 (68–75)		72 (65–78)		0.738
TC, mg/dL	173±37		167±42		0.399*
LDL-C, mg/dL	123±30		120±38		0.694*
HDL-C, mg/dL	41.5±10.4		36±6.67		0.001*
TG, mg/dL	106 (83–163)		133 (93.5–167)		0.348
Glucose, mg/dL	94 (82.8–110)		98 (85.5–149)		0.076
Furin, ng/mL	1.00 (0.65–2.20)		2.11 (1.17–3.50)		0.0001
Hs-TroponinT, ng/L	6 (4–11)		9 (7–18)		0.001
Hs-CRP, mg/L	0.28 (0.11–0.63)		0.34 (0.15–0.79)		0.287

*: p for Independent t-test and values are given as mean±standard deviation; **: p for Pearson Chi-Square Test and values are given as n (%). The remaining variables are given as median with IQR(Q1-Q3) and Mann-Whitney U Test was used for statistical analysis. CAD: Coronary artery disease; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; TG: Triglyceride; Hs-CRP: High sensitive-C reactive protein.

detectable dose of the assay is less than 0.055 ng/mL with an intra-assay CV% of <10% and an inter-assay CV% of <12%.

Statistical analyses

The Kolmogorov-Smirnov test was used to determine whether the variables were normally distributed. The data were presented using mean and standard deviation (mean±SD) for normally distributed variables. The data not scattered normally were expressed as median with interquartile range (Q1-Q3) values. The independent t-test was used to compare the variables that showed normal distribution. Pairwise comparisons were made using the Mann-Whitney U test for non-normal distribution. The data comparisons for mild, moderate, and severe CAD (+) patients were performed by the Kruskal-Wallis test. The Chi-Square test was used for categorical variables. Binary logistic regression analysis was performed to determine independent predictors of CAD. A binary logistic regression model was established using age, gender, diabetes mellitus, smoking, HDL-C, and furin that were significant in the univariate analysis as independent variables. The model's goodness of fit was evaluated using the Hosmer-Lemeshow test. The correlation coefficients (r) and their significance were assessed by Pearson correlation analysis, and log transformation was performed for non-normally distributed variables. Statistical analyses were performed using SPSS version 23.0 software (SPSS, Inc., Chicago, USA). Receiver operating characteristics (ROC) curves were constructed on MedCalc version 9.6.4 software (MedCalc Software BVBA, Belgium) to assess sensitivity, specificity, and respective areas under the curves (AUCs) with 95% CI. P-values <0.05 were considered statistically significant.

Results

The main characteristics of demographic, anthropometric, and biochemical variables of the study population are represented in Table 1. The mean age of CAD (–) subjects was 58±14 years, and the mean age of CAD (+) patients was 62±12 years (p=0.043). Male individuals constituted 80% of CAD (+) patients and were significantly different from CAD (–) subjects (p=0.0001). The body mass index (BMI), waist circumference, systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate values were not different between CAD (+) patients and CAD (–) subjects. The history of diabetes mellitus and smoking was found to be higher in CAD (+) patients (p=0.005). In addition, no significant difference was found in fasting TC, LDL-C, TG, and glucose levels, but HDL-C levels showed a significant decrease in CAD (+) patients (p=0.001). The median serum furin concentration in CAD (+) patients (2.11 ng/mL) was approximately twofold higher compared to CAD (–) subjects (1.00 ng/mL) (p=0.0001) (Table 1 and Fig. 1a). Hs-TroponinT concentration was found to be significantly

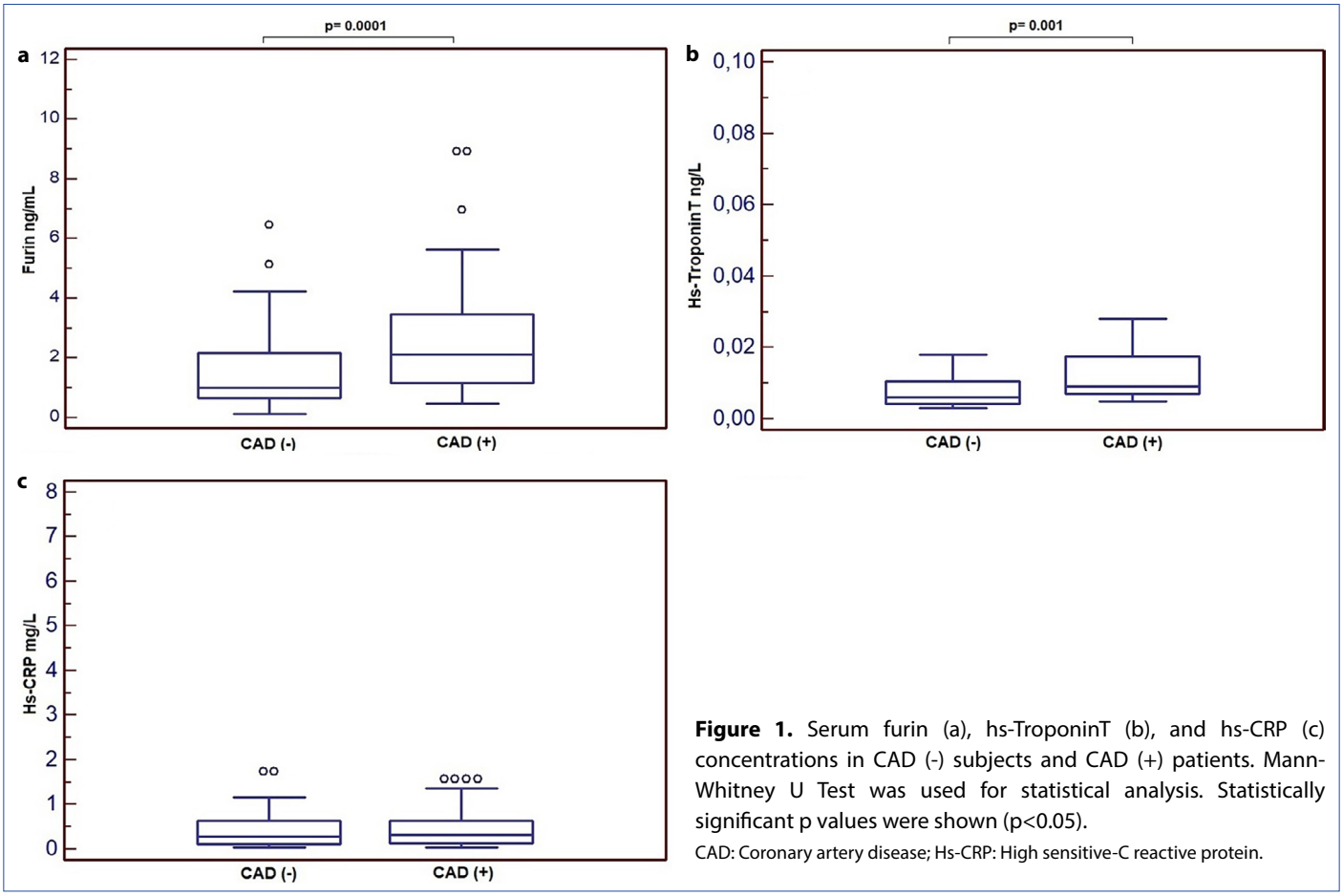

Figure 1. Serum furin (a), hs-TroponinT (b), and hs-CRP (c) concentrations in CAD (-) subjects and CAD (+) patients. Mann-Whitney U Test was used for statistical analysis. Statistically significant p values were shown (p<0.05).

CAD: Coronary artery disease; Hs-CRP: High sensitive-C reactive protein.

higher in CAD (+) patients versus CAD (–) subjects (p=0.001) (Table 1 and Fig. 1b). Although there was an increase in hs-CRP concentrations in CAD (+) patients, it did not reach a statistically significant level (p=0.287) (Table 1 and Fig. 1c).

In order to assess the potential of serum furin concentrations to discriminate between CAD (+) patients and CAD (–) subjects, ROC curve analysis was performed. The ROC curves for furin, hs-TnT, and hs-CRP were evaluated, and the area under the curve (AUC) values, 95% confidence intervals (CI), and p-values were provided in Figure 2. Serum furin concentrations had significant discriminating ability between CAD (+) patients and CAD (–) subjects with the highest AUC value of 0.730 (95% CI=0.646–0.804, p=0.0001). In addition, the serum furin cut-off value corresponding to the maximum of the Youden index was 1.03 ng/mL (86.4% sensitivity, 54% specificity).

The results of the binary logistic regression analysis for CAD risk factors are presented in Table 2. The analysis showed that serum furin concentrations (OR=1.399, 95% CI=1.075–1.822, p=0.013), gender (OR=7.322, 95% CI=2.767–19.376, p=0.001), and age (OR=1.040, 95% CI=1.003–1.078, p=0.034) were independent predictors for CAD.

CAD (+) patients were divided into tertiles as mild, moderate, and severe according to their Gensini scores (Table 3). The median furin levels of mild, moderate, and severe CAD (+) patients were 1.53 ng/mL, 2.01 ng/mL, and 3.03 ng/mL, respectively (Table 3).

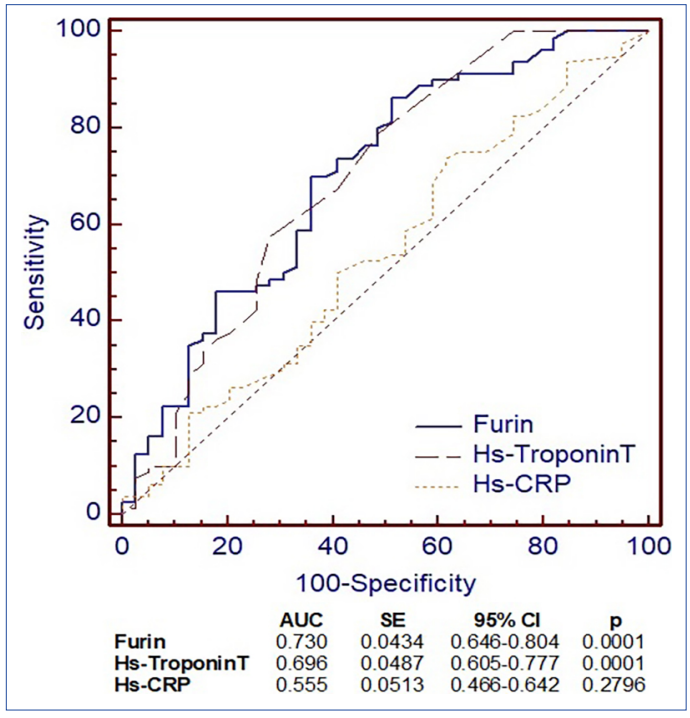

Figure 2. Receiver operating characteristic curve of furin, hs-TroponinT and hs-CRP concentrations for discrimination of CAD (+) patients from CAD (-) subjects.

Table 2. Odds ratios (ORs) and 95% confidence intervals (CIs) for coronary artery disease by using binary logistic regression analysis

Variables	OR	95% CI	p
Gender	7.322	2.767–19.376	0.001
Furin	1.399	1.075–1.822	0.013
Age	1.040	1.003–1.078	0.034
Diabetes mellitus	5.178	0.957–28.024	0.056
Smoking	2.888	0.565–14.762	0.203
HDL-C	0.967	0.912–1.025	0.254

Although it tended to increase, it was not statistically significant among tertiles (p=0.131). On the other hand, the serum furin levels of mild, moderate, and severe CAD (+) patients were found significant according to CAD (–) subjects (p=0.018, p=0.002, and p=0.0001, respectively, Fig. 3a). Moreover, hs-TroponinT concentrations of mild, moderate, and severe CAD (+) patients were found significant according to CAD (–) subjects (p=0.025, p=0.006, and p=0.002, respectively, Fig. 3b), but hs-CRP levels of the tertiles were not significant compared to CAD (–) subjects (p>0.05, Fig. 3c). There were no statistically significant alterations between the tertiles in terms of other variables (Table 3).

The results of ROC curve analysis of the potential diagnostic value of serum furin concentrations for severe CAD patients were provided in Figure 4. For the analysis, mild+moderate CAD patients (n=54) were considered as one group. Serum furin levels had discriminating ability between severe CAD patients and mild+moderate CAD patients (AUC=0.635, 95% CI=0.520–0.739, p=0.045). The serum furin cut-off point corresponding to the maximum of the Youden index was 2.61 ng/mL (59.3% sensitivity, 74.1% specificity).

The associations between serum furin levels and other variables were also evaluated. In the whole study population, we observed a positive correlation between serum furin levels and hs-CRP levels (r=0.179, p=0.041), but serum furin levels showed a negative correlation with serum HDL-C levels (r=–0.203, p=0.020). Moreover, furin levels did not correlate with the Gensini score in CAD (+) subjects (r=0.091, p=0.420).

Discussion

The main findings of the present study were that the concentrations of serum furin increased in stable CAD patients and showed a tendency to increase with coronary artery lesion severity.

There were limited investigations about circulating furin levels in CAD patients. To our knowledge, there is only one study examining plasma furin levels in stable CAD patients. This study by Langnau et al. [18] focused on the association between plasma furin levels and the prognosis of COVID-19 patients with preexisting stable CAD. Additionally, they did not observe any difference in furin levels between healthy controls (n=39) and stable CAD patients (n=28) [18]. We observed

Table 3. Demographic, anthropometric and biochemical variables according to Gensini score tertiles in CAD (+) patients

Variables	Mild n=27		Moderate n=27		Severe n=27		p
	n	%	n	%	n	%	
Age, years	62 (53–70)		62 (53–68)		64 (55–70)		0.751
Male	19	70	24	89	22	82	0.228*
Hypertension	14	52	13	48	14	52	0.952*
Diabetes Mellitus	3	11	9	33	6	22	0.145*
Smoking	8	30	7	26	3	11	0.223*
Family history	13	48	9	33	14	52	0.377*
BMI, kg/m^2	27 (25–32)		29 (25–32)		29 (27–32)		0.444
Waist cimcumference, cm	105 (90–110)		102 (95–110)		100 (94–108)		0.952
SBP, mm/Hg	125 (120–130)		120 (110–130)		130 (120–140)		0.312
DBP, mm/Hg	80 (75–80)		70 (65–80)		70 (65–80)		0.028
Heart rate, beats/min	72 (67–76)		68 (60–78)		71 (65–82)		0.503
TC, mg/dL	167 (153–191)		154 (127–175)		162 (138–190)		0.174
LDL-C, mg/dL	127 (104–138)		106 (85–134)		115 (104–142)		0.176
HDL-C, mg/dL	36 (31–41)		36 (31–39)		36 (29–44)		0.898
TG, mg/dL	124 (81–154)		136 (109–167)		136 (88–179)		0.724
Glucose, mg/dL	95 (86–122)		102 (89–166)		90 (83–149)		0.409
Furin, ng/mL	1.53 (1.05–3.37)		2.01 (1.17–2.61)		3.03 (1.45–3.87)		0.131
Hs-TroponinT, ng/L	6 (9–18)		7 (9–17)		7 (11–18)		0.629
Hs-CRP, mg/L	0.25 (0.12–0.52)		0.33 (0.21–0.63)		0.46 (0.18–1.36)		0.206

*: p for Pearson Chi-Square Test and values are given as n (%). The remaining variables are given as median with IQR(Q1-Q3) and Kruskal Wallis Test was used for statistical analysis.

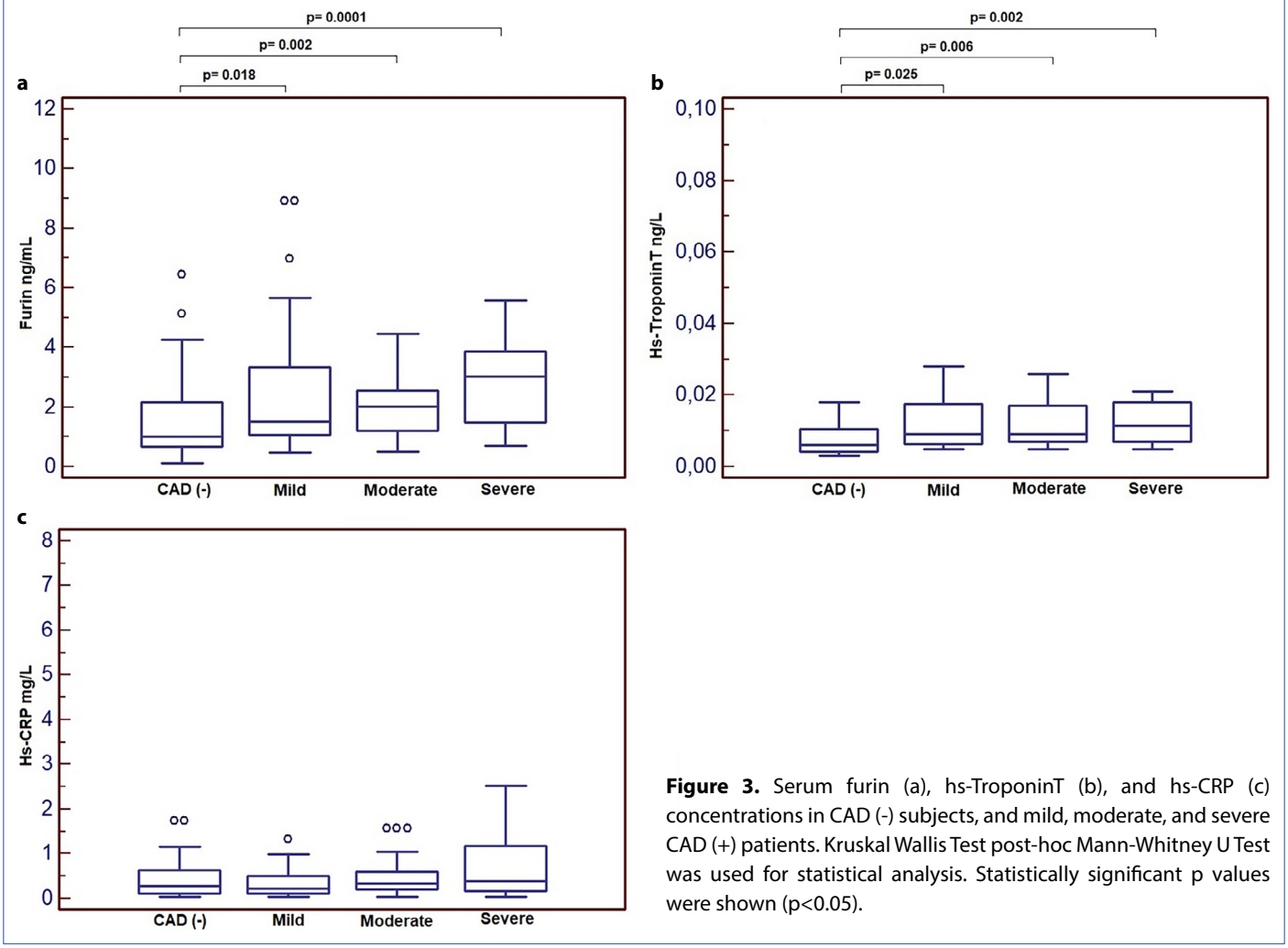

Figure 3. Serum furin (a), hs-TroponinT (b), and hs-CRP (c) concentrations in CAD (-) subjects, and mild, moderate, and severe CAD (+) patients. Kruskal Wallis Test post-hoc Mann-Whitney U Test was used for statistical analysis. Statistically significant p values were shown ($p<0.05$).

approximately twofold increased serum furin levels in stable CAD patients compared to CAD (–) subjects. The difference in findings may be due to the characteristics of the study population. In the current study, the increased serum furin levels in CAD patients might be explained by its increased cellular activity. Furin has roles in the transformation of many zymogen substrates to their functionally active form, which participate in atherogenesis, and thus CAD. Furin modulates lipid metabolism by inactivating endothelial lipase (EL) [19] and lipoprotein lipase (LPL) [20]. It regulates plasma LDL-C levels by cleaving PCSK9 [21]. Furin promotes the activation of pro-inflammatory tumor necrosis factor (TNF) superfamily cytokines [22], is involved in the MT1-MMP driven proteolytic cascade of pro-MMP2 activation [23], and regulates blood pressure by cleaving the (pro)renin receptor [24]. Thus, furin is an important regulator of cell functions associated with the initiation and progression of atherosclerosis [11]. In the present study, ROC curve analysis of furin for stable CAD patients showed discriminating power, and its value was better than hs-TnT. Also, binary logistic regression analysis indicated that serum furin concentrations, gender, and age were independent predictors for CAD. Therefore, it could be proposed that increased levels of furin may indicate progression of atherosclerosis and serve as an independent predictor for CAD risk.

When evaluating furin levels in relation to coronary artery lesion severity, there was a tendency for serum furin levels to increase from the bottom tertile to the upper tertile. One of the most important findings of the current study was that severe CAD patients had the highest serum furin levels. Furthermore, serum furin levels discriminated severe CAD patients from mild and moderate CAD patients. These findings may be clinically important because of the potential role of furin as a circulating biomarker of coronary artery disease lesion severity. However, in the current study, serum furin levels did not correlate with the Gensini score in CAD patients. Wang et al. [25] investigated the prognostic value of furin in acute myocardial infarction (AMI) patients [25]. They observed that patients with higher furin levels had an increased risk of major adverse cardiac events (MACE), all-cause mortality, recurrent MI, and hospitalization for heart failure. Conversely, another study found that plasma furin was not associated with the risk of MACE, but higher levels of plasma furin might be related to a higher risk of recurrent MI in AMI patients [26]. Moreover, Liu et al. [26] found a slight increase in cTNT levels in patients with higher

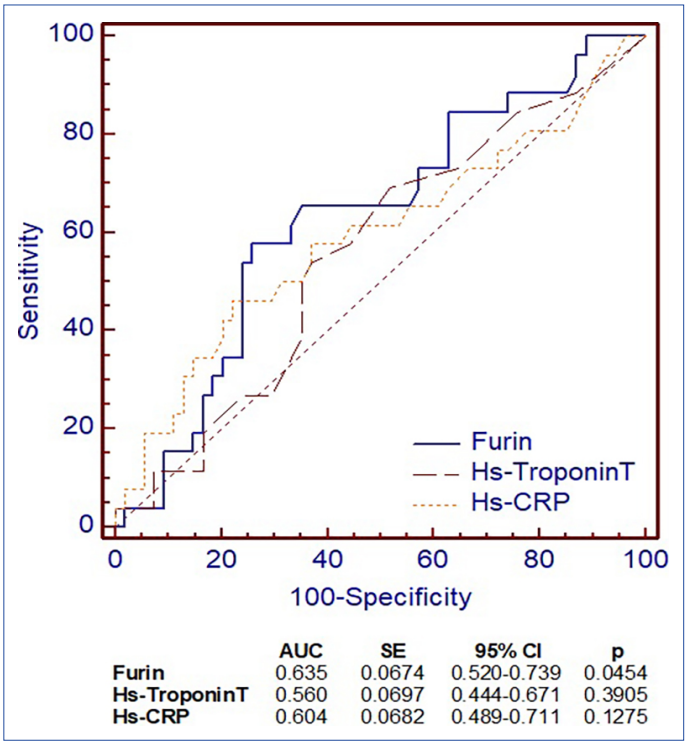

Figure 4. Receiver operating characteristic curve of furin, hs-TroponinT and hs-CRP concentrations for discrimination of severe CAD (+) patients from mild and moderate CAD (+) patients.

plasma furin concentrations. They suggested that higher circulating furin concentrations may imply progression of atherosclerosis and more severe or vulnerable plaque lesions.

The main limitation of the current study was that the number of CAD patients in tertiles was small. Further studies with larger sample sizes on the clinical effectiveness of using furin as a circulating biomarker for CAD risk and prognosis need to be conducted.

It was concluded that serum furin levels were increased in stable CAD patients, predicted CAD risk, and discriminated the severity of CAD. Therefore, furin might be used as a candidate molecule for determining CAD risk.

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

Ethics Committee Approval: The study was approved by The Karadeniz Technical University Faculty of Medicine Scientific Researches Ethics Committee (No: 2011/3, Date: 17/10/2011).

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Research Article

Biomarkers of a Football Match-play - Internal load analysis using Technical Soccer Specific Aerobic Field Test (TSAFT^{90})

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Abstract

Objectives: The study measured the magnitude of physiological, immune, endocrine, and muscle damage markers of exercise-induced fatigue using the Technical Soccer Specific Aerobic Field Test (TSAFT^{90}). We examined the effect of fatigue on performance and recovery at 24 hours post-exercise using biochemical indices.

Methods: Professional football players (n=30) with a mean age of 19.20 years participated in the study during their preseason. To induce fatigue, participants underwent a 90-minute fatigue simulation program, TSAFT^{90}. Venous blood samples were collected at baseline, 0-hour, and 24 hours post-fatigue. Analyzed markers included fatigue metabolites (lactate and uric acid), endocrine response marker (cortisol), muscle damage marker (creatine kinase), immunological markers (leukocytes, lymphocytes, neutrophils, and monocytes), inflammatory marker (CRP), hydration indicator (serum osmolality), and recovery marker (magnesium). Ball velocity and a 7-point Likert scale for muscle soreness were recorded to assess performance and perception of fatigue, respectively.

Results: All biomarkers studied were significantly elevated ($p<0.05$) at 0 hours post-fatigue. Uric acid, creatine kinase, leukocytes, monocytes, CRP, serum osmolality, and magnesium remained altered at 24 hours. Ball velocity significantly reduced post-fatigue ($p=0.04$) from 94.67 km/hr to 90.47 km/hr, whereas there was no change in the soreness scale.

Conclusion: The failure of the biomarkers to return to baseline levels within 24 hours indicates disrupted homeostasis. Monitoring the internal load with biomarkers aids in formulating strategies that can delay or mitigate fatigue and help achieve optimal performance and recovery, thus reducing the likelihood of injury.

Keywords: Athletic performance, biomarkers, fatigue, football, recovery, sports injury

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Playing a 90-minute football match leads to a decrease in physical performance with associated disturbances in psychophysiological and biochemical parameters. Neuromuscular fatigue is defined as an exercise-induced reduction in the maximal voluntary force that a player can exert. Fatigue causes a breakdown in internal homeostasis due to an increase in energy production demanded by an external stimulus [1]. This incomplete force restoration can significantly influence performance. Most football injuries occur in the final 15 minutes of each half, with a higher injury risk in the second half than in the first [2]. Fatigue monitoring and management are crucial for controlling athletes' training adaptations and reducing

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their susceptibility to injury as they face higher physical demands coupled with fixture congestion [3]. Among the tools available to monitor fatigue, the serum biochemical profile reflects the ideal internal load. Biomarker fluctuations could affect performance, and recovery is thought to be influenced by these changes [4]. Immunosuppression tends to occur following a rapid increase in training load, leading to a higher risk of illness and injury for athletes who do not return to baseline levels within the latency period [5].

Existing literature categorizes the peripheral biomarkers of exercise-induced fatigue into several groups: Fatigue metabolites (lactate and uric acid) [6, 7], endocrine response markers (cortisol and testosterone) [7, 8], muscle damage markers (creatine kinase and myoglobin) [7–10], immunological markers (lymphocytes, leukocytes, neutrophils, and monocytes)[4, 8, 11], oxidative stress and inflammatory markers (reactive oxygen species, TAS, CRP, and interleukin 6) [6, 7, 11], hydration indices (hematocrit, haemoglobin, and serum osmolality) [12, 13], and recovery markers (magnesium) [12, 14]. Measuring these biomarkers helps understand the effects of training load, nutrition, hydration, and recovery methods on an athlete's homeostasis.

Although various football-specific simulation programs for fatigue are mentioned in the literature, such as free running, motorized, and non-motorized treadmill programs [15], those involving technical actions like passing, dribbling, shooting, and jumping are essential for a simulation protocol's success. The Technical Soccer Specific Aerobic Field Test (T-SAFT90) is one such program with proven external validity [16]. Data related to fatigue in football in the Asian sub-continent is scarce. This study is the first in the region using TSAFT^{90}, a novel football-specific simulation program, to analyze fatigue biomarkers and their influence on kicking velocity and recovery in Indian professional football players. The primary objective was to measure the magnitude of physiological, immune, endocrine, and muscle damage markers of exercise-induced fatigue at baseline, 0 hours, and 24 hours post-TSAFT^{90}. The secondary objective was to assess the effect of fatigue on performance using kicking velocity and recovery from muscle soreness, thereby drawing recommendations to monitor and manage match-induced fatigue in footballers.

Materials and Methods

Study participants

Professional male football players (n=30) representing the first division league level with a mean age of 19.20 (1.56) years and training experience of 7.90 (2.6) years consented to participate in this quasi-experimental study. Ethical clearance was obtained from the Institutional Ethical Committee board before the start of the study (IEC-NI/21/APR/78/82). A medical clearance and injury screening were conducted by a sports physician prior to participation. The study was conducted during the pre-season. Questions on the number of weekly training sessions, strength and conditioning sessions, and matches played were recorded to maintain homogeneity. Additionally, players' recovery strategies post-match/training, average sleep hours, and a 24-hour dietary recall were documented. All players were familiarized with the TSAFT^{90} well before the test day. A training-free period of 48 hours was observed before the fatigue testing. All tests were conducted at the same time of day for participants in batches. Participants were asked to fill in a 7-day food recall diary. Food and water intake on the test day and the following day were standardized for all participants.

TSAFT 90 setup

The players underwent a 90-minute audio-guided football-specific fatigue simulation program, TSAFT^{90} [16]. A schematic representation of the fatigue program is depicted in Figure 1. The protocol involves a 20-meter shuttle circuit, with poles, barriers, and a goalpost positioned strategically for players to navigate through utility movements. The movements were directed by verbal signals from an audio file at both the 0-meter ("outbound") and 20-meter cones ("return activities"). Each 15-minute protocol segment represents the rhythm and various types of activities demanded (13 in total; "stand still", "walk," "jog," "run," "sprint," "jump," "passes," "shots on target", "ball dribbling", and other combinations) [16]. Six cycles of the program were completed to achieve 90 minutes of simulation (45 minutes [3 cycles] - 15-minute rest - 45 minutes [3 cycles]), inducing fatigue.

Blood sampling and processing

Venous blood samples were collected at baseline, 0-hour post-fatigue, and 24 hours post-fatigue. The baseline sample was collected one week before the test day, following a non-training period of 48 hours. The laboratory analyzed blood samples for the following serum markers: lactate, uric acid, cortisol, Creatine Kinase, magnesium, osmolality, leukocytes, neutrophils, monocytes, lymphocytes, and CRP. Hematological markers, namely leukocytes, neutrophils, monocytes, and lymphocytes, were analyzed using fluorescence flow cytometry with Sysmex SP-50 (Sysmex Co., Kobe, Japan). The AU 5800 and AU 680 (Beckman Coulter, Brea, California, USA) measured serum uric acid, magnesium (serum xylidyl blue), creatine kinase (IFCC, NAC activated), and lactate (enzymatic method). Serum osmolality was calculated using the Smithline and Gardner formula (Serum osmolality = 2(Na) + glucose/18 + BUN/2.8). Cortisol was measured using an electrochemiluminescent immunoassay (ECLIA) on a Cobas 8000 module e602 (Roche Diagnostics, Mannheim, Germany). CRP levels were assayed by rate turbidimetry on an Immage 800 Immunochemistry System CRPH (Beckman Coulter, Brea, CA, USA).

Performance indicators

Post-strike ball velocity is an established performance variable [17]. Each player kicked a ball from a distance of 11 meters

Figure 1. TSAFT[90] (Technical Soccer Specific Aerobic Field Test) set up in the indoor training area.

from the goal post. Kicking velocity was measured before and after TSAFT[90] using a handheld radar speed gun (Pocket Radar Ball Coach, Pro-Level Speed Training Tool, USA) from 1 meter behind the goal post. The perception of lower limb muscle soreness was noted at 0 and 24 hours post-fatigue using a lower limb-specific 7-point Likert scale, adapted from Impellizzeri 2007 [18]. Heart rate and rate of perceived exertion were monitored throughout the protocol.

Statistics

Data were analyzed using R statistical software version 4.0.2. An unpaired t-test was used for performance indicators and one-way ANOVA followed by Tukey HSD (Honestly Significant Difference) post hoc test to compare the mean difference across groups for fatigue biomarkers. Statistical significance was set at a p-value of <0.05.

Results

Among the participants, 31% were forwards, 36% were midfielders, and 33% were defenders. Fatigue metabolites, namely lactate and uric acid, the endocrine response marker cortisol, the muscle damage marker creatine kinase, immunological markers leukocytes, lymphocytes, neutrophils, and monocytes, the inflammatory marker CRP, all remained significantly elevated at the end of 0 hours post-fatigue compared to baseline ($p<0.05$). The hydration status indicator, serum osmolality, and the recovery marker, serum magnesium, were also deranged and did not return to normal levels post-fatigue (Table 1).

Uric acid, creatine kinase, leukocytes, monocytes, CRP, serum osmolality, and magnesium remained altered at 24 hours. Graphical representations of the biomarkers are depicted in Figure 2a-d. The kicking velocity significantly decreased post-fatigue ($p=0.04$) from 94.67 km/hr to 90.47 km/hr, and the Likert scale showed no significant difference after 24 hours ($p=0.07$). Average heart rate and RPE during all six blocks of TSAFT[90] are shown in Figure 2e, f.

Discussion

A 90-minute football match induces acute physiological changes that stress aerobic metabolism. The utility of biochemical indices to study fatigue in football and its practical implications for managing recovery and preventing injury is still under investigation. This study aimed to measure the magnitude of various subtypes of fatigue biomarkers using a football-specific simulation program and assess the effect of fatigue on performance and recovery over 24 hours. Despite numerous studies on biochemical profiles during fatigue, no data involving Asian football athletes have been found to date. This study utilized a simulation program encompassing

Table 1. Fatigue analysis of various biomarkers at Baseline, 0 hour and 24 hour post fatigue

Biomarkers/ (mean [SD])	Baseline	0 Hour	24 Hour	Baseline vs 0 hr	0 hr vs 24 hr	Baseline vs 24 hr
Fatigue metabolites						
Lactate (mg/dL)	24.63 (7.51)	45.89 (13.04)	24.80 (8.13)	p=0.001 ↑	p=0.001 ↑	p=0.33
Uric acid (mg/dL)	5.58 (1.10)	6.01 (1.20)	5.82 (1.08)	p=0.05 ↑	p=0.002 ↑	p=0.05 ↑
Endocrine response						
Cortisol (µg/dL)	9.34 (2.68)	13.50 (5.68)	9.59 (3.29)	p=0.001 ↑	p=0.001 ↑	p=0.70
Muscle damage						
CK (U/L)	233.17 (122.83)	525.93 (396.45)	658.47 (563.80)	p=0.001 ↑	p=0.004 ↑	p=0.001 ↑
Hydration and muscle contraction						
Serum mg (mg/dL)	2.06 (0.13)	1.92 (0.14)	2.02 (0.13)	p=0.02 ↓	p=0.05 ↓	p=0.02 ↓
Serum osmolality (mOsm/kg)	285.00 (3.01)	292.20 (3.52)	287.77 (3.53)	p=0.02 ↑	p=0.04 ↑	p=0.01 ↑
Immunological state						
Leukocytes (cu/mm)	6.458.00 (1091.13)	11.543.00 (3572.52)	6.920.00 (1584.27)	p=0.001 ↑	p=0.002 ↑	p=0.05 ↑
Lymphocytes	37.22 (9.33)	25.60 (9.89)	37.68 (9.37)	p=0.002 ↑	p=0.001 ↑	p=0.22
Neutrophils	53.74 (10.03)	68.07 (10.73)	53.01 (10.63)	p=0.001 ↑	p=0.01 ↑	p=0.77
Monocytes	4.33 (1.04)	3.63 (0.84)	4.44 (1.32)	p=0.42	p=0.01 ↑	p=0.04 ↑
Eosinophils	3.95 (3.01)	1.79 (2.05)	4.13 (3.06)	p=0.001 ↑	p=0.001 ↑	p=0.07
Inflammatory markers						
C Reactive Protein	0.10 (0.10)	0.19 (0.12)	0.30 (0.25)	p=0.02 ↑	p=0.001 ↑	p=0.01 ↑

↑ Statistical significance set to p <0.05. One-way ANOVA followed by Tukey HSD (Honestly Significant Difference) post hoc test was done. SD: Standard deviation; CK: Creatine Kinase.

all technical activities in an actual football match, showing that the players' internal systems exhibit inadequate physiological recovery adaptation mechanisms to fatigue. At 0 hours post-fatigue, all biomarkers were elevated. Specifically, lactic acidosis impairs contractile function by reducing sarcoplasmic release and reuptake of calcium, myofibrillar sensitivity, the activity of ATPase, and key glycolysis enzymes such as phosphofructokinase and phosphorylase [6]. Blood lactate measurement estimates anaerobic metabolism contribution and interprets an athlete's resistance to fatigue, acting both as a fatigue agent and signaling molecule. Our study indicates a return to baseline for serum lactate at 24 hours. Another fatigue metabolite, uric acid, the end product of purine metabolism, was significantly elevated until 24 hours [5.82 (1.08) mg/dl]. Ascensao et al. [11] 2008 found that uric acid levels in 16 male soccer players were significantly elevated up to 72 hours post-match. Uric acid is associated with total antioxidant status as it scavenges hydroxyl ions and other free radicals produced in human plasma and skeletal muscle after acute strenuous exercise [19], attenuating the rise of plasma oxidative damage due to exercise.

White blood cell (WBC) activity reflects not only the status of the immune system but also the extent of muscle damage caused by exercise. Knight et al. [20] suggested that monitoring the redox status of WBCs is an emerging approach for the long-term management of elite athletes to better prevent overtraining and treat early infections. Our study showed significantly elevated WBC indices at 0-hour vs. 24-hour measurements. After 24 hours, leukocytes [6,920.00 (1584.27) cu/mm] and monocytes [4.44 (1.32) cu/mm] remained elevated, failing to return to baseline, illustrating the effect of fatigue on the immune system. Becatti et al. [21] and Carrera et al. [22] have identified established links between the redox status of leukocyte subpopulations with performance, injury, and training adaptations, demonstrating that training status and antioxidant supplementation impact reactive oxygen species production by WBCs.

Evidence of tissue damage is indicated by the increase in creatine kinase even 24 hours after the fatigue program. The increase in CK is 2-fold (658.47 U/L) in Indian players compared to Europeans (301.4 U/L) [10], which explains the dissimilarity between the study populations regarding nutrition, training load, recovery, and resistance levels to fatiguing conditions. This is consistent with the 24-hour CK values obtained by da Silva CD[16] in Brazilian football players (812±383 U/L). Stajer et al. [9] have elucidated a complex metabolic relationship between creatine and cortisol response to exhaustive exercise training, suggesting a link between cortisol and exercise-induced impaired bioenergetics, specifically the GAA-creatine axis. The biomarkers of creatine metabolism are positively associated with a cortisol stress response to exercise. The study shows a significantly increased stress response with serum cortisol values from 9.34 (2.68) µg/dL at baseline to 13.50 (5.68) µg/dL immediately post-TSAFT[90], but the values seem to be nearing baseline levels 24 hours after fatigue [9.59 (3.29) µg/dL]. The trendline of serum cortisol was found to be similar to that reported in previous studies [16, 23].

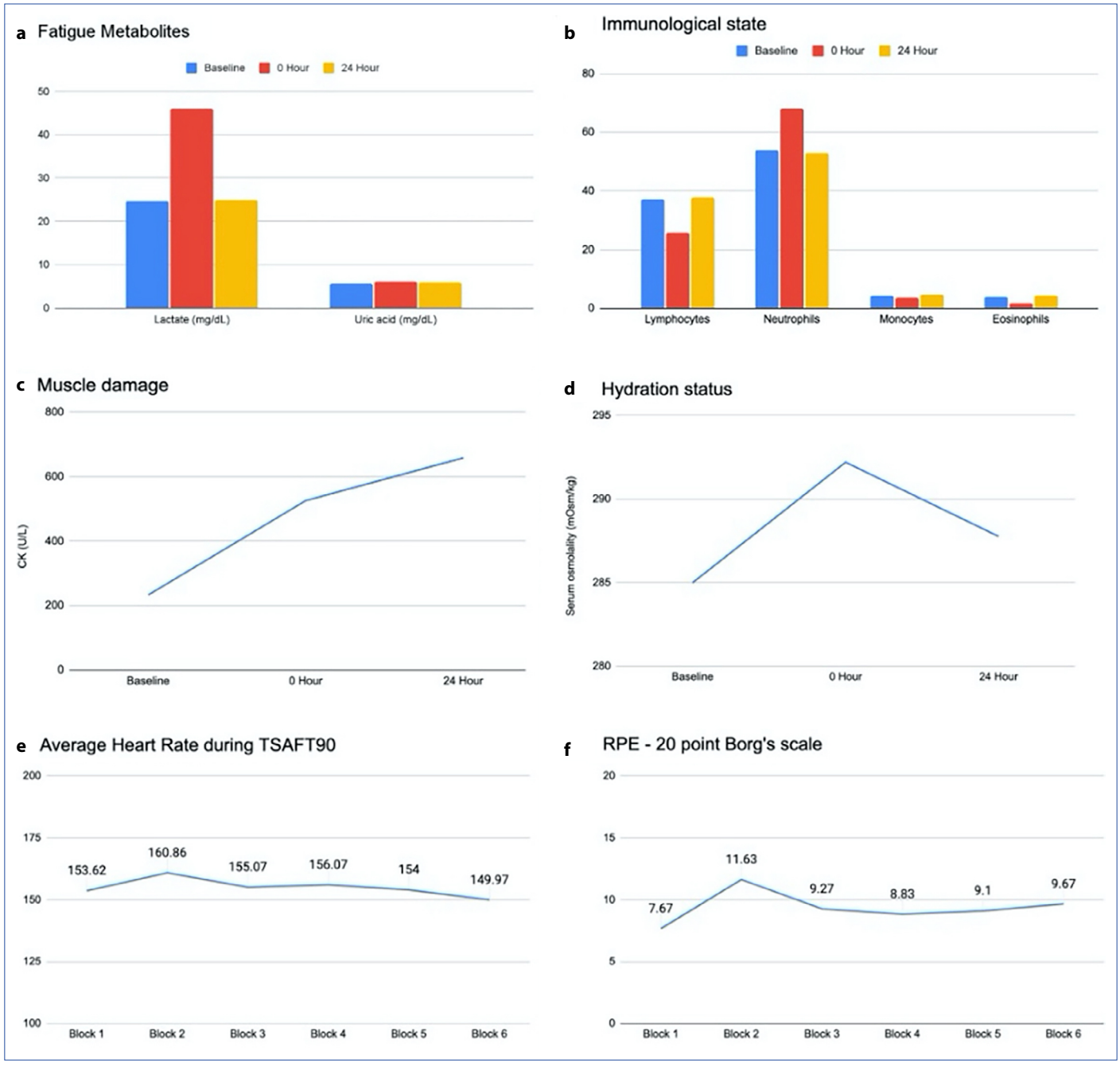

Figure 2. Graphical representation of Biomarkers over three timelines (Baseline, 0 hour, and 24-hour post-fatigue). (a) Fatigue metabolites, (b) immunological markers, (c) muscle damage markers, (d) hydration indicator, (e) average heart rate throughout the fatigue program, (f) RPE using 20-point Borg's scale during six blocks of the fatigue program

RPE: Rate of Perceived Exertion.

Exercise-induced muscle damage leads to an inflammatory response, indicated by increased levels of biomarkers such as CRP, IL-6, IL-1β, and TNF-α [24]. C-reactive protein and interleukins are linked with the physical stress of match-play and an increased susceptibility to injury risk, overreaching, and overtraining [5]. Jatene P et al. [25] have stated that CRP is a reliable biochemical marker for internal load monitoring after training or match-play with high external loads. In this study, CRP levels were significantly elevated at both 0 Hr ($p=0.02$) and 24 Hr ($p=0.01$) post-fatigue. Recent reviews [24] also state that football matches affect the circulating levels of CRP, which tend to peak 24 hours postgame and return to baseline within 72 hours of recovery time. Therefore, a congested match schedule can adversely affect an athlete's physiological system's inflammatory response.

Sweat is hypotonic, and exercise-induced dehydration primarily results in a decrease in extracellular fluid volume due to plasma water loss, a condition known as hypertonic hy-

povolemia. Biomarkers of hemoconcentration have been extensively used as indicators of dehydration. Serum osmolality is the gold standard for assessing acute and dynamic changes in hydration status, particularly in sports [12, 26]. Various studies have identified a dehydration threshold for blood osmolality at 295 mOsm/kg of plasma water [27, 28]. In the current study, football players were within threshold limits at 0 hours and 24 hours, yet there was a significant statistical derangement in hydration status. Magnesium, a crucial micronutrient for recovery [29], plays a vital role in energy production and storage, blood glucose level maintenance, and normal muscle function [14]. Our footballers showed a significant downward trend in magnesium levels post-fatigue. Plasma volume and blood micronutrient concentration, especially magnesium, are interrelated, as demonstrated in this study.

The secondary objective was to evaluate the impact of fatigue on performance through kicking velocity and recovery from muscle soreness. There was a significant decrease in ball velocity post-fatigue ($p=0.04$) from 94.67 km/hr to 90.47 km/hr, reflecting reduced performance. The perceptual measure for grading muscle soreness, Likert's 7-point scale for lower limb soreness, showed no significant change after 24 hours ($p=0.07$). This finding contrasts with the magnitude of disrupted homeostasis in relation to the biochemical internal load of the players. One hypothesis is that when players feel less fatigued, they may train or play at higher intensities, potentially increasing the risk of injuries [30].

Limitations

The levels of biochemical parameters may fluctuate based on an individual's lifestyle, fitness status, training history, nutrition, psychological state, genetics, previous injuries, chronic diseases, and immune status. Additionally, exhausting players for 90 minutes in an indoor environment may differ from an actual outdoor football match. Future studies should aim to bridge the gap between biochemical adaptations to fatigue and their practical applications in preventing injuries and overtraining in football.

Practical implications

- The internal homeostasis of football players remains severely deranged 24 hours after the TSAFT 90 fatigue program. This information can assist in scheduling training sessions and matches to allow for adequate recovery.
- Elevated CK, CRP, UA, and leukocytes may indicate muscle damage and ongoing inflammation. This finding underscores the need for sports scientists to adapt appropriate recovery strategies and monitor the training load to improve the fatigue threshold. These parameters can serve as performance and recovery indicators.
- An increase in serum osmolality and a decrease in magnesium disrupt hydration levels, contributing to fatigue and causing burnout that affects performance. Thus, magnesium supplementation can aid in achieving euhydration and ultimately promote recovery. Tailored planning of pre, during, and post-match nutritional supplementation by sports nutritionists can significantly manage fatigue.
- From an injury prevention and management perspective, these biochemical indices can assist in examining neuromuscular fatigue related to injury risk parameters and in evaluating the late-stage rehabilitation status of players returning from injury. Monitoring the athletes with periodic checks using biomarkers can contribute to longitudinal management to reduce injury and illness risk.
- There was no significant increase in soreness scale scores at 24 hours, which allows players to engage in intensive training or matches, thus increasing the risk of acute and overuse injuries. The mismatch between perceived and physiological differences in fatigue demonstrates that biomarkers may be more reliable than subjective soreness assessments.

Conclusion

All fatigue biomarkers analyzed in the study were significantly elevated immediately post-TSAFT 90 simulation program. The failure of these biomarkers (UA, CK, leukocytes, CRP, serum osmolality, and magnesium) to return to baseline levels within 24 hours suggests disrupted homeostasis. Poor internal recovery at this time can affect performance and increase the likelihood of injury.

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

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Research Article

Exploratory role of serum FGF-8 as a marker of bone metastasis in tumor progression

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Abstract

Objectives: FGF-8, a member of the FGF family, plays a crucial role in cellular processes and has been implicated in cancer progression. The study aims to comprehend FGF-8's involvement in bone metastasis, emphasizing its potential as a diagnostic marker and focusing on its association with Bone-Alkaline Phosphatase (B-ALP) and other biochemical parameters.

Methods: The case-control study spans 12 months, involving 60 participants, including 30 with secondary bone metastases and an equal number without metastasis. FGF-8 levels were quantified using ELISA, and B-ALP, serum ALP, and various biochemical parameters were assessed. The study employed standardized procedures to minimize bias, including matching cases and controls, and obtaining ethical approval.

Results: In patients with bone metastasis, serum ALP levels, particularly B-ALP, were significantly higher. The metastatic group exhibited elevated FGF-8 concentrations, showcasing a positive correlation with B-ALP and serum calcium levels. The study successfully differentiated ALP isoenzymes through heat inactivation and L-phenylalanine inhibition. Additionally, serum calcium levels were markedly elevated in the metastatic group.

Conclusion: The findings suggest that FGF-8 is a potential diagnostic marker for bone metastasis, particularly in breast and prostate cancers. Elevated FGF-8 levels correlate with increased B-ALP and serum calcium, indicating its role in osteoblastic differentiation in metastasis. The study proposes the utility of ELISA-based kits for FGF-8 in serum as a practical and efficient method for assessing bone tumor progression.

Keywords: B-ALP, bone metastasis, diagnostic marker, FGF-8, osteogenesis

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Human FGF is a class of cytokines that significantly impacts tissue disease, metabolism, cell growth, and development. The FGF family comprises 22 members and can be further broken down into paracrine and endocrine subgroups [1]. The FGF family is divided into seven subfamilies, including the FGF-1, FGF-4, FGF-7, FGF-8, FGF-9, FGF-11, and FGF-19 subfamilies. Each subfamily is distinguished by the similarity and specificity of its protein structure [2]. FGF signaling controls the actions of articular chondrocytes, peripheral synoviocytes, and osteoblasts, essential for maintaining joint health and functional balance [3]. The FGF-8 subfamily consists of three proteins: FGF-8, FGF-17, and FGF-18 [4]. FGF-17 is essential for brain development, while FGF-18 and FGF-8 are crucial for chondrogenesis and osteogenesis [5]. Members of the FGF-8 subfamily exhibit distinct tissue distribution patterns and binding affinities for FGF receptors. FGF-8, also known as androgen-induced growth factor (AIGF) [6], plays a role in stimulating physiological cellular processes such as cell proliferation, differentiation, and migration [7]. FGF signaling pathways may be significant in cancer pathophysiology since they have been linked to tumor growth and progression. Cell lines from breast and prostate cancers express FGF-8 [8]. Bone develop-

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ment is controlled by a network of signaling molecules and pathways, with FGF and its receptors shown to be important in bone formation. It has been found that several FGFRs perform complementary roles and show some functional redundancy during osteogenesis [9]. During the metastatic phase, cancer cells subvert these pathways and alter cell-to-cell interactions in bone. Depending on the precise interaction between cancer cells and bone cells, bone metastases can be osteoblastic, like those from prostate cancer, or osteolytic, like those from multiple myeloma. However, both components, osteolytic and osteoblastic, are present in most bone metastases at various levels [10]. In particular, people with lung, breast, and prostate cancer are more likely to develop metastatic bone disease than other cancer patients [11]. Rising evidence suggests that the FGF axis contributes to bone metastases and has been linked to the development of numerous malignancies.

The four isoenzymes of alkaline phosphatase, namely liver, bone, kidney (L/B/K) alkaline phosphatase, and intestinal alkaline phosphatase (I-ALP), placental alkaline phosphatase (P-ALP), and germ cell alkaline phosphatase, are expressed throughout various tissues. In bone diseases, increased osteoblastic activity and/or enhanced hepatocyte production are the main causes of high blood alkaline phosphatase [12]. Bone alkaline phosphatase (B-ALP) is a relatively specific marker for osteogenesis and an indicator of osteoblast metabolism [13]. Therefore, B-ALP measurements may be a helpful addition to identifying individuals with skeletal involvement. Preoperative estimates of predicted survival have a role in determining whether surgical management for bone metastasis patients is appropriate. Several prognostic indicators have been identified in the past to aid in decision-making, but there is currently no precise biomarker for bone metastasis.

To include the ideas of the current understanding of the involvement of the FGF axis in bone metastases, this study was designed to comprehensively characterize the role of Fibroblast Growth Factor-8 (FGF-8) in bone metastasis and to investigate the potential of FGF-8 as a diagnostic marker for bone metastasis. The secondary objective is to correlate FGF-8 levels with Bone-Alkaline Phosphatase (B-ALP) for a more comprehensive understanding of FGF-8's role in bone health and disease.

Materials and Methods

Study design

This study employed a case-control design to investigate the associations between FGF-8 levels and bone metastasis, with a specific study duration of 12 months. The study was conducted within the Department of Biochemistry, and approval for the study protocol was obtained from the Institutional Ethical Committee, ensuring strict adherence to ethical guidelines.

Participants

The participant cohort was meticulously selected based on specific criteria, comprising a total of 60 individuals. This group included 30 participants with secondary bone metastasis and an equal number without metastasis. The identification of bone metastasis was achieved through whole-body MRI, in females diagnosed with breast and males with prostate cancer. All cases of metastasis were recurrences of breast and prostate cancer in females and males, respectively, and enrolled before the commencement of chemotherapy.

Variables

The study incorporated distinct variables, both independent and dependent. The primary focus was on evaluating FGF-8 levels as the independent variable. Dependent variables encompassed Bone-Alkaline Phosphatase (B-ALP), serum ALP levels, and various biochemical parameters such as ALT, AST, total bilirubin, blood urea, creatinine, and calcium.

Data sources/measurement

Data acquisition and measurement were meticulously conducted through standardized procedures. Each participant underwent the aseptic collection of 5 mL venous blood, followed by centrifugation for serum separation. Biochemical analyses were performed on the obtained serum samples using the Beckman Coulter AU-480 analyzer (California, United States). The evaluation of B-ALP levels employed an inhibition method. The initial phase encompassed the quantification of serum ALP levels in individuals, thereby determining the overall ALP concentration. Subsequent heat inactivation at 56°C for 10 minutes resulted in a substantial reduction, yielding B-ALP levels. Further diminution ensued with the addition of L-phenylalanine (10 mmol/L), leading to the inhibition of the intestinal fraction of ALP and the concomitant yield of the L-ALP fraction. The residual portion was designated as the intestinal ALP fraction [14]. FGF-8 levels were quantified using Enzyme-Linked Immunosorbent Assay (ELISA) with a kit from Elabscience®.

Bias

Concerted efforts were made to minimize bias. Matching cases and controls from the same socioeconomic background and age group aimed to control potential selection bias. Standardized procedures for blood collection and analysis were implemented to minimize information bias. Selecting participants with breast and prostate cancer was strategic to control potential confounding factors.

Sample size

The study encompassed a total of 60 participants, with 30 in the metastatic group and 30 in the non-metastatic group.

Statistical methods

Primary statistical tools included Student's independent t-test and Pearson correlation coefficient. The MedCalc® platform from MedCalc Software Ltd in Belgium served as the

analytical tool. A predetermined significance level of $p<0.05$ indicated statistical significance. Calculating the Coefficient of Variation (CV%), interassay and intra-assay variability for FGF-8 measured by an ELISA Kit involved assessing the precision and reliability of the assay. The percentage of coefficient of variation, CV% of intra-assay variability was 2.63% (mean=18.27, SD=0.48), and the CV% of inter-assay variability was 3.39% (mean=18.20, SD=0.62).

Ethics approval and Helsinki declaration

The present study received ethical approval from the Institutional Ethical Committee. The study adheres to the principles outlined in the Declaration of Helsinki, as adopted by the World Medical Association, guiding ethical conduct in biomedical research involving human subjects. Participants were provided with detailed information about the research objectives, procedures, potential risks, and benefits. Informed consent was obtained from all participants, and their confidentiality and privacy were strictly maintained throughout the study.

Results

In the present study, 30 patients diagnosed with secondary bone metastasis were included. Out of these, 10 were females with breast cancer, while 20 were males with prostate cancer. The average age of the metastatic group was 60.86±12.14 years (ranging from 38 to 78 years). Females with breast cancer had an average age of 46.2±5.26 years, whereas males with prostate cancer had an average age of 66.7±8.24 years. Two-way ANOVA was used to analyze the association between age, sex, and metastatic/non-metastatic status, but no significant interaction was found. Out of 20 males with prostate cancer, 6 had a history of smoking, and all 10 females with breast cancer were non-smokers. Initially, the serum ALP level was measured in patients with bone metastasis, which averaged 471.13±135.23 U/L. Following heat inactivation at 56°C for 10 minutes, the mean serum ALP level dropped to 74.55±24.72 U/L. Additionally, after the addition of L-phenylalanine (10 mmol/L), the mean ALP level further decreased to 33.55±11.96 U/L. The inhibition percentage after heat inactivation was calculated to be 84.39%, which increased to 92.59% after the addition of L-phenylalanine. The inhibition study was utilized to calculate the fractions of Bone (B), Liver (L), and Intestinal (I) ALP. The mean B-ALP activity, which was inhibited by heat inactivation, was 396.07±111.84 U/L. The remaining 74.55±24.72 U/L of ALP level consisted of both liver and intestinal fractions. The addition of L-phenylalanine successfully inhibited the intestinal fraction of ALP, resulting in a mean ALP level of 33.66±11.99 U/L, representing the L-ALP fraction. The remaining 38.19±14.34 U/L was considered the intestinal ALP fraction (Fig. 1). In patients with bone metastasis, the percentages of bone, liver, and intestinal ALP in serum were 84.17%, 7.45%, and 8.31%, respectively, whereas in the non-metastatic group, the percentages were 18.42%, 41.26%, and 41.68%, respectively. The B-ALP/total ALP ratio was 0.87 in the metastatic and 0.19 in the non-metastatic. Other biochemical parameters, including serum ALT, AST, total bilirubin, blood urea, creatinine, and calcium, were measured in both groups (Table 1).

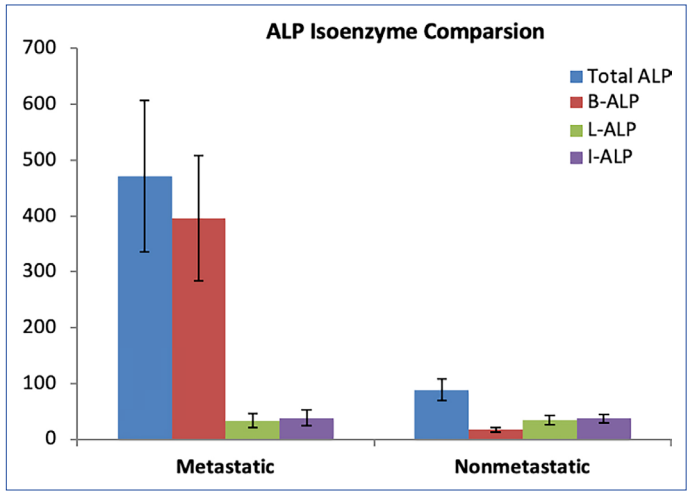

Figure 1. Mean±SD of fractions of ALP in controls and cases.
SD: Standard deviation; ALP: Alkaline phosphatase.

Furthermore, FGF-8 protein levels were measured in the serum of both metastatic and non-metastatic groups. The mean concentration of FGF-8 in the metastatic group was 21.51±3.41 pg/ml (ranging from 15.13 to 28.35 pg/ml), while in the non-metastatic group, it was 5.36±1.79 pg/ml (ranging from 2.52 to 9.08 pg/ml). The metastatic and non-metastatic cases were stratified by gender. In females with metastasis, the mean FGF-8 concentration was 19.39±2.73 pg/ml compared to 6.15±1.90 pg/ml in females without metastasis. For males, the corresponding values were 22.56±3.26 pg/ml for metastatic cases and 4.46±1.16 pg/ml for non-metastatic cases. These higher mean values in metastatic cases for both genders indicated a significant difference compared to the non-metastatic group ($p<0.05$) (Fig. 2). The concentration of FGF-8 in metastatic and non-metastatic groups, comprising two categories, i.e., males and females, was analyzed using two-way ANOVA. The difference between males and females was not significant ($p=0.36$), but there was a significant difference between the metastatic and non-metastatic groups (independent variable) in relation to the dependent variable FGF-8 ($p<0.05$). It was also found that there was an interaction between the two variables, sex and metastatic/non-metastatic status, in relation to the dependent variable FGF-8 ($p<0.05$). A non-significant result between males and females cannot prove that there is no difference between sexes. Since the interaction between sex and metastatic/non-metastatic status was significant, it indicates that sex was important in influencing the dependent variable, i.e., FGF-8 (Fig. 3). In the metastatic group, FGF-8 protein showed a significant positive correlation with B-ALP isoenzyme ($r=0.58$) and serum calcium ($r=0.62$). Serum calcium also displayed a very high positive correlation ($r=0.59$) with B-ALP isoenzyme activity (Fig. 4).

Table 1. Mean±SD of various parameters in non-metastatic and metastatic groups

Parameters	Non-metastatic	Metastatic	p
Age (years)	55.63±11.26	60.86±12.14	0.19
Total serum ALP (U/L)	88.80±18.78	471.13±135.23	≤0.05
Bone-ALP (U/L)	17.05±4.69	396.07±111.84	≤0.05
Liver-ALP (U/L)	34.41±8.33	33.66±11.99	0.59
Intestinal-ALP (U/L)	37.33±7.90	38.19±14.34	0.93
Serum ALT (U/L)	31.16±8.69	49.36±19.94	0.07
Serum AST (U/L)	42.36±8.69	42.07±13.73	0.86
Total bilirubin (µmol/L)	13.17±2.73	12.65±3.93	1.00
Blood urea (mmol/L)	11.44±3.63	17.53±9.54	0.09
Serum creatinine (µmol/L)	68.92±23.86	99.89±54.80	0.16
Serum calcium (mmol/L)	2.02±0.23	2.65±0.17	≤0.05
Serum FGF-8 (pg/ml)	5.36±1.79	21.51±3.41	≤0.05

Data are expressed as Mean±SD and were compared using the Welch's T-test. P<0.05 was considered statistically significant. SD: Standard deviation; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FGF-8: Fibroblast Growth Factor-8.

Discussion

Secondary bone cancer, also known as bone secondaries or bone metastases, occurs when cancer spreads to the bones from its original site. In this study, 30 patients with secondary bone metastasis were enrolled. The research revealed that the age of men with prostate cancer with bone metastasis concurred with the findings of Plym et al. [15]. Similarly, Shoemaker et al. [16] reported that the majority of breast cancer cases occurred in women aged 40–44 and 45–49 years, which aligns with the results of this study. A case-control study by Rao et al. [17] conducted in India found that the mean age of breast cancer patients was 46.2 years.

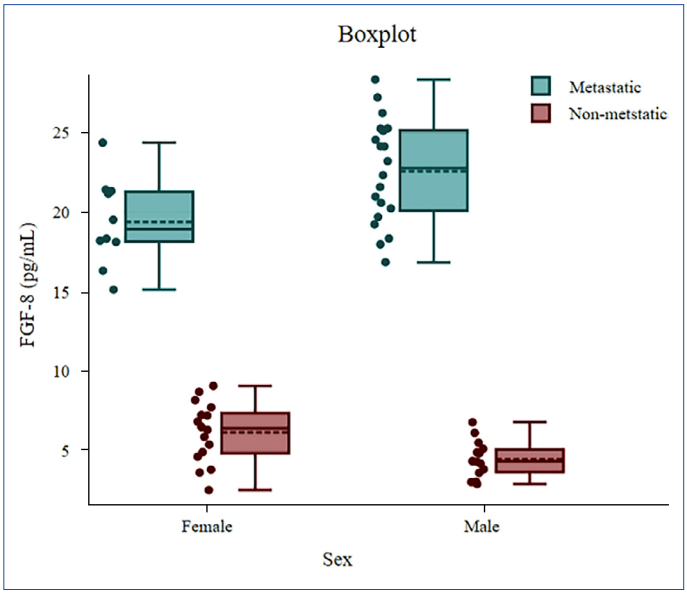

Figure 2. FGF-8 (pg/mL) Concentration in metastatic and non-metastatic stratified by gender.
FGF-8: Fibroblast Growth Factor-8.

The present study indicated that ALP levels in the metastatic group were significantly higher than those in the non-metastatic group. Singh et al. [18] concluded that women with breast cancer generally exhibit higher ALP activities than normal healthy women. The progressive increase in serum ALP activities with breast cancer serves as an indication of metastasis. Measuring this parameter could be a valuable diagnostic tool for monitoring the disease's progression and treatment, especially in areas lacking sophisticated studies. A meta-analysis by Jiang et al. [19] summarized that breast cancer patients had higher ALP levels compared to healthy controls, and elevated levels of both ALP and B-ALP were risk factors for bone metastasis. Another study by Akimoto et al. [20] found that ALP levels showed significant differences concerning the extent of bone metastasis. Researchers have employed heat stability and L-phenylalanine inhibition studies on human serum alkaline phosphatase to

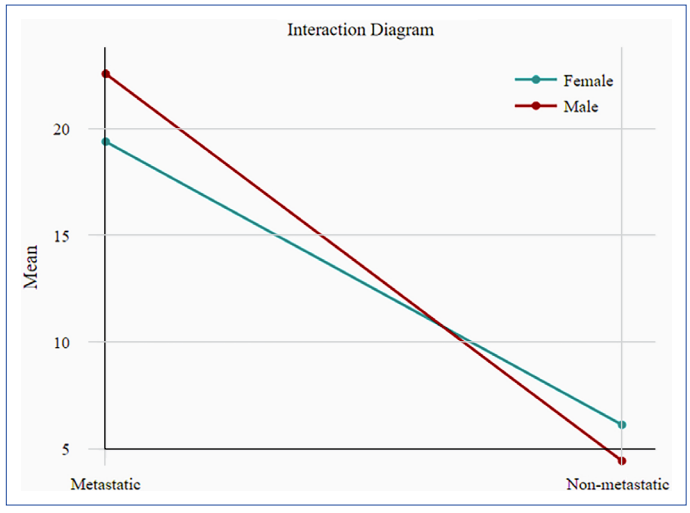

Figure 3. Interaction between the two variables sex and metastatic/non-metastatic in relation to the dependent variable FGF-8.

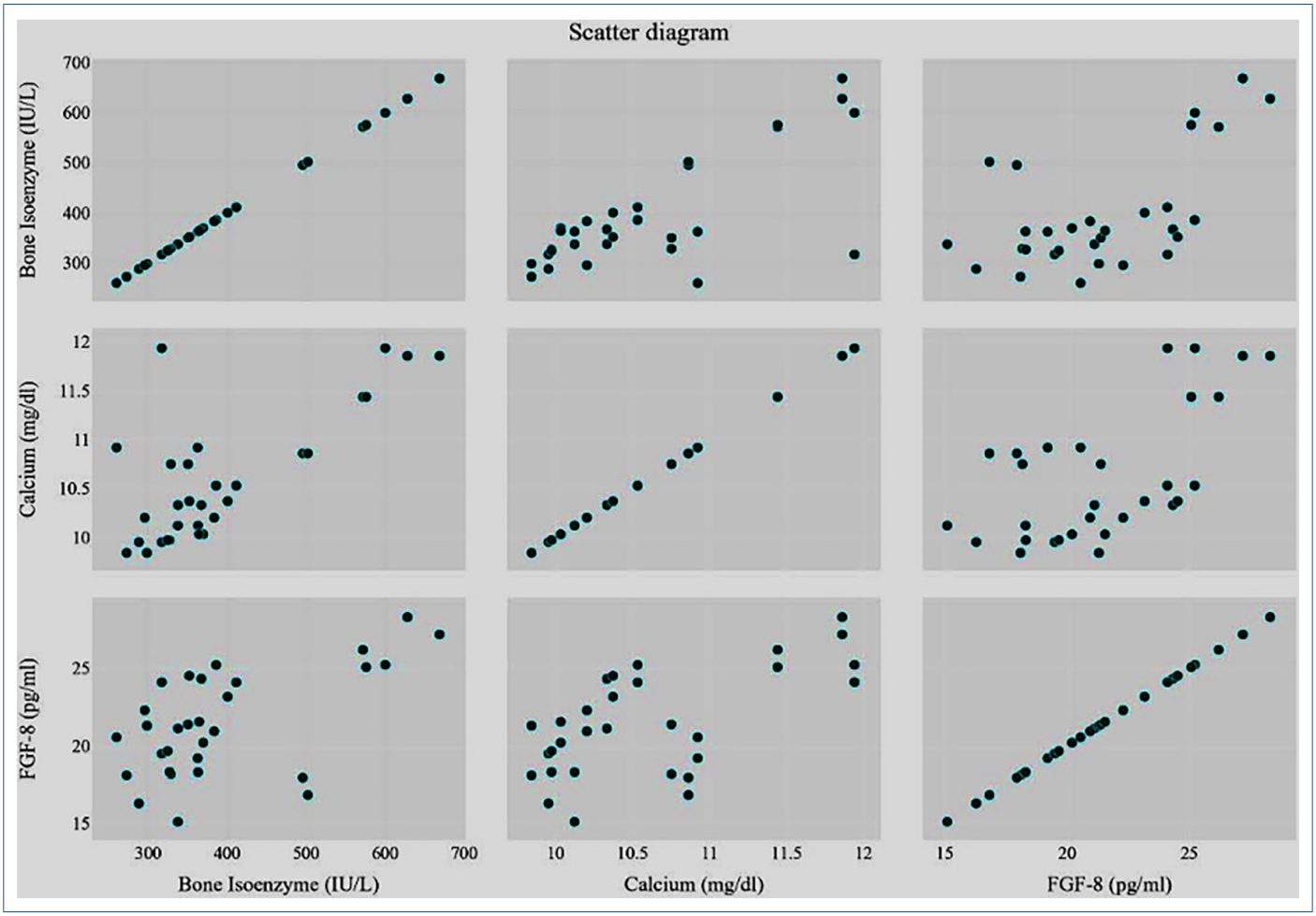

Figure 4. Scatter graph of person`s correlation between FGF-8, serum calcium and bone-ALP isoenzyme.

identify the isoenzyme contributing to the total enzyme activity in the serum. Bone alkaline phosphatase was observed to be more heat-labile than liver and intestinal alkaline phosphatase, allowing for differentiation through pre-incubation of the serum at 56°C for 10 minutes before enzyme assay. After heat treatment, serum alkaline phosphatase levels decreased to less than 14% of the original activity in the metastatic group. The bone ALP fraction was significantly higher in the metastatic compared to the non-metastatic group. In a study by Lorente et al. [21] analyzing bone alkaline phosphatase enzyme concentrations in patients with prostate cancer, it was concluded that the clinical use of bone alkaline phosphatase enzyme measurement is valuable for diagnosing bone metastasis and assessing the progression of prostate cancer due to its good sensitivity and specificity. Other biochemical parameters, including serum ALT, AST, total bilirubin, blood urea, and serum creatinine, did not show significant differences between the metastatic and non-metastatic groups, indicating that there was no invasion of cancer into other tissues except bone.

The serum calcium level was markedly elevated in the metastatic group compared to the non-metastatic group. According to a study by Joeckel et al. [22], the highly expressed calcium-sensing receptor (CaSR) and its downstream signaling pathways promote bone metastasizing cell migration and proliferation. The primary mechanism responsible for approximately 80% of malignancy-related hypercalcemia is PTHrP production. PTHrP's biochemical structure is very similar to that of PTH [23]. PTHrP acts on osteoblasts to increase the production of RANKL, which in turn activates osteoclasts and causes bone resorption, releasing calcium into the circulation. Another pathway through which PTHrP causes hypercalcemia is by increased renal calcium reabsorption. Osteolytic metastases and excessive calcium release from bone account for about 20% of all cases of hypercalcemia. The majority of cases of osteolytic hypercalcemia are caused by breast and prostate cancer [24].

FGF-8 concentration was measured in the serum of both the metastatic group (breast cancer or prostate cancer with bone metastasis) and the non-metastatic group, and it was significantly higher in the metastatic group compared to the non-metastatic group. The difference was statistically significant ($p<0.05$). FGF-8 mRNA expression was found in the cancerous prostatic epithelium by Dorkin et al. [25] using in situ hybridization. FGF-8 expression levels were substantially linked

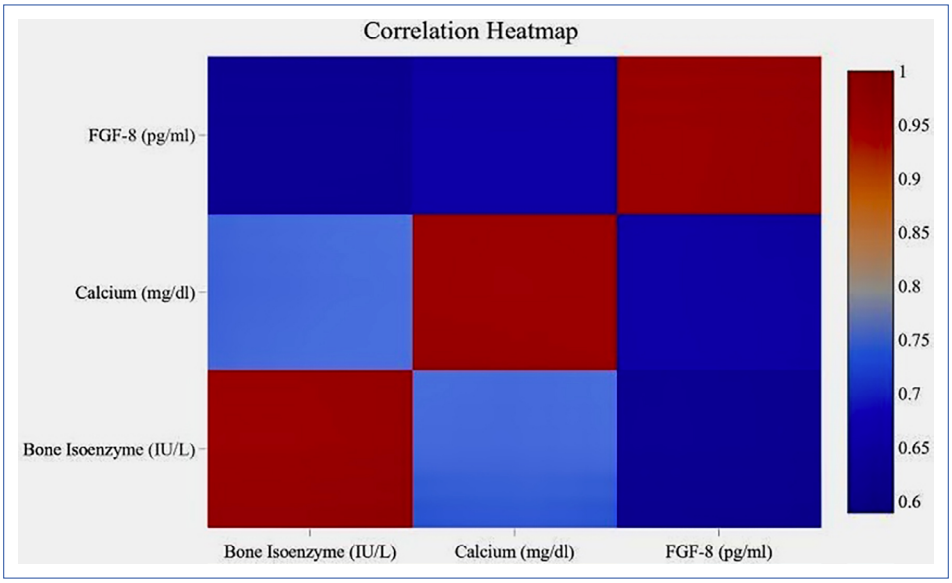

Figure 5. Heatmap diagram of correlation between FGF-8, serum calcium and bone-ALP isoenzyme.

with low survival rates, advanced tumor stages, and higher Gleason scores. Darby et al. [26] studied the expression of hSef, a crucial inhibitory regulator of FGF signaling, in prostate cancer and concluded that siRNA-induced forced downregulation of hSef promoted FGF-8-induced cell migration and invasion. Valta et al. [27] conducted a tissue microarray in patients with prostate cancer with bone metastasis and reported that 76% of samples tested positive for FGF-8. Similar observations were made by Gnanapragasam et al. [28]. Nilsson et al. [29] conducted a cDNA microarray analysis to identify target genes associated with FGF-8b-mediated breast cancer cell proliferation. The study revealed the involvement of several essential regulators of early cell cycle progression, such as Btg2 and cyclin D1, as well as mitosis-related regulators, including cyclin B, Plk1, survivin, and aurora kinase A. These findings suggest that FGF-8 plays a role not only in promoting cell cycle progression through the G1 restriction point but also in regulating critical proteins involved in chromosomal segregation during mitosis and cytokinesis in breast cancer cells. FGF-8 efficiently anticipates the differentiation of bone marrow cells into osteoblasts and boosts bone production *in vitro*. It is conceivable that FGF-8 also increases bone growth *in vivo*. The findings suggest that FGF-8, which is produced in significant amounts by malignant tumors of the breast and prostate, may play a role in the development of osteosclerotic bone metastases [30].

Fibroblast growth factor 8 (FGF-8) emerges as a pivotal player in the insidious choreography of cancer metastasis, wielding its influence through diverse mechanisms. One key act involves triggering epithelial-mesenchymal transition (EMT), a phenotypic metamorphosis where tumor cells shed their epithelial adherence and don mesenchymal traits, granting them enhanced motility and invasiveness [31]. FGF-8 accomplishes this feat by activating signaling pathways like Wnt and MAPK, dismantling epithelial cell junctions, and promoting the expression of mesenchymal markers [32]. Moreover, FGF-8 fosters a pro-metastatic microenvironment by inducing angiogenesis, the construction of new blood vessels that nourish and oxygenate tumors and facilitate their dissemination [33]. Additionally, FGF-8 bolsters the resilience of cancer cells against the hostile conditions encountered during metastasis, such as hypoxia and anoikis (detachment-induced cell death) [34]. This multifaceted orchestration by FGF-8 underscores its critical role in propelling cancer cells along the metastatic odyssey, highlighting its potential as a therapeutic target for curbing cancer's spread.

FGF-8, bone ALP isoenzyme, and serum calcium level all exhibited strong positive correlations. These associations indicate that the FGF-8 protein plays a role in the initial bone metastasis of breast or prostate cancer (Fig. 5). Several lines of evidence point towards a positive correlation between FGF-8 protein levels and bone ALP activity. *In vitro* studies by Mansukhani et al. [35] demonstrated that FGF-8 directly stimulates ALP expression in osteoblast precursors, suggesting a direct regulatory role. In an *in vivo* model of prostate cancer with bone metastasis, FGF-8 expression enhances PC-3 prostate cancer cells' proliferation as intratibial tumors and regulates the occurrence of bone lesions [27]. These observations suggest that FGF-8 acts as a potent stimulator of osteoblast differentiation and maturation, as evidenced by elevated ALP levels. However, the relationship is not unidirectional. Bone ALP may influence FGF-8 signaling through a feedback loop. Recent studies suggest that ALP can dephosphorylate FGF-8, potentially modulating its activity and downstream effects [36]. This intricate interplay highlights the delicate balance between FGF-8 and B-ALP.

The present study suggests that measuring FGF-8 in serum holds promise as a superior marker for bone metastasis compared to B-ALP, due to several key advantages. First, FGF-8 levels are significantly higher in patients with bone metastasis compared to those without, while B-ALP levels can be elevated

due to other bone diseases. This specificity makes FGF-8 a more reliable indicator of bone metastasis. Secondly, FGF-8 may be detectable earlier in the course of bone metastasis compared to B-ALP and may correlate with the extent and severity of bone metastasis, potentially providing valuable prognostic information for treatment planning. FGF-8 plays a direct role in promoting bone metastasis through mechanisms like EMT and angiogenesis, while B-ALP is a downstream marker of bone activity. Understanding the underlying mechanisms of FGF-8 involvement provides more insight into disease progression.

Conclusion

FGF-8 is expressed at a high frequency in bone metastases of hormonal cancers as it is associated with the induction and facilitation of prostate tumorigenesis and increases the growth and angiogenesis of breast and prostate cancer. FGF-8 has demonstrated a robust autocrine growth factor role, fostering osteoblastic differentiation in metastasis, leading to increased B-ALP levels. Previous expression studies have assayed total FGF-8 levels in tissue samples, not particularly in serum. However, affordable, quicker, and more accurate assays like ELISA-based kits that measure FGF-8 in serum might be helpful to assess the progression of bone tumors in patients with breast or prostate cancer for better treatment plans.

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

Ethics Committee Approval: The study was approved by The Pt. B.D. Sharma Post Graduate Institute of Medical Sciences Rohtak, Department of Biochemistry Ethics Committee (No: ECI/21/26, Date: 18/07/2021).

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Case Report

Unmeasurable HbA1c result due to hemoglobinopathy

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Abstract

The aim of this study was to show the interference caused by hemoglobinopathy in the measurement of hemoglobin A1c (HbA1c). In our case presentation, we reported two patients whose HbA1c values were unmeasurable when using our laboratory's cation exchange chromatography. We detected HbSβ+ and HbSC variants by remeasuring the samples with another chromatography instrument. Measurement of HbA1c is a commonly performed procedure for the diagnosis of diabetes and for the assessment of blood glucose control in patients with diabetes. However, various hemoglobinopathies, chronic kidney disease, and abnormalities in red cell turnover rate may interfere with HbA1c quantification.

Keywords: Glycated hemoglobin, HbA1c, hemoglobinopathies, HPLC

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Analysis of glycosylated hemoglobin (A1C, hemoglobin A1C, HbA1c) shows the proportion of hemoglobin to which a glucose molecule has been non-enzymatically adjoined. The production of HbA1c is a spontaneous reaction that is dependent on the prevailing concentration of blood glucose. The irreversible production of HbA1c proceeds for approximately 120 days, which is the red blood cell's lifespan. Therefore, HbA1c levels represent the average glucose levels of the previous 2–3 months. HbA1c is the most common test used in chronic glycemic management [1]. As standardized by the Diabetes Control and Complications Trial (DCCT) and approved by the National Glycohemoglobin Standardization Program (NGSP), the measurement levels of ≥6.5% or ≥48 mmol/mol concentration of HbA1c are crucial parts of the diagnostic criterion for Diabetes Mellitus [2].

HbA1c can be measured by enzymatic assay, immunoturbidimetric assay, boronate affinity, ion-exchange High Perfomance Liquid Chorataography (HPLC), or capillary electrophoresis in clinical laboratories [3].

Testing of HbA1c has some advantages over blood glucose level measurement which is affected by calorie intake and involves the necessity of patient preparation, and specific timing. HbA1c measurement also has more preanalytic stability and less variability between days [1].

However, HbA1c cannot be studied in every laboratory due to its rather high cost. Also, inappropriate correlations between HbA1c measurement results and blood glucose levels can be seen in patients with hemoglobinopathies, thalassemia, etc.

It has been stipulated that HPLC, which is considered the gold standard, should be used for HbA1c testing in every laboratory working on HbA1c in the United States [4].

Case Report

In this case report, hemoglobinopathy variants were detected in two cases in which HbA1c test results couldn't be obtained by cation exchange HPLC. The use of fructosamine testing instead of HbA1c in the follow-up will be discussed.

Patients and Methods

Two male patients (aged 29 and 25) were seen by their general practitioner for their routine checkups. Their biochemistry, hemogram, and HbA1c samples were accepted into

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our laboratory. Test results of the patients are presented in Table 1. The patients' HbA1c tests were performed with a cation-exchange HPLC-based Adams A1C HA-8180V (Arkray, Inc., Kyoto, Japan) instrument. HbA1c values were not obtained by our instrument for both patients (Fig. 1). The samples were reevaluated with a cation-exchange HPLC-based Adams A1C HA-8180T (Arkray, Inc., Kyoto, Japan) instrument (Fig. 2). HbSβ+ and HbSC variants were detected from Case 1 and Case 2, respectively.

Table 1. Test results of the patients

Parameters	Results case 1	Results case 2	Reference value
Glucose (mg/dL)	85	72	74–100
Urea (mg/dL)	31		16.6–48.5
Creatinin (mg/dL)	0.96	0.73	0.70–1.20
AST (U/L)	26	30	0–40
ALT (U/L)	14	38	0–41
Iron (ug/dL)	102	65	33–193
UIBC (ug/dL)	184	228	125–345
Calcium (mg/dL)	9.4	9.2	8.6–10
Ferritin (ug/L)	631	136	30–400
TSH (mU/L)	3.87	2.9	0.27–4.2
Free T4 (ng/L)	12.4	12.1	8.9–17.1
WBC (10^3/uL)	9.78	10.48	3.8–10
RBC (10^6/uL)	2.59	4.82	4.3–5.7
Hemoglobin (g/dL)	95	129	132–173
Hematocrit (%)	28.9	35.7	40–52
MCV (fL)	111.7	74	80–95
MCHC (g/dL)	330	362	310–370
Trombocyte (10^3/uL)	400	283	150–400

AST: Aspartate transaminase; ALT: Alanine aminotransferse; UIBC: Unsaturated iron binding capaticy; TSH: Thyroid stimulating hormone; WBC: White blood cell; RBC: Red blood cell; MCV: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

Discussion

The quantity of HbA1c in patient samples is not only affected by blood glucose levels but also influenced by changes in red blood cell lifetime and globin chain structure. Hence, clinical biochemistry, hematological, and analytical methods are all important when commenting on HbA1c [5].

When A1C values are unexpected or incompatible with blood glucose measurement results, a disorder in red cell turnover, hemoglobin variants, and chronic kidney disease should be considered.

Red cell turnover: Incorrectly elevated HbA1c levels can occur when red cell turnover is low. This situation can occur in megaloblastic anemia (related to folate or vitamin B12 deficiency). Conversely, incorrectly low HbA1c levels can occur with fast red cell turnover. This situation can occur in patients

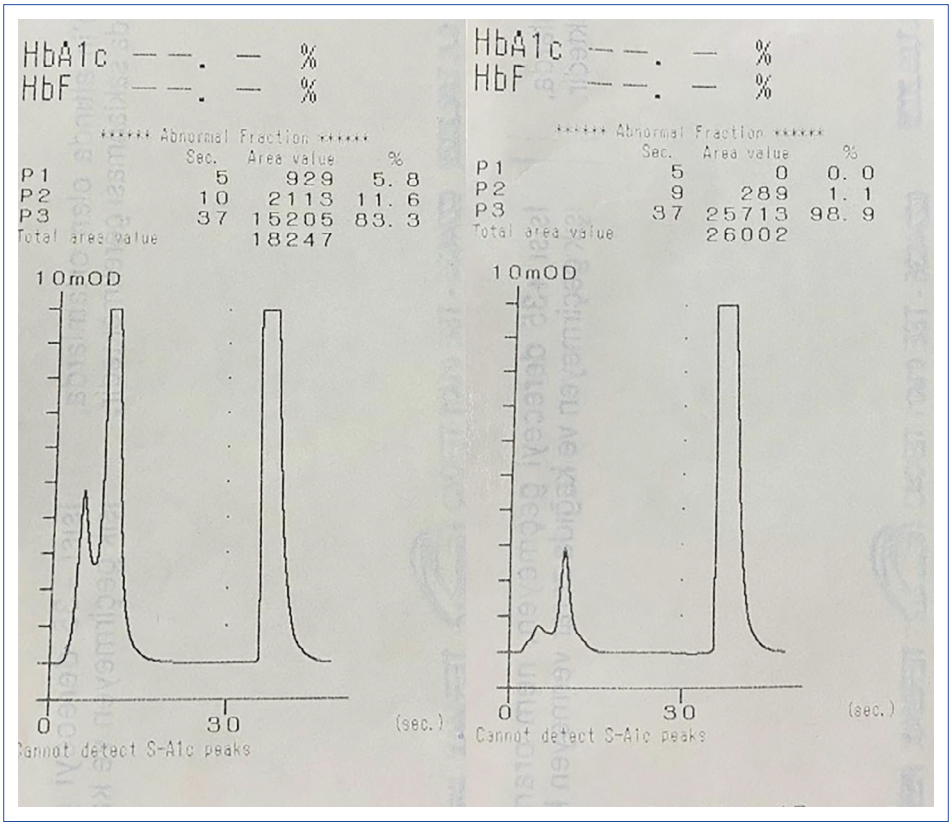

Figure 1. Unmeasurable HbA1c value of cases.
HbA1c: hemoglobin A1C.

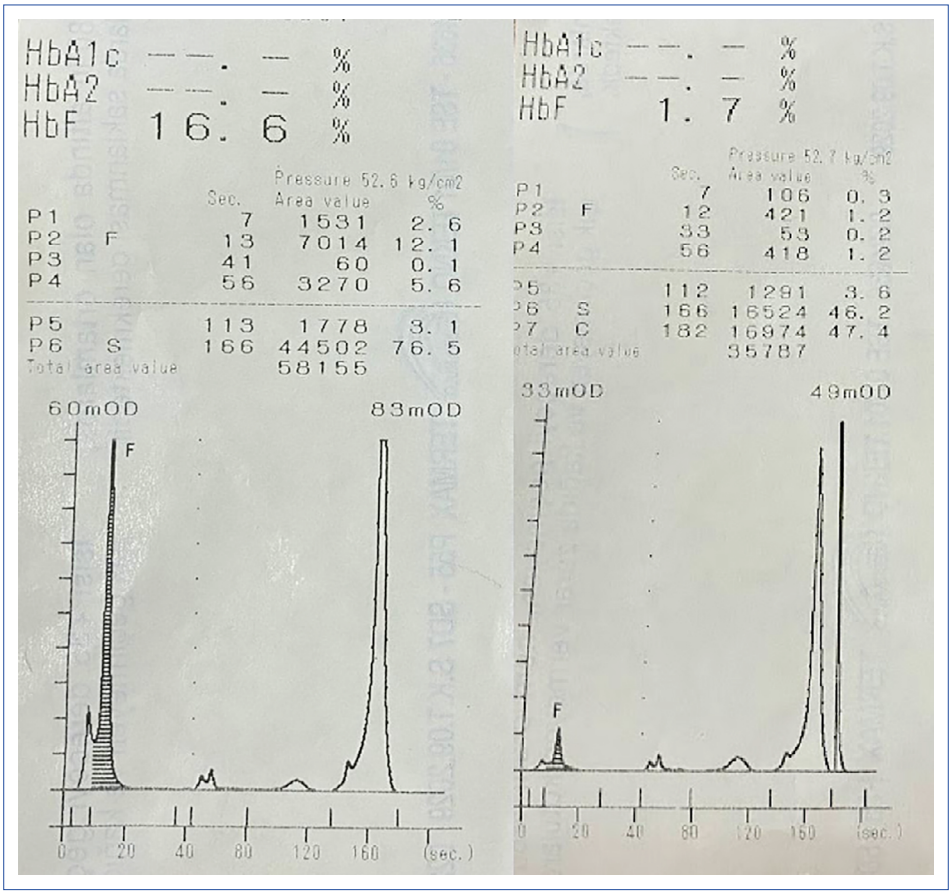

Figure 2. Reevaluated sample of the cases.

with chronic hemolysis (thalassemia, glucose-6-phosphate dehydrogenase deficiency) and patients treated with iron or erythropoietin [6–9].

Hemoglobinopathy: HbA1c levels can be measured high or low in patients with hemoglobin variants depending on the methodology [10].

Chronic kidney disease: HbA1c levels can be altered by advanced chronic kidney disease, hemodialysis, and erythropoietin treatment [11].

All hemoglobin disorders that occur as a result of a genetic disorder are called hemoglobinopathies. Hemoglobinopathies are examined in two main groups; thalassemias and hemoglobin variants. Thalassemia syndromes occur when the defect in the genes causes synthesis disorders without affecting the globin structure. Hemoglobin variants occur when the defect in the genes causes abnormal globin synthesis [12].

Hemoglobin S, associated with sickle cell anemia, is caused by a specific mutation in the beta-globin (HBB) gene (β6 GAG>GTG Glu>Val). This variant can be found in heterozygous (AS, carrier) or homozygous states (SS, patient). The homozygous (SS) form is the most commonly seen form in sickle cell anemia; however, compound heterozygosity of variants of β-thalassemia (Sβ0 and Sβ+) and Hb C (SC) can also be seen in patients showing signs and symptoms of sickle cell anemia [13]. Hemoglobin Sβ-thalassemia occurs with the co-inheritance of HbS and β-thalassemia alleles. HbS and HbA ratios depend on whether the thalassemia allele is β+ or β0 type. Hemoglobin electrophoresis shows 60–90% Hb S, 0–30% Hb A, 1–15% Hb F, and 3.5–6% Hb A2 for Hb Sβ+ thalassemia and 80–95% Hb S, 0% Hb A, 1–15% Hb F, and 3.5–6% Hb A2 for Hb Sβ0 thalassemia [13]. Hemoglobin SC, which has the highest prevalence in West Africa, occurs with the co-inheritance of HbS and HbC alleles. HbSC, which has more target cells and fewer sickle cells in blood smear tests, causes a mild version of sickle cell anemia. Hemoglobin electrophoresis shows 50% Hb S, 0–30% Hb A, 1–7% Hb F, and a normal ratio of HbA2 for HbSC [13]. In our cases, HbA1c values were not measurable by our instrument (Adams A1C HA-8180V), but with another HPLC-based analysis (Adams A1C HA-8180T), HbSβ+ and HbSC were detected in case 1 and case 2, respectively. Genetic analysis was also recommended for the patients to confirm the diagnosis. Macrocytic anemia (Hemoglobin: 95 g/dL and MCV: 111.7 fL) and microcytic anemia (Hemoglobin: 129 g/dL and MCV: 74 fL) were observed in hematology tests from case 1 and case 2, respectively. Tirthankar et al. [14] presented a case whose HbA1c test result was zero by an ion-exchange HPLC method on a Bio-Rad D10 analyzer. They investigated a variant hemoglobin (Hb) by capillary electrophoresis of Hb. They found an elevated peak which was suggestive of HbE disorder. Hegde et al. [15] reported a case whose HbA1c was measured zero by an ion-exchange high-performance liquid

chromatography (HPLC) method on a Bio Rad D10 analyzer. The chromatogram showed a large unknown peak with an area of 82.4% and DNA sequencing confirmed HbD Punjab mutation. Fructosamine should be recommended for clinical use in special populations where HbA1c is less useful. John et al. [16] investigated the clinical use of fructosamine and HbA1c in diabetes mellitus compared to control groups. They found that serum fructosamine should be considered a valid diagnostic marker.

Conclusion

HbA1c results would not be obtained from HbSS, HbSβ+, HbSC, and HbCC patients since these patients would not have any HbA. Alternative test forms such as fructosamine testing should be considered for patients whose HbA is unmeasured in the chromatogram or when HbA1c results are unobtainable by any measurement method.

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

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