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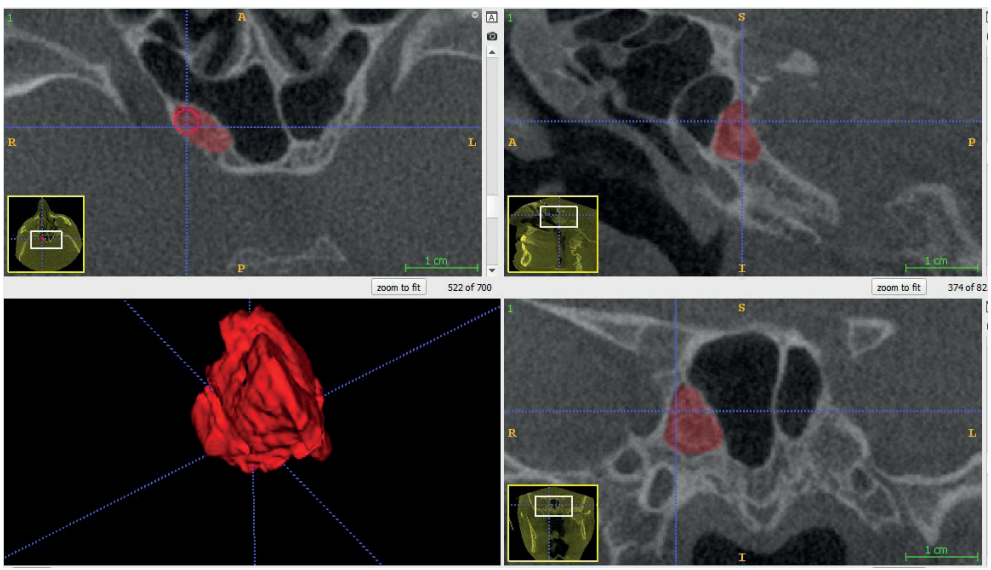

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Journal of Advanced Research in Health Sciences / Sağlık Bilimlerinde İleri Araştırmalar Dergisi 2024
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CORONARY SLOW FLOW AND CHA_2DS_2-VASC-HSF SCORE IN STABLE ISCHEMIC HEART DISEASE

STABİL İSKEMİK KALP HASTALIĞINDA KORONER YAVAŞ AKIM VE CHA_2DS_2-VASC-HSF SKORU

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ABSTRACT

Objective: The coronary slow flow phenomenon (CSFP) and its causes are still not fully explained. We investigated the functionality and usefulness of the CHA_2DS_2-VASc-HSF score in the diagnosis of CSFP in stable ischemic heart disease.

Material and Methods: Patients with no obstructive coronary artery disease (CAD) and CSFP detected as a result of coronary angiography were included in the study. Patients with CSFP were compared with those without. Coronary blood flow velocity was evaluated by calculating the TIMI frame count (TFC) from coronary angiography images. In addition to the traditional CHADS scores of the patients, the CHA_2DS_2-VASc-HSF score was also calculated.

Results: According to our study results, the CHA_2DS_2-VASc-HSF score was higher in patients with CSFP than in those without (3.75±1.27 vs. 2.85±1.11; p<0.001). There was no difference between the $CHADS_2$ and CHA_2DS_2-VASc scores of the two groups. In logistic regression models, Hs-troponin-T and CHA_2DS_2-VASc-HSF scores were determined as independent predictors of CSFP. CHA_2DS_2-VASc-HSF score and TFC were positively correlated in the CSFP group (r=0.848, p<0.001). The sensitivity of CHA_2DS_2-VASc-HSF was determined as 56%, the specificity was 74%, and the cut-off value was 3.5 in detecting the presence of CSFP.

Conclusion: This study shows the association of CHA_2DS_2-VASc-HSF score with CSFP, suggesting that it can be used to predict CSFP and its severity.

Keywords: CHA_2DS_2-VASc-HSF score, coronary slow flow, non-obstructive coronary artery

ÖZ

Amaç: Koroner yavaş akım fenomeni (KYA) ve nedenleri hala tam olarak açıklanamamıştır. Bu çalışmada stabil iskemik kalp hastalığında KYA tanısında CHA_2DS_2-VASc-HSF skorunun işlevselliğini ve kullanışlılığı araştırıldı.

Gereç ve Yöntemler: Çalışmaya obstrüktif koroner arter hastalığı (KAH) olmayan ve koroner anjiyografi sonucunda KYA saptanan hastalar dahil edildi. KYA'lı hastalar olmayanlarla karşılaştırıldı. Koroner kan akış hızı, koroner anjiyografi görüntülerinden TIMI kare sayısı (TFC) hesaplanarak değerlendirildi. Hastaların geleneksel CHADS skorlarına ek olarak CHA_2DS_2-VASc-HSF skoru da hesaplandı.

Bulgular: KYA'lı hastalar olmayan hastalara kıyasla daha yüksek CHA_2DS_2-VASc-HSF skoruna sahipti (3,75±1,27'ye karşı 2,85±1,11; p<0,001). İki grubun $CHADS_2$ ve CHA_2DS_2-VASc skorları arasında fark yoktu. Lojistik regresyon modellerinde, CHA_2DS_2-VASc-HSF skoru ve Hs-troponin-T, KYA'nın bağımsız belirleyicileriydi. CHA_2DS_2-VASc-HSF skoru ve TFC, KYA grubunda pozitif korelasyon gösterdi (r=0,848, p<0,001). CHA_2DS_2-VASc-HSF'nin KYA'nın varlığını tespit etmede duyarlılığı %56, özgüllüğü %74, kesme değeri ise 3,5 olarak belirlendi.

Sonuç: Bu çalışma CHA_2DS_2-VASc-HSF skorunun KYA ile ilişkisini göstererek KYA ve ciddiyetini tahmin etmek için kullanılabileceğini düşündürmektedir.

Anahtar Kelimeler: CHA_2DS_2-VASc-HSF skoru, koroner yavaş akım, obstrüktif olmayan koroner arter

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INTRODUCTION

The delayed opacification observed in non-obstructive epicardial coronary arteries is called the coronary slow flow phenomenon (CSFP) (1, 2). Most patients with CSFP usually describe angina and are referred for coronary angiography. It is suggested that endothelial dysfunction, microvascular disease, or atherosclerosis may be among the causes of CSFP (3-5).

The traditional CHADS scores were created for atrial fibrillation (AF) and they enable evaluation of the risk of thromboembolism and the need for anticoagulant treatment (6). The parameters of these scores are atherosclerosis risk factors. Although these scores are used in clinical practice for atrial fibrillation, they are useful in predicting CAD severity. It has also shown that it may be useful in providing information about the prognosis of acute coronary syndrome (7-9). The CHA2DS2-VASc-HSF score was created by adding important CAD risk factors such as the family history of CAD, smoking, and hyperlipidemia. The gender category was also changed from female to male. Previous studies have shown that CHADS scoring systems may be useful in predicting both CAD severity (10, 11) and no-reflow phenomenon (12). Moreover, the CHA2DS2-VASc-HSF score was more predictive than other CHADS scores. Based on the aforementioned studies, we hypothesized that this score may be associated with CSFP.

MATERIALS and METHODS

Study population

This study included consecutive patients over 18 years of age with CSFP who underwent elective coronary angiography (CA) with suspicion of ischemic heart disease between April 2021 and July 2022. The CSFP group was compared with controls with normal coronary flow based on CA. Non-invasive ischemia tests demonstrated that all patients had stable angina and evidence of myocardial ischemia.

Patients with one of the following were excluded: acute coronary syndromes; presence of obstructive CAD; prior coronary intervention. The Declaration of Helsinki was complied with in our study. The study was approved by the Clinical Ethics Committee of Istanbul University, Istanbul Faculty of Medicine (Date: 21.10.2022, No:19). An informed consent was not obtained from the study group because the design was retrospective.

Calculation of CHADS Scores

Clinical and demographic characteristics, echocardiographic and laboratory data on all patients, were obtained from the patients' medical records.

The CHADS2 score: Congestive heart failure (C)=1 point; Hypertension (HTN)=1 point; Age (A)=1 point; Diabetes (DM)=1 point; Stroke (S)=2 points. The maximum score is 6.

The CHA2DS2-VASc score adds to the CHADS2 score: Vascular disease (V)=1 point; Age 65 to 74 years (A)=1 point; female gender (Sc)=1 point; age>75 years (A2)=2 points. The maximum score is 9.

The CHA2DS2-VASc-HSF score includes Hyperlipidemia (H)=1 point; Smoking (S)=1 point; Family history (F), and male gender (Sc)=1 point; in addition to the CHA2DS2-VASc score. The maximum score is 12.

TIMI frame count measurement

Two independent cardiologists evaluated coronary angiograms retrospectively. Coronary arteries with <50% stenosis were defined as non-obstructive. Thrombolysis in Myocardial Infarction (TIMI) Frame Count (TFC) was calculated by the following technique: The frame count in the left anterior descending coronary artery (LAD) was divided by 1.7 (corrected LAD). The mean TFC was the average of the left circumflex, right coronary, and corrected LAD values. The mean TFC >27 was considered CSFP (2).

Statistical analysis

Statistical Package 26.0 for Windows (IBM SPSS Corp., Armonk, NY, USA) was used for statistical analysis. To prevent selection bias, propensity score matching was performed to inverse probability weight the sample which underwent chart review for co-morbidities. The normality of data was analyzed with the Kolmogorov-Smirnov test. A Mann-Whitney U test was used to compare unpaired samples. Differences in categorical variables between groups were evaluated with the Chi-square test. Pearson or Spearman analysis was used to evaluate correlations. Logistic regression analysis was performed to determine the predictors of CSFP. Results were expressed as relative risk and 95% confidence interval. Receiver operating characteristic analysis was performed for the cutoff value of the score. Significance was defined as two-sided $p<0.05$.

RESULTS

In the present study, 68 consecutive CSFP patients who underwent coronary angiography and did not have obstructive CAD were compared with consecutive controls with normal coronary flow. Gender, age, and body mass index were not different between the two groups. Among the clinical features, smoking, hyperlipidemia, and family history of CAD were more common in the CSFP group than in the normal flow group (p=0.035, p=0.009, p=0.022, respectively). In addition to the diagnosis of hyperlipidemia, the diagnosis of hypertriglyceridemia (defined as triglyceride level >150 mg/dL) was more common in the CSFP group (p=0.004). While HTN was more common (59% vs. 47%) and BMI was higher (28.13±3.80 vs. 26.69±3.23 kg/m^2) in the CSFP group, the difference was not significant. Among the laboratory parameters, D-dimer, Hs-troponin-T (HsTn-T), hemoglobin, leukocytes, monocytes, and the monocytes/HDL ratio were higher in the CSFP group than in the normal flow group (p=0.002, p<0.001, p=0.028, p=0.033, p=0.023, p=0.027, respectively). The CHA2DS2-VASc-HSF score was found to be significantly lower in patients with normal flow than in patients with CSFP (2.85±1.11 vs. 3.75±1.27; p<0.001). CHA2DS2 and CHA2DS2-VASc were not different between groups (Table 1).

CSFP was most common in the LAD artery (41%), followed by the RCA artery (30%). The incidence of ectasia and tortuosity in the coronary arteries was higher in the CSFP group than in the normal flow group (p=0.012, p=0.028, respectively) (Table 2).v

Table 1: Comparison of patients with slow coronary flow and normal flow

Variables	Total patients (n=136)	Patients with normal flow (n=68)	Patients with slow flow (n=68)	p-value
Demographic/clinical parameters				
Age (years)	57.10±11.1	56.91±10.8	57.29±11.5	0.842
Gender				
Male, n (%)	92 (67.6)	45 (66)	47 (69)	0.714
Female, n (%)	44 (32.4)	23 (34)	21 (31)	
Body mass index (kg/m^2)	27.77±3.7	26.69±3.23	28.13±3.80	0.096
Hypertension, n (%)	72 (52.9)	32 (47)	40 (59)	0.168
Diabetes mellitus, n (%)	42 (30.9)	18 (13.2)	24 (17.6)	0.265
Congestive heart failure, n (%)	14 (10.3)	6 (4.4)	8 (5.9)	0.573
Stroke/TIA, n (%)	6 (4.4)	2 (1.5)	4 (2.9)	0.680
Smoking, n (%)	54 (39.7)	21 (15.4)	33 (24.3)	0.035*
Hyperlipidemia, n (%)	59 (43.4)	22 (16.2)	37 (27.2)	0.009*
Hypertriglyceridemia, n (%)	65 (47.8)	24 (17.6)	41 (30.1)	0.004*
Family history, n (%)	53 (39)	20 (14.7)	33 (24.3)	0.022*
AF, n (%)	18 (13.2)	6 (4.4)	12 (8.8)	0.129
Chronic kidney disease, n (%)	11 (8.1)	8 (5.9)	3 (2.2)	0.116
Malignancy, n (%)	4 (2.9)	3 (2.2)	1 (0.7)	0.310
Laboratory parameters				
Creatinine (mg/dl)	0.85 (0.5-13.6)	0.8 (0.5-13.6)	0.88 (0.5-7.7)	0.974
Total cholesterol (mg/dL)	194.59±41.9	192.72±43.1	196.89±40.7	0.332
High density lipoprotein (mg/dL)	43.0±11.7	43.39±11	42.62±12.3	0.405
Low density lipoprotein (mg/dL)	121.36±35.3	117.34±31.8	125.32±38.2	0.189
Triglyceride (mg/dL)	160.25±79.1	158.40±93.9	162.08±61.9	0.191
C-reactive protein (mg/L)	2.4 (0.2-69)	2.25 (0.25-69)	3.8 (0.2-39)	0.159
D-dimer (µg/L)	546.78±341.6	474.41±341.9	619.141±328.8	0.002*
Hemoglobin (gr/L)	13.32±1.7	13.01±1.6	13.63±1.6	0.028*
WBC (10^3/µL)	7.24±1.7	6.95±1.8	7.54±1.7	0.033*
Neutrophile (10^3/µL)	4.39±1.4	4.36±1.3	4.42±1.4	0.848
Lymphocyte (10^3/µL)	2.18±0.7	2.11±0.8	2.23±0.6	0.135
Monocyte (10^3/µL)	0.58±0.2	0.55±0.2	0.62±0.2	0.023*
Platelet (10^3/µL)	240.65±67.86	235.54±73.7	245.75±61.7	0.382
HbA_1C (%)	6.46±2.4	6.32±2.6	6.65±2.2	0.394
AST (U/L)	21.09±13.5	20.78±12.3	21.38±14.6	0.630
ALT (U/L)	19 (2.7-128)	18 (4-128)	19.5 (2.7-87)	0.482
Uric acid (mg/dL)	5.68±1.7	5.63±1.8	5.73±1.7	0.534
Hs-troponin-T (pg/mL)	3.5 (3-193)	3 (3-25)	10 (3-193)	<0.001*
Pro-BNP (pg/mL)	135.5 (20-5406)	139 (20-2615)	133 (20-5406)	0.825
Monocyte/HDL-C ratio	14 (4-34)	13 (4-29)	15 (4-34)	0.027*
Scores				
$CHADS_2$ score	1 (0-4)	1 (0-3)	1 (0-4)	0.233
CHA_2DS_2-VASc score	1 (0-5)	1 (0-4)	1 (0-5)	0.566
CHA_2DS_2-VASc-HSF score	3 (1-7)	3 (1-5)	4 (1-7)	<0.001*

AF: Atrial fibrillation; hs-troponin-T: Highly sensitive troponin-T, HbA_1C: Glycated hemoglobin, AST: Aspartate aminotransferase, ALT: Alanine transaminase, pro-BNP: Pro brain natriuretic peptide, *: p<0.05

Table 2: Coronary angiographic features of the study group

Variables	Total patients (n=136)	Patients with normal flow (n=68)	Patients with slow flow (n=68)	p-value
Slow flow vessel, n (%)				
Left main	14 (10.3)	-	14 (10.3)	-
Left anterior descending	56 (41.2)	-	56 (41.2)	-
Left circumflex	38 (27.9)	-	38 (27.9)	-
Right	41 (30.1)	-	41 (30.1)	-
Coronary Ectasia, n (%)	23 (16.9)	6 (4.4)	17 (12.5)	0.012*
Coronary Tortuosity, n (%)	11 (8.1)	2 (1.5)	9 (6.6)	0.028*

*: p<0.05

Table 3: Correlation of TIMI frame count with laboratory parameters, age, and scores in the coronary slow flow phenomenon group

	Variable	R	p-value
TIMI frame count	Age	0.214	0.080
	Total cholesterol	0.017	0.909
	HDL	-0.094	0.445
	LDL	0.164	0.182
	Triglyceride	0.392	0.001*
	CRP	-0.022	0.868
	D-dimer	0.037	0.797
	Hemoglobin	0.057	0.642
	WBC	0.113	0.357
	Neutrophile	0.129	0.354
	Lymphocyte	0.087	0.534
	Monocyte	0.091	0.460
	Platelet	-0.004	0.971
	HbA_1c	0.289	0.152
	AST	-0.095	0.488
	ALT	0.035	0.799
	Uric acid	-0.006	0.962
	Hs-troponin-T	0.165	0.291
	Pro-BNP	0.050	0.724
	$CHADS_2$ score	0.523	<0.001*
	CHA_2DS_2-VASc score	0.424	<0.001*
	CHA_2DS_2-VASc-HSF score	0.848	<0.001*

Hs-troponin-T: Highly sensitive troponin-T, HbA_1C: Glycated hemoglobin, AST: Aspartate aminotransferase, ALT: alanine transaminase, pro-BNP: Pro brain natriuretic peptide, TIMI: Thrombolysis in myocardial infarction, *p<0.05, R: Correlation coefficient

In the Pearson correlation analysis, the CHA2DS2-VASc-HSF score showed a stronger correlation with TFC than CHADS2 and CHA2DS2-VASc scores in the CSFP group (r=0.848, r=0.424, r=0.523; respectively). The correlation analysis of TFC, including laboratory parameters, age, and scores in the CSFP group, is presented in Table 3.

Table 4: Multivariate regression analyses to predict slow flow phenomenon

4A

	OR	95% CI	p-value
Tortuosity	11.131	0.607-204.158	0.104
CHA2DS2-VASc-HSF score	1.798	1.062-3.045	0.029*
Pro-BNP	0.999	0.998-1.001	0.467
Hs-troponin-T	1.113	1.006-1.231	0.037*

4B

	OR	95% CI	p-value
Ectasia	4.000	0.395-40.549	0.241
CHA2DS2-VASc-HSF score	1.731	1.039-2.884	0.035*
Pro-BNP	1.000	0.999-1.001	0.920
Hs-troponin-T	1.100	1.001-1.208	0.047*

Hs-troponin-T: Highly sensitive troponin-T, pro-BNP: Pro brain natriuretic peptide, OR: Odds ratio, CI: Confidence interval, *p<0.05

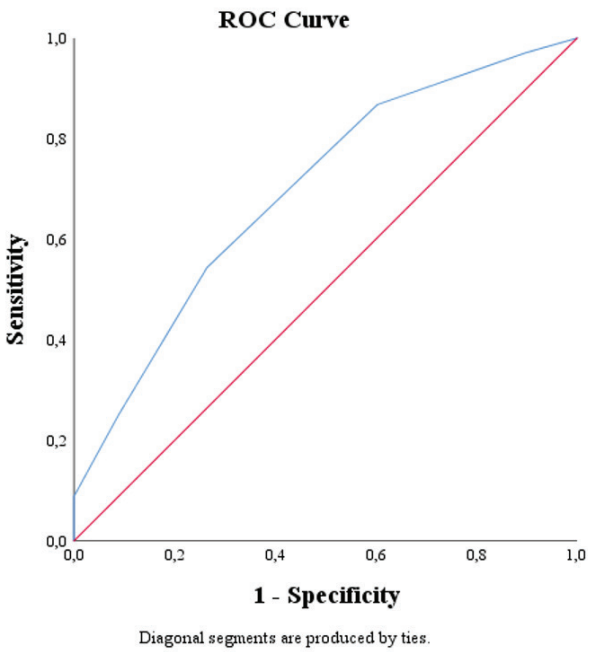

Figure 1: ROC curve analysis demonstrating the prediction of CSFP by the CHA2DS2-VASc-HSF score

The parameters affecting CSFP were evaluated by including clinic-demographic characteristics, laboratory parameters, and CHADS scores in logistic regression analyses. Although the incidence of ectasia and tortuosity in the coronary arteries was higher in the CSFP group than in the normal flow group, in regression models in which coronary ectasia and tortuosity were added, HsTn-T and CHA2DS2-VASc-HSF score were determined as independent predictors of CSFP (Table 4A and 4B). The sensitivity of CHA2DS2-VASc-HSF was determined as 56%, the specificity was 74%, and the cut-off value was 3.5 in detecting the presence of CSFP (AUC: 0.70, 95% CI 0.61–0.78; p<0.001) (Figure 1).

DISCUSSION

We investigated the possible relationship between CHA2DS2-VASc-HSF scores and CSFP in patients who did not have obstructive CAD via elective CA. The results showed that the CHA2DS2-VASc-HSF score was higher in CSFP patients. TCF was correlated with the CHA2DS2-VASc-HSF score in patients with CSFP. The CHA2DS2-VASc-HSF score had sufficient cut-off value to distinguish individuals with CSFP. So far, there is no study showing the relationship of CHA2DS2-VASc-HSF score with CSFP in patients with chronic coronary syndrome.

CSFP is defined by the delay of contrast agent in the coronary artery during CA. Although its frequency varies in the literature, it has been reported as 1-7% (13). While patients with CSFP may be asymptomatic, they may present with stable angina pectoris, myocardial infarction, and even sudden cardiac death (14, 15). It is thought that atherosclerosis, microvascular disease, or endothelial dysfunction may play a role in the pathophysiology of this phenomenon (3, 4, 16). However, it is still not fully explained. Regardless of the cause, patients with CSFP are at high risk for cardiovascular events and often experience poor clinical outcomes (17). In our study, we found a statistically significant increase in HsTn-T in CSFP patients with stable ischemic heart disease. Also, HsTn-T was an independent predictor of CSFP. CSFP may reflect impaired coronary vasomotor reflex and cause myocardial injury in patients at rest. The patients with CSFP may not respond adequately to situations requiring high coronary flow demands (18). The poor prognostic results in CSPF may be explained by this. However, our study is not a follow-up study. Larger follow-up studies are needed to evaluate the prognosis of patients.

The CHA2DS2-VASc scoring system is recommended by the guidelines to evaluate stroke risk in patients with AF (19). DM, age, and HTN, which are the components of this score, are the main risk factors for CAD. Based on this, it has been shown that the CHA2DS2-VASc score can be an indicator of CAD and CAD severity (10, 11). In addition, male gender, hyperlipidemia, smoking, and family history, which are considered other major risk factors for the development of CAD, are among the factors that comprise the CHA2DS2-VASc-HSF score.

Modi et al. showed that CHADS scores were significantly associated with the Gensini score and that the CHA2DS2-VASc-HSF score was superior to other scores in predicting CAD severity with a cut-off value >3. A recent study showed that the new CHA2DS2-VASc-HSF score with a cut-off point of ≥4 predicted the no-reflow phenomenon in STEMI patients and was superior to the other two scores (12). Most patients with CAD have more than one atherosclerosis risk factor. The combination of these multiple risk factors increases the risk and severity of

CAD. Thus, the fact that the CHA2DS2-VASc-HSF score includes more risk factors may explain its better predictive value compared to other scores.

The pathophysiology of CSFP is not completely clear. Some studies investigating the mechanisms underlying CSFP have suggested that one or more of the definitive risk factors for CAD may play a role in its development. Studies have shown that HTN (20-23), obesity (23-25), or smoking (22, 26) are mainly responsible for CSFP, while male gender (23, 27, 28), family history of CAD (27), and hyperlipidemia (22,29) were also shown to be risk factors. In contrast to studies in which obesity was blamed, another study showed that low BMI is a predictor of CSFP (21). It has also been reported that patients with CSFP have higher triglyceride levels (27, 28, 30) or lower HDL levels (21). In our study, smoking, hyperlipidemia, and family history of CAD were more common in patients with CSFP than in the normal flow group. In addition to hyperlipidemia, hypertriglyceridemia was more common in the CSFP group.

The implementation of CHA2DS2-VASc-HSF risk scoring by physicians does not require additional cost in routine practice and is quite easy. The results of this study support that it can be used as a predictive score in the diagnosis of CSFP.

This study has many limitations. To list the most important, it was a single-center study and the number of patients analyzed was small. As the study design was retrospective, data are based on a review of patients' previous clinical histories. This may affect the calculation of scores and there is a possibility of bias.

CONCLUSION

The CHA2DS2-VASc-HSF score can be calculated easily and used in clinical practice to predict patients at risk for CSFP. Larger and prospective studies are needed to support the results of our study.

Ethics Committee Approval: This study was approved by Istanbul University, Istanbul Faculty of Medicine (Date: 21.10.2022, No:19).

Informed Consent: Since the study was in a retrospective design, informed consent was not required.

Peer Review: Externally peer-reviewed.

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INVESTIGATION OF *DICER1* AND *BAFF* GENE MUTATIONS IN B-CELL NON-HODGKIN LYMPHOMA

B HÜCRELİ NON-HODGKİN LENFOMADA *DICER1* VE *BAFF* GEN MUTASYONLARININ ARAŞTIRILMASI

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ABSTRACT

Objective: *DICER1* and *BAFF* gene mutations are effective in T-cell lymphoma progression. Therefore, *DICER1* and *BAFF* genes may have a role in the progression of B-cell lymphomas. For this reason, it was aimed to determine the role of *DICER1* and *BAFF* genes in the development of B-NHL.

Materials and Methods: The study included DNA samples from 60 patients diagnosed with B-NHL who had applied to the Istanbul University, Institute of Oncology, Department of Clinical Oncology between 1991 and 1997. DNA materials obtained from lymphocytes of 30 healthy individuals matched with the patients in terms of age, gender, and race were used as a control group. The c.+3473A>G (rs3742330) polymorphism in the DICER1 gene and the c.-871C>T (rs9514828) single nucleotide polymorphism in the BAFF gene were examined using the PCR-RFLP method. In addition, the presence of mutations in the 11th and 25th exons of the DICER1 gene was evaluated by SANGER sequencing. The results of the control and patient groups were analyzed for polymorphism and mutation presence using Chi-square and Fisher tests.

Results: The polymorphic regions of c.+3473A>G(rs3742330) in the *DICER1* gene and c.-871C>T (rs 9514828) in the *BAFF* gene were examined in the patient and control groups, but no statistically significant relationship was found. When the exon 11 and exon 25 of the *DICER1* gene were investigated, no statistically significant relationship was found between the patient and control groups (p>0.05).

Conclusion: The absence of a difference between the patient and control groups suggests that different genetic mechanisms may be involved in the formation of B-NHL. The small population in the study is one of the reasons why no significant difference was found between the results. Additional studies are needed in larger patient and control groups.

Keywords: B-cell non-Hodgkin lymphoma, *DICER1*, *BAFF*, mutation, SNP

ÖZ

Amaç: *DICER1* ve *BAFF* geni mutasyonlarının T hücreli lenfoma progresyonunda etkili olduğu bilinmektedir. Bu durum B hücreli lenfomaların progresyonunda da *DICER1* ve *BAFF* genlerinin rolünün olabileceğini düşündürmüştür. *DICER1* ve *BAFF* geninin ekspresyon ve mutasyonlarını araştıran birçok çalışma bulunmasına rağmen, B hücreli non-Hodgkin lenfoma progresyonunda tümör baskılayıcı etkisinin nasıl oluştuğu ile ilgili olarak yeterli bilgi bulunmamaktadır. Bu sebeple çalışmada, *DICER1* ve *BAFF* genlerinin B-NHL gelişimindeki rolünün ne olduğunun belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Çalışmaya 1991-1997 yılları arasında İstanbul Üniversitesi, Onkoloji Enstitüsü, Klinik Onkoloji Anabilim Dalı'na başvurmuş ve B-NHL tanısı almış 60 hastaya ait DNA örnekleri dâhil edilmiştir. Kontrol grubu olarak hastalarla yaş, cinsiyet ve ırk olarak eşleştirilmiş 30 sağlıklı kişinin lenfositlerinden elde edilen DNA materyalleri kullanılmıştır. *DICER1* geninde yer alan c.+3473A>G(rs3742330) polimorfizmi ile *BAFF* genindeki c.-871C>T(rs9514828) tek nükleotid polimorfizmi PCR-RFLP yöntemiyle incelenmiştir. Ayrıca *DICER1* geninin 11. ve 25. ekzonları mutasyon varlığı açısından SANGER dizi analizi yöntemiyle değerlendirilmiştir. Kontrol ve hasta grubunun sonuçları polimorfizm ve mutasyon varlığı açısından Ki-kare ve Fisher testleriyle analiz edilmiştir.

Bulgular: *DICER1* genindeki c.+3473A>G(rs3742330) ile *BAFF* genindeki c.-871C>T(rs 9514828) polimorfik bölgeleri hasta ve kontrol grubunda incelenmiş ancak istatistiksel olarak anlamlı bir ilişki bulunamamıştır. *DICER1* geninin 11. ve 25. Ekzonları araştırıldığında ise yine hasta ve kontrol grubu arasında istatiksel olarak anlamlı bir ilişki bulunamamıştır (p>0,05).

Sonuç: Hasta ve kontrol grupları arasında bir farkın bulunmaması, B-NHL oluşumunda farklı genetik mekanizmaların etkisinin olabileceğini düşündürmektedir. Çalışmadaki popülasyon sayısının az olması, sonuçlar arasında anlamlı bir fark bulunamamasının nedenlerinden biridir. Daha geniş hasta ve kontrol grubunda ek çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: B hücreli non-Hodgkin lenfoma, *DICER1*, *BAFF*, mutasyon, SNP

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INTRODUCTION

Cancer covers more than a hundred different types of diseases that originate from various organs and many cell types in the human body and are characterized by the uncontrolled proliferation of cells and metastasis to different organs (1).

Non-Hodgkin lymphoma (NHL) is the most common hematological malignancy worldwide and accounts for approximately 3% of cancer diagnoses and deaths (2). According to GLOBACAN 2020 data, 544,352 people in the world are newly diagnosed with NHL and 259,793 of these patients die from NHL. Although NHL is more common in men than in women worldwide, mortality rates are also higher in men. According to the incidence rates for both sexes, 44.3% of the cases are seen in the Asian population. The 5-year prevalence in the adult population was 2.6% in both sexes, while the incidence of NHL was slightly higher in males than females (3). The incidence in Turkey is 6.5 per 100,000 in men and 4.4 in women (4).

NHL is a neoplasm of lymphoid tissues arising from B cell precursors, mature B cells, T cell precursors, and mature T cells. NHL consists of several subtypes, each with different epidemiologies, etiologies, immunophenotypic, genetic, clinical features, and response to therapy. The most common mature B-cell neoplasms are Follicular lymphoma, Burkitt lymphoma, diffuse large B-cell lymphoma, Mantle cell lymphoma, marginal zone lymphoma, and primary CNS lymphoma (5). B-cell lymphomas are clonal tumors of mature and immature B cells that make up the majority (80-85%) of NHLs. NHLs are a heterogeneous group of lymphoproliferative malignancies with different behavioral patterns and responses to therapy. NHL usually originates in lymphoid tissues and can spread to other organs (6).

In the literature, it has been shown that *DICER1* and *BAFF* genes have important roles in cancer development. Although there are many studies investigating the expression and mutations of *DICER1* and *BAFF* genes, there is not enough information about how these genes act as tumor suppressors in B- NHL progression. For this reason, it was aimed to determine the role of *DICER1* and *BAFF* genes in the development of B- NHL and to contribute to the literature.

MATERIAL and METHOD

Patient population

DNAs isolated from blood samples collected at that time belonging to 60 Caucasian patients with a diagnosis of B- NHL who applied to Istanbul University, Institute of Oncology, Department of Clinical Oncology between 1991-1997 and never received any treatment were used.

The control group consisted of 30 white people who were selected by matching the patient group in terms of age, gender, and ethnicity between 2014 and 2015, and who did not have a family history of cancer and systemic disease. Peripheral blood samples were taken after the subjects who volunteered for the control group were informed about the study before they were included in the study, and after the informed consent form was signed.

The study was approved ethically at the Ethics Committee meeting dated 18.04.2014 and numbered 08 and was carried out at Istanbul University, Institute of Oncology, Department of Basic Oncology, Cancer Genetics Research Laboratory (Date: 18.04.2014, No: 08).

DNA isolation

Since the DNAs of the patient group were used for other research and thesis studies before, they were ready. Peripheral blood samples of the control group were collected within the scope of this study. After 10 mL of peripheral blood sample taken from healthy individuals in EDTA tubes was transferred to 50 mL DNA tubes, the DNA isolation process was started. DNA was isolated from lymphocytes obtained from blood samples using the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. Measurement of the concentrations of the isolated DNAs was carried out using the Nanodrop 2000 Spectrophotometer [Thermo Scientific].

Genetic changes analyzed in patient and control group

Within the scope of the study, the c.+3473A>G (rs3742330) polymorphism in the *DICER1* gene and the c.-871 C>T (rs9514828) single nucleotide polymorphism in the *BAFF* gene were examined by the PCR-RFLP method, while the exon 11 and exon 25 mutations of the *DICER1* gene were evaluated by Sanger Sequencing. The genetic changes analyzed in the patient and control groups are shown in Table 1.

Table 1: Genetic changes analyzed in patient and control group

Study group	Analyzed genetic change
Patient Group (n=60) ve Control Group (n=15)	*DICER1* gene exon 11 and exon 25 mutations
Patient Group (n=60) ve Control Group (n=30)	*DICER1* gene c.+3473A>G (rs3742330) polymorphism and *BAFF* gene c.-871C >T (rs9514828) polymorphism

DICER1: Dicer 1, Ribonuclease III, *BAFF:* B cell activation factor

Primer selection

For the study, forward and reverse primer sequences of the *DICER1* and *BAFF* genes were designed using the IDT Oligo Analyzer computer program (www.itdna.com). The primer sequences of the gene regions and the properties of these primer sequences are given below.

BAFF c.-871C >T (rs 9514828) SNP İçin Kullanılan Primerler [Forward (5'- GGC ACA GTC AAC ATG GGA GT- 3'); Reverse (5'- CCT TGA AGG AAG TGT GGA AGT A- 3')]

Primers Used for *DICER1* c.+ 3473A>G (rs 3742330) SNP [Forward (5-' TGG CGT CTC CAA CAA CTT TA - 3'); Reverse (5-' CCT GCC TTG ACA ACA TGA AA- 3')]

Primers Used for *DICER1* gene Exon 11 [Forward (5'- GTA CAG AGG CAG ACA GCA TAC - 3'); Reverse (5'- GAC TTA AAC TGT GCA ACA TTC CC- 3')]

Primers Used for *DICER1* gene Exon 25 [Forward (5'- AGA AAC TAC ATC TGT GGA CTG C - 3'); Reverse (5'-GGC AGT TTC TGG TTC CAT TTC - 3')].

Restriction enzymes and enzyme recognition sites
Restriction endonucleases to be used in the study were selected by planning to reveal the nucleotide change in the relevant SNP region of the *DICER1* and *BAFF* genes, and the following enzymes were used. In the literature, it was determined that the same enzymes were used for the examination of these sites (17, 25).

BanI restriction endonuclease:

BanI enzyme recognition site 5'... G↓ G Y R C C.... 3' R: A or G

3'... C C R Y G↑ G.... 5' Y: C or T

BsrBI restriction endonuclease:

BsrBI enzyme recognition site5'...C C G ↓ C T C..... 3'

3'...G G C ↑G A G..... 5'

Sequencing of gene regions by The Sanger Method
Firstly, Polymerase Chain Reaction (PCR) was performed from the obtained DNAs. Then, purification, DTCS (Dye Terminator Cycle Sequencing Reaction), and Ethanol precipitation processes were performed, respectively. The obtained samples were thawed and loaded into a sequence analysis device (Beckman Coulter, Ceq 8000 Genetic Analyzer) to perform sequence analysis.

Polymerase chain reaction - Restriction fragment length polymorphism (PCR-RFLP)
In order to examine the presence of c.+3473A>G (rs3742330) in the *DICER1* gene and c.-871C>T (rs9514828) SNPs in the *BAFF* gene in DNA samples isolated from the peripheral blood of the patients and control groups, first the relevant gene regions were amplified by the PCR method. After PCR, amplified DNA products were cut with BanI restriction enzyme to detect c.+3473A>G polymorphism in the *DICER1* gene and with BsrBI restriction enzyme to detect c.-871C>T polymorphism in the *BAFF* gene. In order to determine the size of the DNA fragments fragmented from the recognition sites by restriction endonucleases, 1 µL of 6 X Orange-Dye loading buffer and 5 µL of DNA sample were mixed and 5 µL of this mixture was loaded into the wells on a 2.5% agarose gel and accompanied by a Molecular Weight Marker. After the agarose gel electrophoresis, the bands of the DNA fragments were visualized with the UV imaging system. Samples cut and uncut with restriction enzymes were determined.

Statistics
SPSS v.18 computer program was used in the statistical evaluation of the results of the patient and control groups. Statistical differences between groups were evaluated with the Chi-square test, Yates Chi-square test, and Fisher test.

RESULTS

Distribution of patients with B- NHL according to subgroups, 49 patients with Diffuse Large B-cell Lymphoma (DLBCL) (81.6%), 2 patients with Small-cell Lymphocytic Lymphoma (SLL) (3.33%), 5 patients with Diffuse Mixed Small and Large Cell Lymphoma (8.33%), 1 patient with Marginal Zone Lymphoma (MZL) (1.6%), 2 patients with Malt Lymphoma (3.33%), 1 patient with Burkitt Lymphoma (BL) (1.6%). While the mean age of the patient groups by gender was 51.55 (±15.42) for male patients, the mean age of female patients was 48.3(±18.45). The mean age of the healthy control group was 51.25(±14.79) in men and 45.3(±14.27) in women for the group consisting of 30 people. In the control group consisting of 15 individuals selected from the same healthy control group, the mean age was 48.6(±17.09) in males and 46.6 (±14.94) in females. There is no statistical difference between the patient group and the healthy control groups in terms of age. The characteristics of the patient and control groups are shown in Table 2.

Table 2: Distribution of patient groups by NHL subtypes

Patient groups	Female n (%)	Male n (%)	Total n (%)
DLBCL	18 (36.7%)	31 (63.3%)	49 (81.6%)
SLL	0 (0%)	2 (100%)	2 (3.33%)
Diffuse Mixed Small and Large Cell Lymphoma	2 (40%)	3 (60%)	5 (8.33%)
MZL	0 (0%)	1 (100%)	1 (1.6%)
Malt Lymphoma	0 (0%)	2 (100%)	2 (3.33%)
Burkitt Lymphoma	0 (0%)	1 (100%)	1 (1.6%)
Total	20 (33.3%)	40 (66.7%)	60 (100%)

DICER1: Dicer 1, Ribonuclease III, BAFF:,B cell activation factor, NHL: Non-Hodgkin Lymphoma, DLBCL: Diffuse Large B-cell Lymphoma, SLL: Small-cell Lymphocytic Lymphoma, MZL: Marginal Zone Lymphoma

In the evaluation of the *DICER1* gene exon 11 sequence analysis of 60 patients, a heterozygous change, indicated as Heterozygote 1827C>T(rs150087634), was detected in one patient sample. No mutation or genetic change was observed in the exon 11 sequence analysis results of the other patients. The results of the sequence analysis evaluations for the exon 11 of the *DICER1* gene of the individuals in the patient group are shown in Figure 1.

The DICER1 gene of the healthy control group was not detected in any of the individuals in the healthy group in the evaluation of exon 11 sequence analysis. The results of the sequence analysis evaluations for the exon 11 of the *DICER1* gene of the individuals in the control group are shown in Figure 2.

No mutations or genetic changes were detected in the sequence analysis performed for exon 25 of the *DICER1* gene in the DNA samples of 60 individuals in the patient group. The results of the sequence analysis evaluations for the exon 25 of the *DICER1* gene of the individuals in the patient group are shown in Figure 3.

When DNA samples of 15 individuals in the healthy control group were evaluated by sequence analysis for the presence of a mutation in the exon 25 of the *DICER1* gene, no mutation

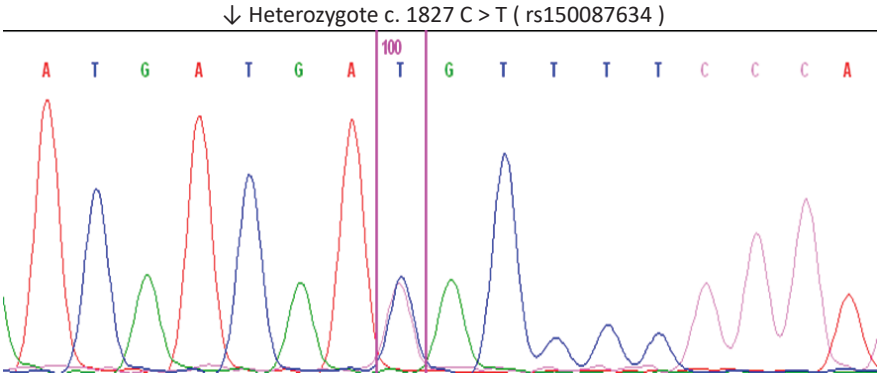

Figure 1: Sequence analysis result of *DICER1* gene exon 11 in patient group

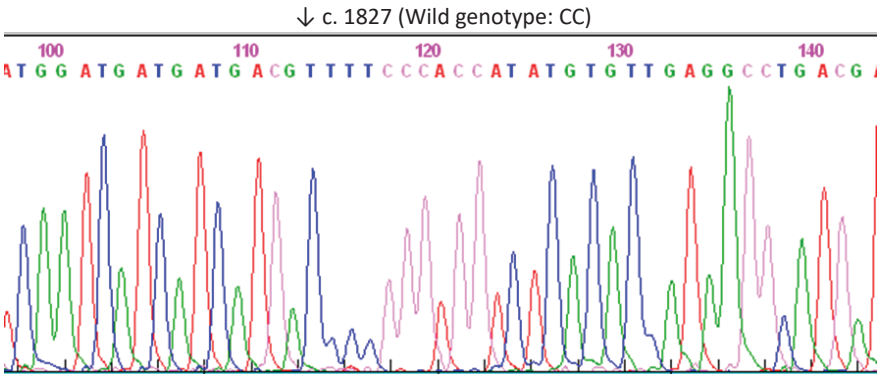

Figure 2: Sequence analysis result of *DICER1* gene exon 11 in control group

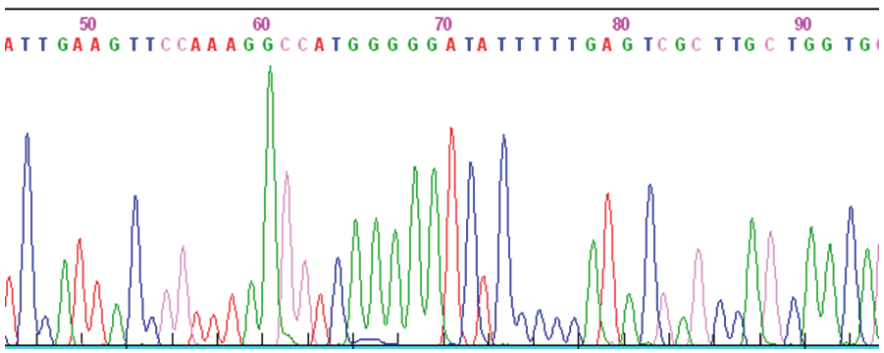

Figure 3: Sequence analysis result of *DICER1* gene exon 25 in patient group

or genetic change was detected. The results of the sequence analysis evaluations for the exon 25 of the *DICER1* gene of the individuals in the control group are shown in Figure 4.

When DNA samples of the patient group were evaluated by PCR-RFLP method, it was determined that 44 patients were wild genotype (AA) and 16 patients were heterozygote genotype (AG) for the c.+3473 region of the *DICER1* gene. *DICER1* gene c.+3473 PCR-RFLP imaging of the patient group is shown in Figure 5.

When the DNA samples of 30 individuals in the healthy control group were evaluated by PCR-RFLP method, it was determined that 24 individuals were in the wild genotype (AA) and 6 individuals were heterozygote genotype (AG) for the c.+3473 region of the *DICER1* gene. c.+3473 PCR-RFLP imaging of the *DICER1* gene of the control group is shown in Figure 6.

When DNA samples of 60 individuals in the patient group were analyzed by PCR-RFLP method, 20 patients were found to be wild genotype (CC), 29 patients were heterozygote genotype (CT), and 11 patients were homozygote genotype (TT) for the c.-871 region in the *BAFF* gene. PCR-RFLP imaging of the c.-871 region in the *BAFF* gene of the patient group is shown in Figure 7.

When DNA samples of 30 individuals in the control group were evaluated by PCR-RFLP method for the c.-871 region of the *BAFF* gene, 10 individuals were found to be wild genotype (CC),

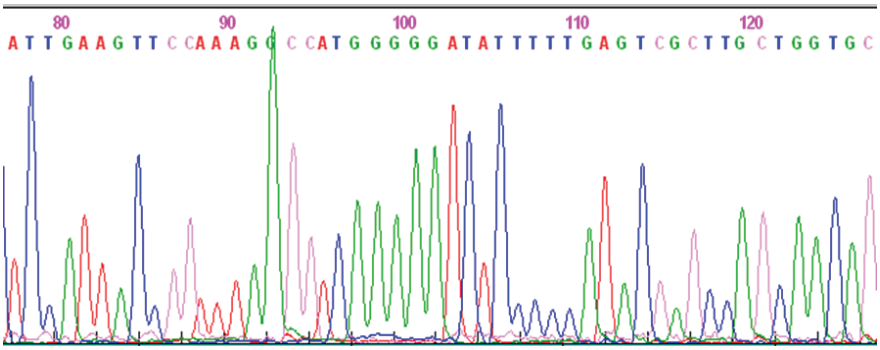

Figure 4: Sequence analysis result of *DICER1* gene exon 25 in control group

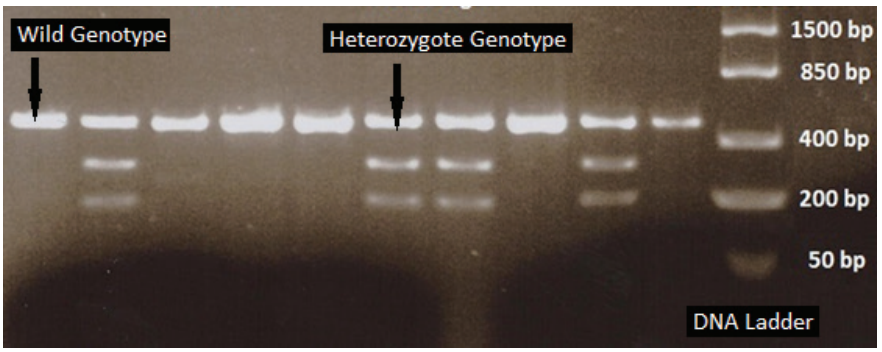

Figure 5: PCR-RFLP imaging of *DICER1* gene c.+3473 region for the patient group

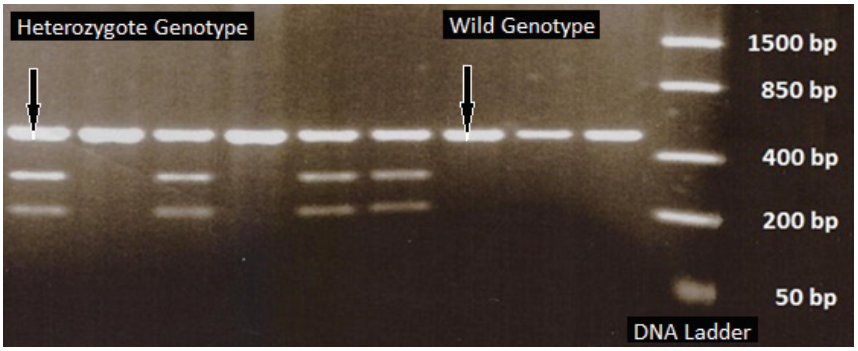

Figure 6: PCR-RFLP imaging of *DICER1* gene c.+3473 region for the control group

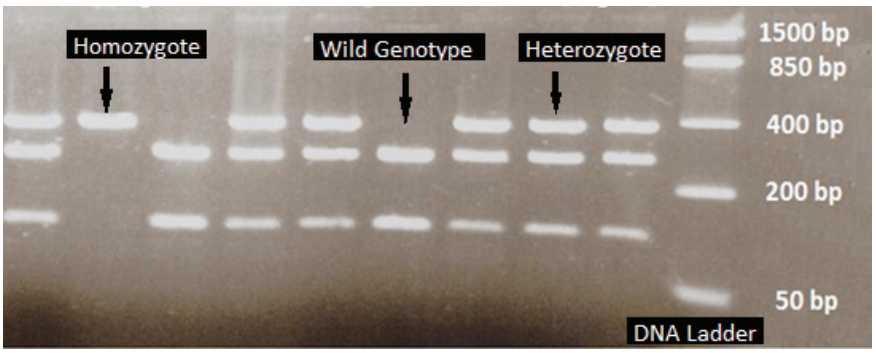

Figure 7: PCR-RFLP imaging of the c.-871 region in the *BAFF* gene of the patient group

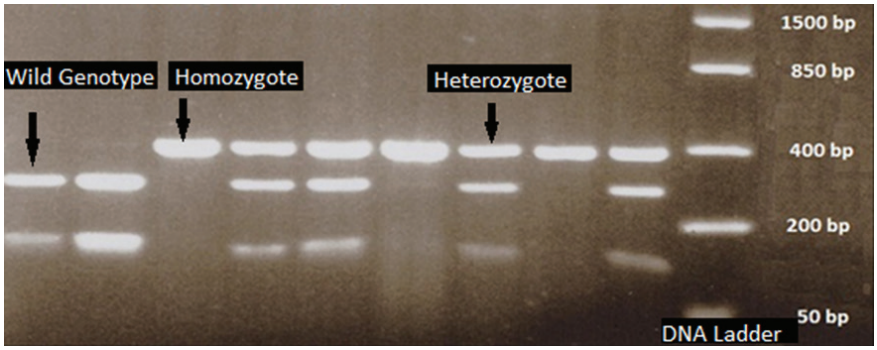

Figure 8: PCR-RFLP imaging of the c.-871 region in the *BAFF* gene of the control group

12 individuals were heterozygote genotype (CT), and 8 individuals were homozygote genotype (TT). PCR-RFLP imaging of the c.-871 region in the *BAFF* gene of the control group is shown in Figure 8.

DNA samples of the patient group (n=60) and control group (n=30) were evaluated for the *DICER1* c.+3473A>G polymorphism with the Yates Chi-Square test. χ2yates=0.1880, degrees of freedom=1 and p= 0.66. Since p>0.05, no statistically significant relationship was established between the patient group and the healthy group in terms of the c.+3473A>G polymorphism of the *DICER1* gene (Table 3).

When the *BAFF* gene c.-871C >T polymorphism in 60 patients with B- NHL and 30 healthy control groups was evaluated statistically by "Chi-square" test, χ2=0.963, degrees of freedom=2, p= 0.618 data were obtained. . Since P was >0.05, no significant relationship could be established between the patient group and the healthy control group in terms of *BAFF* gene c.-871C >T polymorphism (Table 4).

When the *DICER1* gene was evaluated statistically for the c.+3473A>G polymorphism with the Yates Chi-square test, the results obtained were as follows, respectively:

1. There was no statistical significance between patients with DLBCL (n=49) and patients with other B- NHL(n=11) in terms of the c.+3473A>G polymorphism of the *DICER1* gene (χ2yates=1,397, s.d=1, p=0.237 and p>0.05) .

2. There was no statistical significance between patients with DLBCL (n=49) and the healthy control group (n=30) in terms of *DICER1* gene c.+3473A >G polymorphism (χ2yates=0.066, s.d=1, p=0.791 and p>0.05).

3. There was no statistical significance between the patients with other B-NHL (n=11) and the healthy control group (n=30) in terms of the c.+3473A>G polymorphism of the *DICER1* gene (χ2 yates=1.518, s.d=1 , p=0.217 and p>0.05).

Statistical evaluation of *DICER1* gene c.+3473A>G polymorphism according to patient subgroups and control group is shown in Table 5.

Statistical evaluation of the *BAFF* gene c.-871C >T polymorphism between patients with DLBCL (n=49) and healthy controls (n=30) using the chi-square test χ2=0.693, degrees of freedom= 2 and p=0.707 results were obtained. Since P>0.05, there was no statistical significance between patients with DLBCL and the healthy control group in terms of *BAFF* gene c.-871C>T polymorphism (Table 6).

The exon 11 and exon 25 regions of the *DICER1* gene were evaluated for the presence of mutation between the patient group (n=60) and the healthy control group (n=15). No mutation was found in exon 25 in the sequence analysis evaluations of the patient group and the healthy group. Heterozygous c.1827C >T genetic change was detected in the exon 11 region of 1 patient with MALT lymphoma from the samples belonging to

Table 3: Statistical evaluation of patient and control group results of DICER1 gene c.+3473A>G polymorphism

Polymorphism	Patient group n=60 n (%)	Control group n=30 n (%)	Total
Wild Genotype_AA	44 (64.7%)	24 (35.3%)	68 (80%)
Homozygote Genotype_GG	0 (0%)	0 (0%)	0 (0%)
Heterozygote Genotype_AG	16 (72.7%)	6 (27.3%)	22 (20%)
Total	60 (66.6%)	30 (33.3%)	90 (100%)

DICER1: Dicer 1, Ribonuclease III

Table 4: Statistical evaluation of patient and control group results of BAFF gene c.-871C>T polymorphism

Polymorphism	Patient group n=60 n (%)	Control group n=30 n (%)	Total
Wild Genotype_CC	20 (66.7%)	10 (33.3%)	30 (33.3%)
Homozygote Genotype_TT	11 (57.9%)	8 (42.1%)	19 (21.1%)
Heterozygote Genotype_CT	29 (70.7%)	12 (29.3%)	41 (45.6%)
Total	60 (66.6%)	30 (33.3%)	90 (100%)

BAFF: B cell activation factor

Table 5: Statistical evaluation of DICER1 gene c.+3473A>G polymorphism according to patient subgroups and control group

Patient subgroups and control group	Wild Genotype_AA	Homozygote Genotype_GG	Heterozygote Genotype_AG	Total
DLBCL	38 (77.5%)	0 (0%)	11 (22.5%)	49 (54.5%)
Other B-cell non-Hodgkin lymphoma	6 (54.5%)	0 (0%)	5 (45.5%)	11 (12.2%)
Control group	24 (80%)	0 (0%)	6 (20%)	30 (33.3%)
Total	68 (75.6%)	0 (0%)	22 (24.4%)	90 (100%)

DICER1: Dicer 1, Ribonuclease III, DLBCL: Diffuse Large B-cell Lymphoma

Table 6: Statistical evaluation of BAFF gene c.-871C>T polymorphism by patient subgroups and control group

Patient subgroups and control group	Wild Genotype_CC	Homozygote Genotype_TT	Heterozygote Genotype_CT	Total
DLBCL	15 (30.6%)	10 (20.4%)	24 (48.9%)	49 (54.5%)
Other B-cell non-Hodgkin lymphoma	5 (45.5%)	1 (9%)	5 (45.5%)	11 (12.2%)
Control group	10 (33.3%)	8 (26.7%)	12 (40%)	30 (33.3%)
Total	30 (33.3%)	19 (21.1%)	41 (45.6%)	90 (100%)

BAFF: B cell activation factor, DLBCL: Diffuse Large B-cell Lymphoma

Table 7: Statistical evaluation of the sequence analysis results of the *DICER1* gene exon 11 and exon 25 regions

Patient and control groups	Exon 11 Region of *DICER1* gene (n)	Exon 25 Region of *DICER1* gene (n)
B-NHL		
Malt Lymphoma	**Heterozygous c.1827C >T (1)**	**Normal (60)**
Other-NHLs	**Normal (59)**	
Control group	**Normal (15)**	**Normal (15)**
Total	**75**	**75**

DICER1: Dicer 1, Ribonuclease III, B-NHL: B-cell Non-Hodgkin Lymhoma, NHL: Non-Hodgkin Lymhoma

the patient group. When the patient group and the healthy control group were analyzed with the "Fisher" test in terms of genetic changes observed in the exon 11 region, a p=1 value was obtained. Since P>0.05, there was no statistical significance between both groups (Table 7).

DISCUSSION

It has been shown in previous studies that the *DICER1* gene, whose role in the development of B-NHL was investigated within the scope of the study, has many mutations at both the germline and somatic levels. There are 133 germline mutations and 95 somatic mutations reported on the *DICER1* protein construct (7). The results of the studies conducted to date have shown that mutations and single nucleotide polymorphisms that may cause errors in the normal function of the *DICER1* gene may play a role in the formation of a number of malignancies, including pleuropulmonary blastoma (PPB), ovarian cancer, nasopharyngeal cancer, breast cancer and T-cell lymphomas (8). In addition, it is known that miRNAs have roles in the regulation of hematopoiesis and the developmental stages of B cells, and non-Hodgkin B-cell lymphomas originate significantly due to the differentiation of B cells (9). In the results of the study conducted by Li et al. in 2014, it was shown that there is a positive correlation between the c.+3473A>G polymorphism of the *DICER1* gene in patients with T-cell lymphoma and the overall survival of these patients (10). Again, in the results of the study conducted by Li et al. in patients with T-cell lymphoma in 2012, it was observed that there was a significant increase in 5-year survival of patients carrying the c.+3473A>G polymorphism of the *DICER1* gene (8). Murray et al. defined a germline mutation in the 11th exon of the *DICER1* gene of a patient with familial PPB in the results of a study they conducted in 2014, and this mutation was found to have a pathogenic effect since it resulted in a frameshift in the relevant gene sequence (11). When the same patient was evaluated in terms of somatic mutations in the *DICER1* gene; A somatic hotspot mutation expressed as c.5425G>A[p.Gli1809Arg] has been found in exon 25 of the *DICER1* gene (11). When they analyzed the serum samples of patients with PPB with mutations in the *DICER1* gene, they found that miR 125a-3p, miR-125b-2-3p, let 7a-3p, let-7b-3p and six other miRNA levels were approximately 40 times higher than the healthy control group (11).

The *BAFF* gene is expressed by immune system cells, including monocytes, macrophages, dendritic cells, a subset of T lymphocytes, and some of the immune system cells such as B lymphocytes (12). In addition, the *BAFF* gene plays an important role in the selection, homeostasis, and transformation of B cells into malignant cells (13). In the literature, it has been shown that the *BAFF* gene is abnormally expressed in malignant B cells of B-NHL patients. It has been understood that this situation protects the malignant cells from apoptosis by spontaneous or drug stimulation and provides the activation of NF-kB, a transcription factor belonging to the Rel gene family, via autocrine or paracrine pathways (14, 15). It has been reported that exogenous *BAFF* upregulates the c-Myc gene, which stimulates B cell proliferation, downregulates the cell proliferation inhibitor p53, and increases the expression of the B cell differentiation inhibitor, BCL6, in some NHLs (14). B-cell tumors were found to express little or no membrane-dependent *BAFF*, but it was reported that serum *BAFF* levels were significantly higher in most patients with NHL whose serum *BAFF* levels were compared with healthy controls, and higher serum *BAFF* levels were associated with aggressive disease and poor response to therapy (14). Novak et al. examined the expression of *BAFF* and *BAFF* receptors in biopsy samples of tumor tissue from patients with B-cell NHL in 2004 and reported that there is a relationship between the increase in *BAFF* gene expression and the aggressiveness of the tumor tissue (16). In 2012, Zhai et al. investigated the *BAFF* gene c.-871C>T(rs9514828) polymorphism in patients with T-cell lymphoma. They found that those with homozygote genotype for c.-871C>T(rs9514828) polymorphism had a significantly higher 5-year survival rate than those with wild type or heterozygote genotype (17).

The results of these studies investigating *DICER1* gene and *BAFF* gene polymorphisms and *DICER1* gene mutations showed that single nucleotide polymorphisms and mutations in *DICER1* and *BAFF* genes may result in changes in gene expression, and this may contribute to the formation of B-cell lymphomas. Considering the studies in the literature, we aimed to determine the role of *DICER1* and *BAFF* genes in the development of B-cell non-Hodgkin lymphomas.

In our study, when the DNA samples of the patient group and control group were analyzed for the c.+3473A>G polymorphism of the *DICER1* gene, no individual carrying the GG genotype was found in the patient and control groups. When the study results were compared between healthy and patient groups with GA and AA genotypes, no statistically significant relationship could be established (p>0.05). The absence of a significant difference between the two groups suggested that different genetic mechanisms may also be involved in the formation of B-cell non-Hodgkin lymphoma. It was thought that the small number of the population participating in the study may be one of the reasons why no significant difference could be found between the results.

In our study, when the patient group and the healthy control group were analyzed for the presence of a mutation in the 11th exon of the *DICER1* gene, a heterozygote c.1827C>T [p.Asp609Asp]-rs150087634 - change was found in a patient with MALT lymphoma. As a result of this base change, the encoded amino acid (aspartic acid) does not change, so this mutation is silent. When the exon 25 region of the *DICER1* gene in the same patient and control group was examined for the presence of mutation, no mutation was found in the exon 25. Our findings were found to be similar to the results obtained by Lee et al. in hematological tumors.

In our study, when the patient and control groups were compared in terms of c.-871C>T polymorphism in the *BAFF* gene, no difference was found between the patient and control groups (p>0.05). Although the contribution of this polymorphism seen in the *BAFF* gene to the development of B cells and B-cell lymphoma was emphasized in previous studies, the c.-871C>T polymorphism in the *BAFF* gene in our study group was not statistically significant in the patient and control groups. The reason why this difference did not occur is thought to be due to the sample size of our study group.

In summary, the polymorphic regions of c.+3473A>G(rs3742330) in the *DICER1* gene and c.-871C>T(rs 9514828) in the *BAFF* gene were examined in patients with B-cell NHL and healthy control groups. There was no significant relationship between patients and subgroups of patients. When the exon 11 and exon 25 regions of the *DICER1* gene were investigated for the presence of mutations, no statistically significant relationship could be established between the patient and control groups (p>0.05). Conducting the study in a larger sample and adding the expression profiles of the genes in question will make the results more meaningful. In this context, the study is planned to be expanded and continued.

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Ethics Committee Approval: This study was approved by Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 18.04.2014, No: 08).

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

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EFFECT OF MEDICAL NUTRITION KNOWLEDGE ON GLYCEMIC REGULATION IN PATIENTS WITH DIABETES MELLITUS

DIABETES MELLITUS TANILI HASTALARDA TIBBİ BESLENME TEDAVİSİ HAKKINDAKİ BİLGİ DÜZEYLERİNİN GLİSEMİK REGÜLASYON ÜZERİNE ETKİSİ

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ABSTRACT

Objective: To evaluate the effect of medical nutrition knowledge on glycemic control in patients with Diabetes Mellitus (DM).

Material and Methods: Type 1 and Type 2 DM patients (n: 105) who had received medical nutrition therapy (MNT) education at least once were recruited. Nutritional knowledge scores (NKS) were obtained from the NKS evaluation form and patients were classified into Group 1 (NKS ≤60, n:24) and Group 2 (NKS>60, n:81). Patients' socio-demographic characteristics, biochemical parameters, nutritional habits, anthropometric measurements, and 24-hour food consumption data were recruited.

Results: Mean age in Group 1 and Group 2 was 50.5±12.1 and 45.5±15.5, respectively. Mean NKS scores were higher in patients with type 1 DM (p=0.02). There was no significant relation between NKS and HbA1c (p=0.3). NKS was significantly associated with higher educational degrees, higher HDL, and lower frequency of neuropathy (p=0.03; 0.01; 0.01, respectively). NKS was negatively correlated with age, triglyceride, and neuropathy frequency (p=0.001, r=-0.35, p=0.004, r=-0.36; p=0.01, r=-0.24, respectively); positively correlated with HDL, educational degree, health literacy and presence of leisure time activities (p<0.001, r=0.38; p<0.001, r=0.53; p=0.01, r=0.26 and p<0.001, r=0.31, respectively). Logistic regression analysis revealed the relationship between NKS and educational degree (p=0.01, OR:0.17, CI:0.04-0.68).

Conclusion: Adequacy of nutritional knowledge may not be sufficient in achieving better glycemic regulation and patients should be motivated to reflect their nutritional knowledge in their daily living activities.

Keywords: Diabetes mellitus, HbA1c, medical nutrition therapy, nutrition knowledge level

ÖZ

Amaç: Diabetes mellitus (DM) tanılı hastalarda tıbbi beslenme tedavisi ile ilgili bilgi düzeyinin glisemik kontrol üzerine etkilerinin araştırılması.

Gereç ve Yöntemler: Diyabet Polikliniğinde takip edilen ve en az bir kez tıbbi beslenme tedavisi (TBT) eğitimi almış olan DM tanılı hastalar (n:105) çalışmaya dahil edilmiştir. Hastalar; beslenme bilgisi değerlendirme formundan (BBDF) elde edilen beslenme bilgisi skorlarının (BBS) ≤60'ın altında ya da >60 üzerinde olmasına göre sırasıyla Grup 1 (n:24) ve Grup 2 (n:81) olarak sınıflandırıldıktan sonra; sosyodemografik özellikleri, biyokimyasal bulguları, beslenme alışkanlıkları, antropometrik ölçümleri, TBT bilgisi ve 24 saatlik besin tüketim kayıtları değerlendirilmiştir.

Bulgular: Grup 1 ve Grup 2'de yaş ortalaması sırasıyla 50,5±12,1 ve 45,5±15,5 izlendi. Tip 1 DM tanılı hastalarda BBS ortalamaları daha yüksek tespit edildi (p=0,02). BBS ile HbA1c ortalamaları arasında anlamlı bir ilişki izlenmedi (p=0,3). BBS düzeyi yüksek olan hastalarda eğitim düzeyi ve ortalama HDL düzeylerinin daha yüksek, nöropati sıklığının ise daha düşük olduğu görüldü (p değerleri sırasıyla; 0,03, 0,01, 0,01). BBS ile ortalama yaş ve trigliserid düzeyleri ve nöropati sıklığı (p=0,001, r=-0,35; p=0,004, r=-0,36; p=0,01, r= -0,24; sırasıyla); ortalama HDL düzeyleri, eğitim düzeyi, sağlık okur yazarlığı ve hobilerle ilgilenme arasında ise pozitif korelasyon (p<0,001, r=0,38; p<0,001, r=0,53; p=0,01, r=0,26 ve p<0,001, r=0,31; sırasıyla) izlendi. Lojistik regresyon analizinde BBS ile eğitim düzeyi arasında ilişki görüldü (p=0,01, OR:0,17, CI:0,04-0,68).

Sonuç: Diyabetli hastalarda glisemik regülasyonun iyileştirilmesinde tıbbi beslenme bilgisinin iyi olması tek başına yeterli olmayabileceğinden hastaların beslenme ile ilgili bilgilerini günlük yaşam aktivitelerine yansıtabilmeleri açısından motive edilmesi hedeflenmelidir.

Anahtar Kelimeler: Diabetes Mellitus, HbA1c, tıbbi beslenme tedavisi, beslenme bilgi düzeyi

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INTRODUCTION

Diabetes mellitus (DM) is a chronic condition with hyperglycemia that occurs due to the insufficiency, absence, or ineffectiveness of the insulin hormone secreted by beta cells in the pancreas. In addition to acute metabolic complications, the disease causes vascular, renal, retinal, or neuropathic changes in the long term (1). It is a common disease with high morbidity and early mortality risk representing a significant medical and economic burden on both the society and the individual (2). According to the 2021 data of the International Diabetes Federation (IDF); the total number of diabetic patients around the world was announced as 537 million people, representing 10.5% of the global adult population (aged 20–79). It was estimated that this number may rise to 643 million by 2030 (11.3% of the population) and 783 million (12.2%) by 2045 (3). It is predicted that by the year 2045, Turkey will be among the top 10 countries with the highest number of diabetics in the adult population (3). DM is among the top causes of death and is responsible for 6.7 million deaths in 2021(3). According to the results of the "Turkey Diabetes Epidemiology study (TURDEP-II)" in 2013, the prevalence of DM in adults over the age of 20 was %13,7 translating to 6.5 million adults with DM indicating that the 2030 expectations regarding diabetes prevalence had already been exceeded (4).

Many risk factors affect the emergence of diabetes. While age, genetics, and race are non-modifiable risk factors; being overweight or obese, unhealthy dietary habits, smoking, and insufficient physical activity are modifiable risk factors. Additionally, a significant inverse relationship between educational level and the prevalence of diabetes especially among women has already been proposed (4). By modification of these risk factors, the management of diabetes can be improved, and diabetes complications may be reduced (3). It has been shown that lifestyle changes such as weight control, diet, and exercise may be beneficial for diabetes management (5,6). Medical nutrition therapy is the most important part of diabetes treatment and diabetes management, and it is recommended that individuals with diabetes be directed to a dietitian as soon as possible after diagnosis (7). According to the definition by the American Academy of Nutrition and Dietetics, medical nutrition therapy (MNT) is the provision of one or more of the stages of nutritional evaluation and intervention, resulting in the prevention, delay, or optimal of diseases (8).

Nutrition therapy aims to provide necessary information for self-management of individuals with diabetes, to develop self-management skills, solutions for existing problems, and as a result, to provide metabolic control and improve quality of life (9). HbA1c is one of the most important criteria in the diagnosis of diabetes. It reflects the average glucose value of diabetic patients in the last three months and indicates the risk of developing complications (10). In a study conducted in England, it was determined that a 1% decrease in the HbA1c values of diabetic individuals resulted in a 37% reduction in diabetes-related microvascular complications and a 21% reduction in mortality (11). Strong evidence supports the effectiveness of MNT interventions provided by RDNs for improving A1C, with absolute decreases up to 2.0% (in type 2 diabetes) and up to 1.9% (in type 1 diabetes) at 3-6 months (12). Various acute and chronic complications of diabetes can be prevented or delayed by controlling hyperglycemia (3,14,15). For this reason, to ensure glycemic regulation and prevent chronic complications of diabetes, having adequate knowledge of medical nutrition therapy and trying to eliminate potential information gaps by periodically reviewing medical nutrition training plays a very important role in management of DM. In this study, we aimed to evaluate the effect of medical nutrition knowledge on glycemic control in patients with DM.

MATERIALS and METHODS

This study was conducted in patients diagnosed with Type 1 and Type 2 diabetes mellitus (DM) between the ages of 18-65, who were being followed up in the Endocrinology and Diabetes Outpatient Clinic of Istanbul Medical Faculty between January and June 2022. A total of 105 DM patients (Type 1 DM: 33, Type 2 DM: 72) who had received medical nutrition therapy education at least once and who agreed to participate in the study were recruited.

Data collection tools

Interviewer-administered questionnaires were used to collect the socio-demographic characteristics of the patients, diabetes-related information, nutritional habits, 24-hour food consumption records, and nutritional information evaluation forms. Anthropometric measurements and body compositions of the patients were measured with the TANITA BC-418 MA analyzer. Patients were classified into two groups according to their nutritional knowledge scores (NKS) which they obtained from the nutritional knowledge evaluation form (NAKED) (13). Patients with scores below ≤60 or above >60 points were classified as Group 1 (n:24) and Group 2 (n:81), respectively. Socio-demographic characteristics, HbA1c, LDL, Triglyceride, HDL, LDL levels, frequency of hypoglycemia, retinopathy, nephropathy, neuropathy, diabetic foot ulcer, hypertension, and presence of cardiovascular disease were analysed through retrospective data. Blood glucose measurement data, HbA1c, LDL, HDL, and triglyceride levels were obtained from the registry records retrospectively during the last routine outpatient clinical control visit. Data on nutritional habits were obtained from the patient evaluation forms, 24-hour food consumption records, and three-day food consumption questionnaires which were completed by the patients. The patients' total daily energy intake, carbohydrate, protein, fat ratios, and nutritional components were calculated using the Nutrition Information System (BeBIS) 7.2 program.

This study was approved by the Clinical Research Ethics Committee of Istanbul Faculty of Medicine (Date: 24.12.2021, No: 23).

Statistical analysis

Data entry and analysis were performed by using SPSS version 23. Descriptive statistics, including frequency, percentages,

Table 1: Demographical and clinical features of the patients according to the nutritional knowledge scores

	Nutritional knowledge Score ≤60 (n:24)	Nutritional knowledge Score >60 (n:81)	p
Nutritional knowledge score	54.4±6.1	76.2±8.8	**0.001**
Gender			
Female (n)	11	44	0.3
Male (n)	13	37	
Age (mean±SD)	50.5±12.1	45.5±15.5	0.1
BMI (mean±SD)	28.1±5.2	27.0±5.1	0.3
DM Type			
Type 1 (n)	3	30	**0.02**
Type 2 (n)	21	51	
Educational status			
Elementary school (n)	14	22	
High school (n)	8	31	**0.03**
University (n)	2	28	
Family history of DM			
Absent (n)	8	21	0.6
Present (n)	16	60	
Diabetes duration (Year)	12.7±8.3	11.8±7.9	0.6
Marital status			
Single (n)	8	27	0.6
Married/Partnership (n)	16	54	
Occupational status			
Employed/Student (n)	6	25	0.4
Unemployed/Retired (n)	18	56	
Working Hours			
<8 hours	3	8	0.1
≥8 hours	3	17	
Smoking			
Absent (n)	17	59	0.3
Present (n)	7	22	
Alcohol			
Absent (n)	2	15	0.3
Present (n)	22	66	
Who do you live with	4	6	
Living alone (n)	20	75	0.4
Living with partner/family (n)			
Socioeconomic status			
Middle income (n)	20	75	0.2
Low income (n)	4	6	
Diagnosis of depression			
Absent	20	77	0.2
Present	4	4	
Regular physical activity			
Absent (n)	13	42	0.6
Present (n)	11	39	
Leisure time activity			
Absent	14	33	0.3
Present	10	48	
Health literacy			
Absent (n)	14	40	0.40
Present (n)	10	41	

Retinopathy			
Absent (n)	11	54	0.1
Present (n)	13	27	
Nephropathy			
Absent (n)	20	71	0.3
Present (n)	4	10	
Neuropathy			
Absent (n)	9	53	**0.01**
Present (n)	15	28	
Foot ulcers			
Absent (n)	18	69	0.3
Present (n)	6	12	
Hypertension			
Absent (n)	16	58	0.2
Present (n)	8	23	
Cardiovascular disease			
Absent (n)	16	55	0.2
Present (n)	8	26	
Dyslipidemia			
Absent (n)	13	48	0.3
Present (n)	11	33	
DM treatment			
OAD (n)	5	18	0.4
Insulin (n)	7	37	
OAD+ Insulin (n)	12	26	
Frequency of SMBG			
Irregular (Less than once a month) (n)	8	22	0.8
Once-twice a week (n)	3	12	
Once- twice a day (n)	13	47	
Frequency of hypoglycemia in the last month			
None (n)	15	37	
1-3 times (n)	8	34	
≥4 times (n)	1	10	0.3
Hospitalisation in previous year			
Absent	20	74	0.4
Present	4	7	
Regular sleeping schedule			
Absent	4	21	0.3
Present	20	60	
Unhealthy nutritional habits			
Skipping main meals (n)	7	15	0.4
Skipping snacks (n)	6	32	0.6
Nocturnal eating (n)	5	12	0.2
Fast food consumption (n)	12	46	0.6
Emotional eating (n)	8	42	0.2
HbA1c (mean±SD)	7.9±1.8	8.4±1.9	0.3
LDL (mean±SD)	113.0±39.8	97.6±43.3	0.1
HDL (mean±SD)	42.5±14	52.6±16.3	**0.01**
Triglyceride (mean±SD)	169.4±130.8	144±89.4	0.2

SD: Standard deviation, BMI: Body Mass Index, DM: Diabetes mellitus, OAD: Oral antidiabetic drugs, SMBG: Self-monitoring of blood glucose, LDL: Low density lipoprotein, HDL: High density lipoprotein, n: Number of participants, p significance <0.05

mean, and median values were used to evaluate the distribution of data. Pearson correlation analysis method was used to evaluate correlations between parameters. Student t-test was used to evaluate the normally distributed variables in the comparative statistical analysis, and the chi-square test was used to compare the categorical variables. Logistic regression analysis was performed in the evaluation of the effect of independent variables. The results were accepted as statistically significant when p level was <0.05.

RESULTS

The mean age for the patients in Group 1 and Group 2 was 50.5±12.1 and 45.5±15.5, respectively. Mean Nutritional Know-

Table 2: Correlation analysis of clinical features based on nutritional knowledge score

	p	r
Age	**0.001**	**-0.35**
BMI	0.3	-0.1
Diabetes duration	0.1	0.3
HbA1c	0.3	-0.14
LDL	0.2	0.11
HDL	**0.001**	**0.38**
Triglyceride	**0.004**	**-0.36**
Educational status	**0.001**	**0.53**
Frequency of SMBG	**0.6**	**0.23**
Health literacy	**0.01**	**0.26**
Leisure Time Activities	**0.001**	**0.31**
Regular physical activity	0.1	0.22
Regular sleeping schedule	0.8	0.02
Diagnosis of depression anxiety	0.06	0.18
Unhealthy nutritional habits	0.2	0.12
Hospitalization in previous year	0.8	-0.02
Retinopathy	0.6	0.21
Nephropathy	0.1	0.05
Neuropathy	**0.01**	**-0.24**
Socioeconomic status	0.2	0.13
Working hours	0.3	0.04
Diabetes management education in previous year	0.6	0.04

BMI: Body Mass Index, LDL: Low density lipoprotein, HDL: High density lipoprotein, SMBG: Self-monitoring of blood glucose, p significance <0.05 , r: Correlation coefficient

Table 3: Logistic regression analysis of the factors affecting nutrition knowledge score

	p	OR	CI
DM type	0.06	7.2	0.89-38
Educational status	**0.01**	**0.17**	**0.04-0.68**
Age	0.1	0.3	0.06-1.5
Health literacy	0.9	1.0	0.35-3.0
Leisure time activities	0.8	0.9	0.32-3.0
Frequency of SMBG	0.3	0.46	0.1-1.7
Neuropathy	0.7	0.38	0.13-1.0

DM: Diabetes mellitus, SMBG: Self-monitoring of blood glucose, OR: Odds ratios, 95% CI: Confidence intervals, p significance <0.05.

ledge Scores were higher in patients with type 1 DM (p=0.02). NKS levels were significantly associated with higher educational degrees, higher HDL levels, and lower frequency of neuropathy (p=0.03, 0.01, 0.01, respectively). Demographic and clinical features of the patients according to the nutritional knowledge scores (NKS) are demonstrated in Table 1. NHS levels were negatively correlated with age, triglyceride levels, and frequency of neuropathy (p=0.001, r=-0.35, p=0.004, r=-0.36, p=0.01, r=-0.24, respectively); and positively correlated with HDL, educational degree, health literacy and presence of leisure time hobbies (p<0.001, r=0.38, p<0.001, r=0.53, p=0.01, r=0.26 and p<0.001, r=0.31, respectively) Correlation analysis of

Table 4: Correlation analysis of clinical features based on HbA1c Levels

	p	r
Age	0.4	-0.1
BMI	0.8	0.02
Nutritional knowledge score	0.6	-0.6
Diabetes duration	0.6	0.05
Working hours	**0.01**	**0.27**
Educational status	0.1	-0.25
Socioeconomic status	0.1	-0.21
Frequency of SMBG	0.4	-0.1
Regular sleeping schedule	0.4	-0.1
Diagnosis of depression anxiety	0.1	0.15
Health literacy	0.2	-0.1
Regular physical activity	**0.03**	**-0.22**
Unhealthy nutritional habits	**0.02**	**0.23**
Presence of diabetes management education in the previous year	**0.003**	**-0.3**
Retinopathy	**0.001**	**0.32**
Nephropathy	**0.004**	**0.27**
Neuropathy	**0.02**	**0.22**
LDL	0.2	0.1
HDL	0.4	-0.1
Triglyceride	0.2	0.13

BMI: Body Mass Index, SMBG: Self-monitoring of blood glucose, LDL: Low density lipoprotein, HDL: High density lipoprotein, p significance <0.05, r: Correlation coefficient.

Table 5: Logistic regression analysis of the factors affecting HbA1c levels

	p	OR	CI
Regular physical activity	**0.01**	**0.3**	**0.12-0.73**
Unhealthy nutritional habits	**0.4**	**0.7**	**0.31-1.01**
Presence of diabetes management education in previous year	**0.03**	**0.2**	**0.03-0.88**
Educational status	**0.5**	**0.7**	**0.4-1.3**
Socioeconomic status	**0.7**	**0.2**	**0.05-1.12**
Hospitalizations in previous year	**0.6**	**0.9**	**0.3-2.1**

OR: Odds ratios, 95%, CI: Confidence intervals, p significance <0.05.

clinical features based on NKS is revealed in Table2. Logistic regression analysis revealed that the strongest factor affecting the NHS was educational degree (p=0.01; OR:0.17; CI:0.04-0.68) (Table 3). Correlation analysis of clinical features based on HbA1c levels and logistic regression analysis of the factors affecting Hba1c levels are demonstrated in Table 4 and Table 5, respectively.

DISCUSSION

The most important aspect of treating and managing diabetes is medical nutrition therapy (16). Type 2 diabetes constitutes 90-95% of diabetes cases in the world and Turkey (17). In line with these facts, we investigated the effect of NKS on glycemic regulation in our patient group which consisted of 68% type 2 DM patients with a diabetes duration of 1-10 years in 52% of the study group. It is known that there is a strong relationship between family history and diabetes risk (17-19). It was seen that 72.4% of the patients in this study had diabetes in their families.

In 2020, 3.2 million diabetics (approximately 1% of the total diabetic population) were reported to have moderate or severe visual impairment due to diabetic retinopathy (20). As the duration of diabetes increases, the frequency and degree of retinopathy increase. In a multicenter study, the frequency of diabetic retinopathy was found to be 20% (21). Diabetic neuropathy is the most common form resulting in pain, poor quality of life, gait disturbances, and depressive symptoms in approximately 30% of people with type 2 diabetes (22,23). Studies are reporting the prevalence of neuropathy in DM is between 5% and 60% (24). Clinical and subclinical diabetic neuropathy can be seen in 10% of diabetic patients (25). In a study conducted in Izmir, 34.8% of the most common complications were neuropathy, and 28.1% were retinopathy (26). In our study, the relationship between diabetic complications and NKS was only significant for the presence of diabetic neuropathy. The presence of neuropathy is inclined to be less frequent in patients with higher NKS. However, the association was no longer statistically significant after adjustment with the other confounding factors in regression analysis. The most effective factor in NKS was found to be the educational degree of the patients. The lack of association between NKS and glycemic regulation indices such as HbA1c and presence of diabetic complications may be interpreted as that patients might not be regulating their nutritional habits in line with their level of nutrional knowledge.

Studies have also reported controversial results about the effect of lifestyle changes and regular physical activity on better glycemic control and improved triglyceride levels in individuals with DM (27-29). In this study, as the NKS of the patients increased, HDL levels tenden to be higher and triglyceride levels were lower. Although HbA1c levels were not significantly affected with better NKS degree of dyslipidemia tended to be positively affected in our study. The NKS of the patients in our study was positively correlated with their educational levels and negatively correlated with age. This may be interpreted as that elderly patients might need more intensive and more frequent repetition in diabetes education. In the study of Ozkarabulut et al, there was a significant relationship between the NKS and employment status, and income levels of the patients (30). In this study, NKS was not significantly associated with occupational status or income levels. This might be related to the similar employment status and socioeconomic levels in the study population. However, the most influential factor on NKS was found to be educational status which may be interpreted as diabetes education should be emphasized and reviewed more frequently in people with lower educational degrees to ensure better NKS levels.

Tulek et al demonstrated a significant correlation between NKS and HbA1c levels, where patients with higher NKS had lower HbA1c levels (31). However, in this study, there was no significant correlation between NKS and HbA1c levels. The controversial findings might be related to the content and frequency of the diabetes education program. Patients in this study were selected randomly from the outpatient clinic who had received medical nutrition therapy education at least once in the previous year. However, the patient population in Tulek et al.'s study consisted of patients who had actively participated and completed a diabetes patient school (31). It might be possible that a single diabetes education session has a limited impact on HbA1c levels and periodical repetition of diabetes education may be more helpful to ensure patients' comprehension in building healthy lifestyle habits that would restore better glycemic regulation.

In this study, there was no significant relationship between the patient's HbA1c and educational status, employment status, and income levels which was also compatible with previous findings in the literature (32). However, in our study, HbA1c levels were significantly correlated with regular physical activity which was also previously reported (33). In our study, the most influential factors on HbA1c levels were regular physical activity and the presence of diabetes management education in the previous year. Although NKS is not significantly effective on HbA1c levels, rehearsal of diabetes education once a year might improve glycemic regulation and HbA1c levels. This might particularly be valid in elderly patients or patients with lower educational status who have lower NKS.

The limitation of this study is that the number of the patient population was limited, and the reproducibility of these results may be investigated through larger-scale studies.

In conclusion, our study has shown that adequate levels of nutritional knowledge are not sufficient to maintain good glycemic regulation in patients with DM. Patients probably experience difficulties in applying their theoretical knowledge into their daily life practices. Therefore, it may be beneficial if the clinicians would pay special attention to motivating DM patients to build regular physical activity and healthy nutritional habits, reflecting their nutritional knowledge in daily living activities to improve glycemic regulation and diabetes management.

Ethics Committee Approval: This study was approved by the Clinical Research Ethics Committee of Istanbul Faculty of Medicine (Date: 24.12.2021, No: 23).

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DIRECTED EVOLUTION OF AN ONCOLYTIC VESICULAR STOMATITIS VIRUS ADAPTED TO HUMAN MALIGNANT MENINGIOMA CELLS

İNSAN MALIGANT MENİNJİOM HÜCRELERİNE UYUMLANMIŞ BİR ONKOLİTİK VEZİKÜLER STOMATİT VİRÜSÜNÜN YÖNLENDİRİLMİŞ EVRİMİ

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ABSTRACT

Objectives: Recombinantly-engineered versions of the oncolytic virus VSV are currently under clinical investigation for the treatment of several different types of cancer. Here we aim to enhance the cancer-killing oncolytic phenotype of VSV-1'GFP toward human malignant meningioma cells using a directed evolution approach.

Material and Methods: Two independent trials of repeated growth of VSV-1'GFP on cultures of meningioma IOMM-Lee cells were performed. This adaptation procedure allows for the selection of viral mutants that display an enhanced oncolytic phenotype. A fluorescent viral plaque assay was used to measure changes in plaque size indicative of enhanced viral growth on these cancer cells. Sanger sequencing was used to identify the viral mutations responsible.

Results: Adapted VSV-1'GFP from each of the growth trials yielded larger fluorescent plaques than control virus, indicating the emergence of viral mutants with increased growth on these meningioma cells. Plaques from adapted virus were 184%±9% (Trial 1) and 166%±7% (Trial 2) larger than control (n=60; p<0.001; ANOVA). Sequencing determined that adapted virus from Trial 1 harbored 3 mutations: a silent mutation Y178Y in the M gene, an E92K mutation in the G gene, and a K152R mutation in the L gene. Trial 2 yielded 3 mutations in the G gene: N36T, E92K, and E254K.

Conclusion: The E92K mutation of the viral G-protein emerged independently in both growth trials, suggesting that this change may play a role in producing the enlarged-plaque phenotype and enhanced oncolytic propagation in IOMM-Lee cells. Further investigations of the prospect for treating malignant meningiomas using VSV-based oncolytic virotherapy appear warranted and, to the best of our knowledge, the present study appears to be the first directed evolution experiment involving an oncolytic virus adapted to human meningioma cells.

Keywords: Vesicular stomatitis virus, oncolytic virus, meningioma, mutagenesis

ÖZ

Amaç: Onkolitik virüs VSV'nin rekombinant olarak tasarlanmış çeşitleri birkaç farklı kanser türünün tedavisi için klinik olarak araştırılmaktadır. Bu çalışmada, yönlendirilmiş evrim yaklaşımı kullanarak VSV-1'GFP' nin insan malign meninjiyom hücrelerine yönelik kanser öldürücü onkolitik fenotipini geliştirmek hedeflenmektedir.

Gereç ve Yöntemler: Meninjiyom IOMM-Lee hücrelerinin kültürleri üzerinde VSV-1'GFP'nin tekrarlanan büyümesine ilişkin iki bağımsız deneme gerçekleştirildi. Bu adaptasyon prosedürü, gelişmiş bir onkolitik fenotip gösteren viral mutantların seçimine olanak tanır. Bu kanser hücrelerinde viral büyümenin arttığını gösteren plak boyutundaki değişiklikleri ölçmek için bir floresan viral plak tahlili kullanıldı. Sorumlu viral mutasyonları tanımlamak için Sanger dizilimi kullanıldı.

Bulgular: Büyüme denemelerinin her birinden uyarlanmış VSV-1'GFP, kontrol virüsünden daha büyük floresan plaklar verdi; bu, meninjiyom hücrelerinde artan büyüme ile viral mutantların ortaya çıktığını gösterir. Uyarlanmış virüsten alınan plaklar kontrolden (n=60; p<0,001; ANOVA) %184±%9 (Deneme 1) ve %166±%7 (Deneme 2) daha büyüktü. Dizileme, Deneme 1'den uyarlanan virüsün 3 mutasyon barındırdığını belirledi: M geninde sessiz bir Y178Y mutasyonu, G geninde bir E92K mutasyonu ve L geninde bir K152R mutasyonu. Deneme 2, G geninde 3 mutasyon ortaya çıkardı: N36T, E92K ve E254K.

Sonuç: Viral G-proteininin E92K mutasyonu, her iki büyüme denemesinde de bağımsız olarak ortaya çıktı; bu değişiklik, IOMM-Lee hücrelerinde genişlemiş plak fenotipinin ve gelişmiş onkolitik yayılımın üretilmesinde rol oynayabileceğini düşündürmektedir. Malign meninjiyomların VSV bazlı onkolitik viroterapi kullanılarak tedavi edilmesi olasılığına ilişkin daha fazla araştırma gerekli görülmektedir ve bilgimiz dâhilinde, mevcut çalışma, insan meninjiyom hücrelerine uyarlanmış bir onkolitik virüsü içeren ilk yönlendirilmiş evrim deneyi olarak literatüre katkı sunmaktadır.

Anahtar Kelimeler: Veziküler stomatit virüs, onkolitik virus, meninjiom, mutagenez

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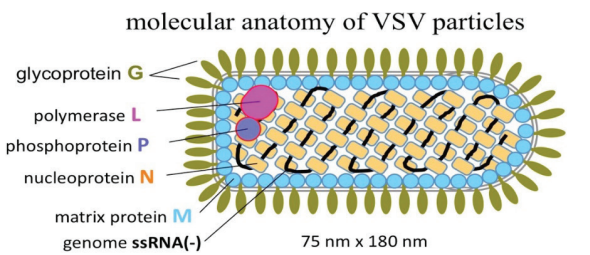
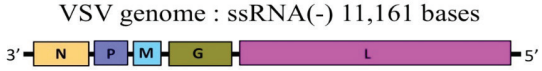
INTRODUCTION

Oncolytic viruses are a heterogenous collection of recombinant viruses that have been engineered to selectively target, infect, and ultimately kill cancer cells; hence the term oncolytic (onco=cancer; lytic=lysis) that is commonly used to describe them (1, 2). Several different oncolytic viruses are currently under investigation for clinical application in the treatment of cancer (1, 2). In these experimental oncolytic virotherapy treatment regimens, the virus is typically injected directly into the solid tumor mass, where it initially infects a small fraction of tumor cells. Over the course of several hours, the virus replicates and new daughter viruses are released from these infected cells to continue further cycles of viral infection and replication throughout the tumor mass. Since the virus seizes control of all the intracellular translation machinery to make more copies of itself, infected cancer cells ultimately die (oncolysis). Recombinantly-engineered poliovirus, measles, and herpes simplex viruses have all been the focus of clinical trials aimed at examining the safety and efficacy of these agents in targeting and eliminating glioblastoma, multiple myeloma, and melanoma, in addition to several other types of cancer (3-5). In 2015, the Food and Drug Administration (FDA) of the United States approved the first oncolytic virus, talimogene laherparepvec (T-Vec), for use in the treatment of inoperable late-stage melanoma (6). Another oncolytic virus with therapeutic prospects that has been the focus of much interest is vesicular stomatitis virus (VSV) (7, 8). VSV is an enveloped, non-segmented ssRNA(-) virus from the family *Rhabdoviridae* with human infections generally presenting as either asymptomatic or with mild flu-like symptoms (Figure 1) (9). To date, a wide assortment of recombinant VSVs have been generated and tested for their oncolytic properties *in vitro*, with several showing therapeutic potential *in vivo* using both immune-compromised animals implanted with human tumors and immune-competent (syngeneic) animal tumor models (10-14). Efforts have also been made to exploit the highly-mutagenic nature of this class of ssRNA(-) virus by adapting recombinant oncolytic VSVs for growth on cultures of specific types of cancer cells, e.g. pancreatic cancer, breast cancer, and glioblastoma cells (15-18). These directed evolution strategies of viral mutagenesis aim to select mutant cancer-specific variants of VSV that exhibit an enhanced oncolytic phenotype targeting the cancer of interest.



VSV genome : ssRNA(-) 11,161 bases



gene	length (bases)	protein (a.a.)	protein (kDa)
N	1269	422	47.4
P	798	265	29.9
M	690	229	26.0
G	1536	511	57.5
L	6330	2109	241.0

Figure 1: Molecular characteristics of vesicular stomatitis virus (VSV). Illustration showing molecular anatomy of VSV particles (top) and organization of VSV genome (below)

Here we describe a directed evolution experiment utilizing the recombinant oncolytic virus VSV-1'GFP to target cultures of the immortalized human malignant meningioma cell line IOMM-Lee. Meningiomas are the most frequently encountered type of primary brain tumor and although the majority of these tumors are benign, a small subset is highly-aggressive and result in significant morbidity (19, 20). Several genes have been identified as playing a role in meningioma oncogenesis and include *NF2, TRAF7, KLF4, AKT1*, among others (21, 22). The IOMM-Lee cell line utilized in the present study was initially established from a freshly-resected intraosseous malignant meningioma of the skull taken from a 61-year old male (23, 24). Multiple passages of VSV-1'GFP on cultures of these cells resulted in the emergence of an enhanced oncolytic phenotype accompanied by several mutations of the viral genome that were identified through Sanger sequencing.

MATERIALS and METHODS

Cell cultures and recombinant virus

The human malignant meningioma IOMM-Lee cell line utilized in the present study was a kind gift from the laboratory of M. Gunel (Yale University School of Medicine). Cells were cultured using Dulbecco's Modified Eagle Medium cat no. 11965-092 (Gibco/ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% FBS cat no. 16000-044 (Gibco) and 1% Pen-Strep solution cat no. 15140-122 (Gibco) (23-25). Cultures were maintained in an incubator with a humidified atmosphere at 37° C supplemented with 5% CO_2.

The recombinant oncolytic virus VSV-1'GFP was a kind gift from the laboratory of S. Whelan (Washington University School of Medicine in St. Louis). This virus was engineered to expresses green fluorescent protein (GFP) from the first (1') genomic position. Virus was grown and harvested as described previously (26, 27).

Repeated passage experiments

The day before infection, IOMM-Lee cells were seeded into 35 mm dishes or wells at a density of 5.0 x 10^5. The following day, nearly confluent cultures were inoculated with virus and incubated 1 hr to allow time for viral adsorption and infection. After incubation, the inoculum was removed, the cells were washed with PBS, and fresh medium was added before returning the cultures to the incubator. After 24 hrs, cultures were observed under fluorescent microscopy and a sample of media containing newly generated viral progeny was drawn and used as inoculum to infect a fresh set of cultures. Remaining medium was harvested and stored at -80 °C for later analysis. This repeated passage procedure was continued for 22-24 cycles of infection.

The initial VSV-1'GFP inoculum used to infect the first culture (p0) consisted of a 15 µL volume containing $2.4x10^6$ infectious virions. In subsequent passages, the volume of viral inoculum was varied in response to the observed viral propagation of the previous passage.

Plaque-size assay and fluorescent imaging

VSV-1'GFP expresses GFP as a reporter protein, thus, fluorescent imaging was used to determine the size of viral plaques that develop on infected cell monolayers in a plaque assay (12, 28). Briefly, confluent monolayers of IOMM-Lee cells grown in 6-well plates were inoculated using 1 mL volumes of serially-diluted virus and allowed to incubate at 37 °C for one hour. After adsorption, the viral inoculum in each well was aspirated and cell monolayers were overlaid with 2 ml of 0.5% (wt/vol) Ultrapure GPG/LE agarose cat no. AB00972 (American Bioanalytical, Natick, MA, USA) that had been melted and mixed with growth medium. After 3-5 min of solidification, the overlaid plates were returned to the 37 °C incubator for 24 hrs to allow time for viral plaque development.

Visualization of fluorescent plaques and infected cell monolayers was performed using an inverted IX71 fluorescent microscope system (Olympus, Tokyo, Japan) fitted with a GFP filter set. Images were captured using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and further processed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA). Individual fluorescent plaques were measured across their diameter directly from a computer monitor and 60 plaques were randomly measured from each experimental condition to determine the mean plaque-size of the population.

Viral genome sequencing

Viral genomic RNA for sequencing was isolated from the culture medium of infected cells using the QIAamp Viral RNA Mini Kit cat no. 52904 (QIAGEN, Germantown, MD, USA). This RNA was then used as template for generating cDNAs in a reverse-transcription reaction with SMARTScribe Reverse Transciptase cat no. 639537 (Takara Bio, San Jose, CA, USA). The oligos AF00001 and DF04742 were used in these reactions (RT1, RT2). Viral genomic cDNAs were then used to generate a series of seven overlapping PCR products (A, B, C, D, E, F, and G), covering the length of the VSV-1'GFP genome. These PCR products ranged in size from 1.5 – 2.3 kb and were generated using the Phusion High-Fidelity PCR Master Mix kit cat no. M0531S (New England Biolabs, Ipswich, MA, USA). The annealing temperatures used for Phusion High-Fidelity PCR were calculated using the online NEB Tm Calculator at the New England Biolabs website (tmcalculator.neb.com). After amplification, PCR products were purified using the QIAquick PCR Purification kit cat no. 28104 (QIAGEN), mixed with sequencing primer, then submitted to the Keck DNA Sequencing Facility (Yale University School of Medicine, New Haven, CT, USA) for automated DNA sequencing. All oligos were synthesized by IDT (Integrated DNA Technologies, Inc., Research Triangle Park, NC, USA) . The numbering associated with each oligo is indicative of the nucleotide position in the original recombinant VSV genome (29). Recombinant VSV reference sequence used in the design of oligos and primers was obtained from plasmid # 31833 available on the Addgene website (www.addgene.org). VSV glycoprotein sequence of the wild-type Orsay strain of Indiana serotype VSV was obtained from GenBank Accession: M11048 (30). Initial sequencing of the G gene of the founder stock of VSV-1'GFP immediately after arrival in the lab found that there are five variations in the founder sequence relative to the GenBank M11048 sequence, i.e. c.3918A>G, (p.Gln26Arg); c.4115A>G, (p.Lys92Glu); c.4440C>T, (p.Thr200Met); c.5022T>C, (p.Leu394Ser) and c.5247T>G, (p.Phe469Cys). At this time, it is unclear whether these variations were present in the original VSV-1'GFP plasmid construct used to generate the virus or emerged later during standard harvesting and maintenance of viral stocks.

Statistical analysis

Fluorescent plaque-size data were compared using a one-way Analysis of Variance statistical model (ANOVA), followed by post-hoc analysis (Bonferroni's test) and were computed using the InStat version 3.0b software package (GraphPad Software, Boston, MA, USA). P-values <0.05 were considered significant.

RESULTS

Repeated passage of virus using IOMM-Lee cells

Cultures of human malignant meningioma IOMM-Lee cells were inoculated with VSV-1'GFP and incubated for 24 hrs to allow time for viral propagation (Figure 2). Since VSV-1'GFP expresses green fluorescent protein (GFP) as a reporter molecule, the extent of viral infection in IOMM-Lee cultures was easily monitored using fluorescent microscopy. After 24 hrs, a sample of inoculum containing newly generated viral progeny was collected and used to infect fresh IOMM-Lee cultures (Figure 2). Two independent trials (performed serially) of this repeated passage procedure were conducted with Trial 1 consisting of 24 passages and Trial 2 consisting of 22. During the

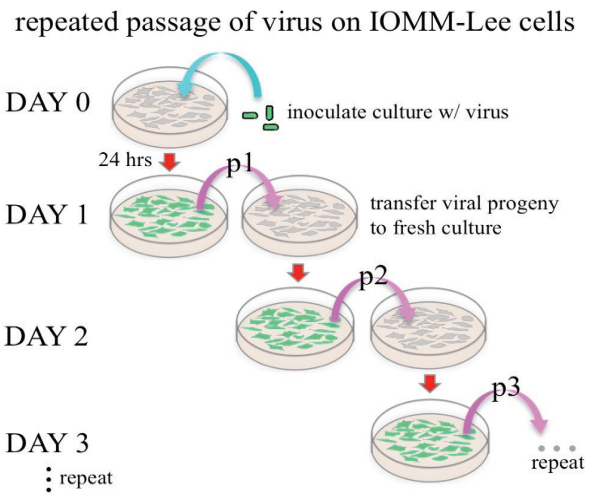


Figure 2: Directed evolution of VSV-1'GFP by repeated passage. Diagram depicting inoculation of IOMM-Lee cells with virus and transfer of newly generated viral progeny to fresh cultures after 24 hrs

course of these trials, robustly infected cultures prompted the use of smaller volumes of inoculum (one-half to one-third) in the next subsequent passage (Figure 3, left). Likewise, weakly infected cultures prompted the use of greater volumes of inoculum (Figure 3, center). The rationale behind this strategy was aimed at maximizing the potential number of cycles of viral replication per passage. A culture that becomes fully infected within the first few hours after inoculation leaves few remaining host cells available for infection and further cycles of viral replication. Thus, an oversupply of infectious virions at the outset of a passage might be expected to slow the emergence of new mutations and their fixation within the viral population.

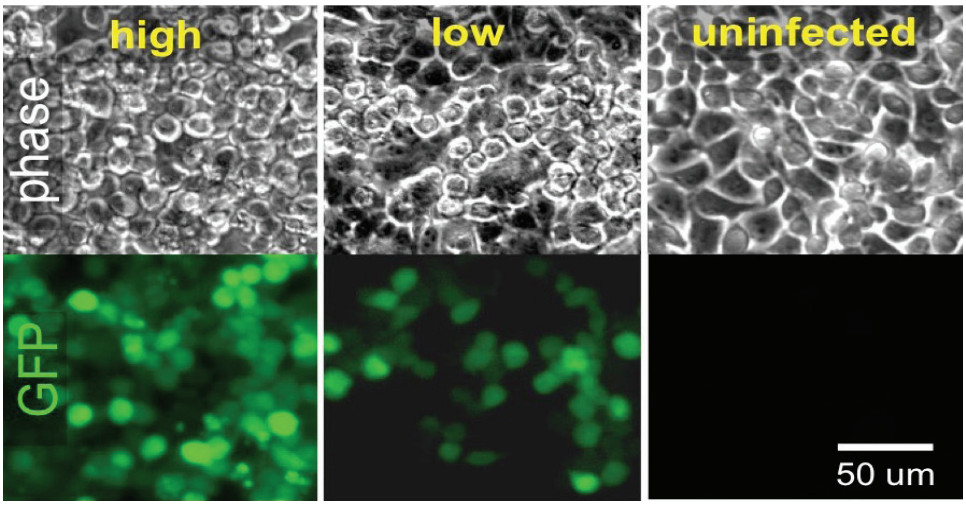


Figure 3: Infection of IOMM-Lee cells with VSV-1'GFP. Phase-contrast (above) and fluorescent images (below) of infected cultures. Highly infected culture (left) displays numerous rounded cells due to cellular cytopathic effects (CPE) of infection. Less infected culture (center) displays fewer CPE and fewer GFP positive cells. Uninfected culture (right) included as control

Altered phenotype: Viral plaque-size

The application of a semi-solid agarose overlay restricts the diffusion of newly generated viral progeny to an area immediately surrounding the initial infected host cell and, over the course of hours, results in the development of a viral plaque or circular-shaped area of robustly infected cells (28). Using confluent cultures of IOMM-Lee cells, we compared the size of fluorescent viral plaques that developed after infection with early- (p1) and late- (p24 and p22) passage viral inoculum harvested from both repeated passage trials (Figure 4). In both Trial 1 and Trial 2, late-passage virus yielded larger fluorescent plaques than early-passage virus; late-passage plaques were 184% ± 9% and 166% ± 7% larger, respectively (n=60; $p<0.001$; ANOVA).

Altered genotype: VSV-1'GFP mutations

Next we decided to perform whole viral genome sequencing to determine what mutations had emerged in VSV-1'GFP that might account for the enlarged fluorescent plaque phenotype (Figure 5). Viral genomic RNA was extracted from both early- (p1) and late- (p24 and p22) passage virus from both trials and reverse-transcribed into two overlapping single-stranded cDNAs. The first cDNA (RT 1) included the GFP reporter gene and the viral N, P, M, and G genes. The second cDNA (RT 2) contained the viral polymerase L gene. RT 1 cDNA was then used as template to generate 3 overlapping PCR products (A, B, C) spanning the length of RT 1; RT 2 was used as template for 4 PCR products (D, E, F, G). Sanger sequencing was then used to sequence all PCR products. After assembly and inspection of the sequencing chromatograms, several mutations were identified in the genomes of the late-passage virus from both trials (Figure 6). Late-passage virus (p24) in Trial 1 harbored 3 mutations: a silent mutation Y178Y in the M gene and two amino acid altering mutations, E92K in the G gene and K152R in the L gene. Trial 2 late-passage virus (p22) also harbored 3 mutations that all altered amino acids within the G gene: N36T, E92K, and E254K. Sequences of the early-passage (p1) virus from both trials matched each other and pre-existing sequence of the original founder stocks. Interestingly, some regions of the late-passage genomic sequence from both trials appeared to harbor what may be additional mutations that have not yet become genetically fixed in the viral population (e.g. Figure 6 p22 in K152R column). However, it is unclear from the sequencing chromatograms alone what fraction of the virions in the population may possess these types of alterations.

DISCUSSION

A common feature among RNA viruses is their ability to mutate very quickly (31). This high frequency of mutagenesis is understood to arise from a lack of proof-reading activity by the virally-encoded RNA polymerases responsible for replicating the viral genome (32, 33). For VSV, this RNA polymerase is encoded by the L gene and has been estimated to yield mutation rates as high as one mutation per genome per replication cycle (34, 35). Additionally, measurements of recombinant VSV growth kinetics *in vitro* indicate that the time between initial cellular

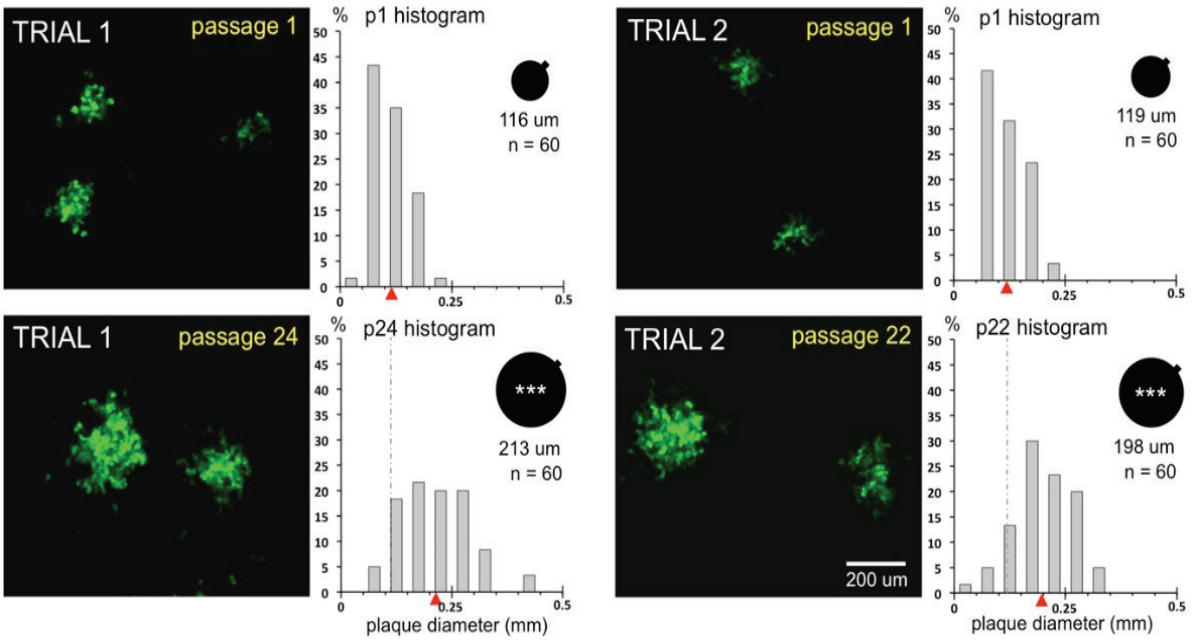


Figure 4: Viral plaque-size assay of early- and late-passage VSV-1'GFP. Fluorescent plaques were imaged 24 hrs after infection and agarose overlay of IOMM-Lee cells. Trials 1 and 2 are shown (left and right, respectively). Plaques from later passages (p24, p22; bottom) are larger than initial passage (p1; top). Size measurements (n=60 plaques per condition) indicate a significant increase ($p<0.001$; ANOVA) in plaque size. Circles depict mean plaque-size with standard error (small projection) and histograms depict plaque-size distribution (red markers indicate mean)

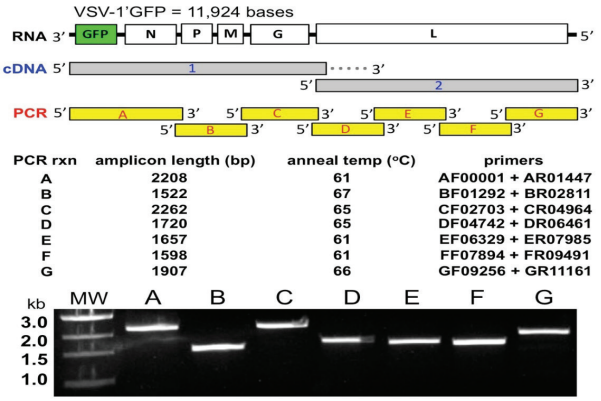


Figure 5: PCR-based VSV-1'GFP sequencing strategy. Illustration (top) shows VSV RNA genome, cDNA products (#1 and #2 generated by reverse-transcription with oligos AF00001 and DF04742, respectively), and resulting PCR products (A, B, C, D, E, F, G) generated using high-fidelty PCR. PCR reactions and conditions are listed (center). Agarose gel of purified PCR products (bottom)

infection and release of new viral progeny (the latency period) ranges from 2-3 hrs (12, 36). Thus, over the course of a 24 hr period, one might expect 8 to 12 cycles of VSV replication to occur. Finally, single cell analysis suggests that, after infection and prior to cell death, nearly 1,000-2,000 new progeny VSV virions can be released from a single infected host cell (37). The high VSV mutation rate, fast replication kinetics, and robust release of progeny virions all contribute to the production of a genetically diverse VSV population that is highly adaptable and amenable to experiments involving artificial selection, as demonstrated by the directed evolution experiment of the present study.

Of the four amino acid altering mutations that emerged in VSV-1'GFP after repeated passage on IOMM-Lee cultures, three (N36T, E92K, and E254K) were localized to the G gene, with the E92K mutation emerging twice, independently in both trials. While cross-contamination between cultures and experiments is always a concern, this explanation for the presence of E92K in both trials can be excluded due to the presence of the silent Y178Y mutation found in the M gene from Trial 1, yet absent from the sequence of Trial 2. Despite being conducted serially rather than in parallel, if cross-contamination between the trials had somehow occurred, then this silent mutation from the first trial would also have been carried over into the second trial. Thus, the E92K mutation appears to have emerged independently in both trials and may be playing a role in producing the enlarged-plaque phenotype observed in the plaque-size assay.

The VSV glycoprotein or G-protein encoded by the G gene is an integral membrane protein localized to the plasma membrane enveloping the VSV virion. This protein is responsible for the initial attachment and fusion of the virus with the host cell and is critical to host cell entry, infection, and continued propagation (9, 38). Since the G-protein plays such an integral role in the VSV replication cycle, even minor changes to the amino acid sequence might be expected to measurably influence the growth and size of viral plaques in the context of a plaque-size assay. Interestingly, the wild-type sequence of the Orsay strain of Indiana serotype

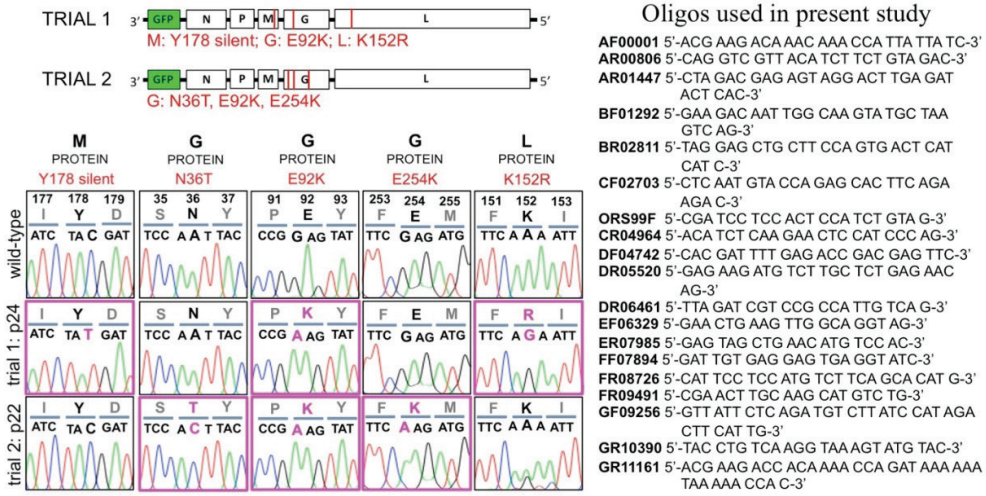


Figure 6: Mutations identified in late-passage VSV-1'GFP genomes. Diagram showing genomic location of mutations that emerged after repeated passage in each trial (top left). Sequence chromatograms (bottom left) from wild-type and late-passage virus covering each mutation site. Nucleotides and amino acids differing from wild-type are highlighted in magenta. Sequences of oligos used for RT, PCR, and automated DNA sequencing are also listed (right)

VSV (GenBank Accession: M11048) from which the VSV-1'GFP G-protein is derived, codes for a lysine (K) at codon 92, the same as that found in both of our IOMM-Lee derived late-passage mutants. Initial sequencing of the G-protein in the founder stock of VSV-1'GFP immediately after arrival in the laboratory indicated several deviations from the M11048 sequence (see Methods), one of which was the substitution of glutamate (E) at codon 92. At this time, it is unclear whether these variations were present in the original VSV-1'GFP plasmid construct used to first generate the virus, or emerged some time later during standard maintenance of viral stocks. To replenish viral stocks, VSV is most frequently grown and harvested from infected cultures of BHK-21 cells (baby hamster kidney cells) or Vero cells (African green monkey kidney cells). Both of these cell lines harbor defects of their innate immune system, making them highly permissive cellular hosts suitable for the maintenance propagation of viruses (39, 40). One possibility is that VSV-1'GFP was originally generated using the wild-type K92 codon and, through repeated maintenance propagation on either BHK-21 or Vero cells lacking a functional innate immune system, mutated to become the E92 variant found in our VSV-1'GFP founder stocks. Repeated passage on IOMM-Lee cells, which appear to display at least a partially functional innate immune response, may have simply re-introduced a wild-type selection pressure that shifted the viral population back to the original wild-type codon of K92 (41). Further work will be necessary to determine what role, if any, innate immune effects might play in the emergence, or loss, of the E92K mutation.

Recently, a directed evolution experiment involving two different oncolytic VSVs (VSV-p53wt and VSV-p53-CC) was reported in which 33 repeated passages were used to adapt each virus to pancreatic cancer cells (18). Interestingly, after sequencing each of the adapted viral genomes, a pair of G-protein mutations (K174E and E238K) were found to have emerged in both of these independently adapted viruses. Whereas, in the context of this single study, one might argue that a pair of mutations involving the same two amino acids, i.e. glutamate (E) and lysine (K), may simply be a coincidence, this same reasoning becomes less plausible when we also consider the E92K and E254K mutations found in our IOMM-Lee adapted VSV-1'GFP. The Orsay VSV G-protein is assembled from 511 amino acids, of which 26 (5.1%) are glutamate (E) and 30 (5.9%) are lysine (K). Given a single E or K residue taken from a pool of the 511 Orsay G-protein residues, the probability of randomly drawing a second E or K is 55/510 or 10.8%. The probability of randomly drawing a pair of E or K residues from two duplicate pools in only two draws from each pool is $[(56/511)\ (55/510)]^2$ or 0.0014%. At neutral pH, both glutamate and lysine are charged molecules with glutamate being negative (-E) and lysine positive (+K). It has been suggested that the K174E and E238K mutations might improve virus entry into pancreatic cancer cells due to their proximity to the region of the G-protein that undergoes a large conformational rearrangement during the process of virion fusion with the endosomal membrane (17). A similar argument could potentially be made for the E92K and E254K mutations and IOMM-Lee cells. Since this fusion event is triggered by acidification of the endosome, mutations in this region of the G-protein that result in a substitution with an oppositely charged amino acid (e.g. -E ⇒ +K) may influence the sensitivity of this triggering mechanism (42, 43). Taken together, the finding that cells from two very different types of cancer, i.e. pancreatic cancer and malignant meningioma, both yielded pairs of E/K mutations localized to the same region of a single protein warrants further investigation. Additional work examining the influence of extracellular pH on these, and similar G-protein mutants may prove informative.

Finally, the N36T mutation of the G-protein in Trial 2 and the K152R mutation of the L-protein in Trial 1 appear to have no

obvious basis for comparison in the literature. However, the sequencing chromatogram for the K152R site from Trial 2 is suggestive of a heterogenous population of virions, some with the K152R mutation and some without (Figure 6 p22 in K152R column). This apparently mixed population of mutant and wild-type virions may indicate the initial emergence of the K152R mutation in Trial 2, prior to becoming genetically fixed in the viral population, as it has in Trial 1. Newly-engineered recombinant VSVs (reverse-genetics) will be required to investigate which of the 4 amino acid altering mutations of the present study are necessary and sufficient for the production of the enlarged-plaque phenotype.

CONCLUSION

The present study demonstrates that *in vitro* directed evolution techniques can be applied to adapt mutation-prone oncolytic viruses for improved growth on cultures of human meningioma cells. After adaptation on IOMM-Lee cells in two independent trials, VSV-1'GFP was found harboring 3 non-synonymous mutations (N36T, E92K, E254K) of the glycoprotein (G) responsible for viral entry and 1 non-synonymous mutation (K152R) of the RNA polymerase protein (L) responsible for RNA synthesis and replication of the viral genome. One of these mutations (E92K) was found to have emerged independently in both adaptation trials, thus suggesting a role for this mutation in the production of the enlarged plaque-size phenotype found on IOMM-Lee cells. Further investigations of the prospect for treating malignant meningiomas using VSV-based oncolytic virotherapy appear warranted.

Ethics Committee Approval: No Ethics Committee Approval is needed for this research.

Informed Consent: No patient data was used.

Peer Review: Externally peer-reviewed.

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PRSS57 GENE EXPRESSION PREDICTS EARLY MOLECULAR RESPONSE FAILURE IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

KRONİK MİYELOİD LÖSEMİLİ HASTALARDA ERKEN MOLEKÜLER YANIT TAHMİNİ İÇİN PRSS57 GEN İFADESİ

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ABSTRACT

Objectives: The molecular response to tyrosine kinase inhibitors in chronic myeloid leukemia (CML) is monitored by quantitative detection of BCR/ABL transcripts. After the initiation of the treatment, patients are followed-up with molecular analysis at three-month intervals. Early molecular response (EMR) is considered achieved when the BCR/ABL international scale (IS) is 10% or below in the three-month follow-up after treatment. This response, which has been reported to have a strong prognostic significance in CML patients, is associated with favorable long-term outcomes. However, the three-month follow-up period may be too long in terms of disease progression and treatment management for patients who fail to achieve EMR. Therefore, additional biomarkers that can predict the prognosis are needed.

Material and Methods: This study investigated the relationship between serine protease 57 (*PRSS57*) gene expression, and EMR. The *PRSS57* gene expression in 20 CML patients was determined by the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) method and its relationship with EMR was analyzed.

Results: The *PRSS57* gene expression was found to be significantly higher in patients who failed EMR (p=0.002) and positively correlated with BCR/ABL IS value (r=0.567, p=0.009). Our results also revealed that the *PRSS57* gene expression was decreased in the post-treatment follow-up sample when compared with the diagnostic sample (p=0.000).

Conclusion: These findings indicate that the *PRSS57* gene expression in diagnosis may be useful for predicting patients at high risk of EMR failure.

Keywords: CML, early molecular response, *PRSS57*

ÖZ

Amaç: Kronik myeloid lösemide (KML) tirozin kinaz inhibitörlerine verilen yanıtın moleküler takibi BCR/ABL transkriptlerine göre yapılmaktadır. Tedavi başlangıcından sonra hastalar üç aylık periyotlarla moleküler analizlerle takip edilmektedir. Tedavi sonrası üç aylık takipte BCR/ABL Uluslararası değeri (IS) %10 ve altına düştüğünde erken moleküler yanıt (EMY) başarılı kabul edilmektedir. KML hastalarında önemli prognostik değeri olduğu bildirilen bu yanıtın uzun vadede olumlu sonuçlarla ilişkili olduğu bulunmuştur. Ancak EMY başarısız olan hastalar için hastalığın ilerlemesi ve tedavi yönetimi için üç aylık takip geç olabilmektedir. Bu nedenle tanı sırasında prognozun tahmini sağlayabilecek ek biomarkerlara ihtiyaç duyulmaktadır.

Gereç ve Yöntemler: Çalışmamızda serin proteaz 57 (*PRSS57*) gen ifadesinin EMY ile ilişkisi araştırıldı. 20 KML hastasında *PRSS57* gen ifadesi kantitatif ters transkriptaz polimeraz zincir reaksiyonu (qRT-PZR) yöntemiyle belirlendi ve EMY ile ilişkisi analiz edildi.

Bulgular: *PRSS57* gen ifadesi EMY başarısız hastalarda anlamlı derecede yüksek olduğu (p=0,002) ve BCR/ABL IS değeri ile pozitif yönde bağlantılı olduğu bulundu. Sonuçlarımız aynı zamanda *PRSS* gen ifadesinin tedavi sonrası izlem örneklerinde azaldığını gösterdi.

Sonuç: Bu bulgular tanıda *PRSS57* ifadesinin EMY başarısızlığı riski yüksek hastaların tahmininde yararlı olabileceğini göstermektedir.

Anahtar Kelimeler: KML, erken moleküler yanıt, *PRSS57*

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INTRODUCTION

Chronic myeloid leukemia (CML) accounts for 15% of all leukemia and 0.5% of all cancers. The BCR/ABL fusion gene which stimulates tyrosine kinase activity is present in 95% of patients with CML (1-4). Therefore, synthetic tyrosine kinase inhibitors (TKI) should decrease tyrosine kinase activity in CML patients. The National Comprehensive Cancer Network (NCCN) and the European Leukemia Net (ELN) are two of the main international committees, which develop evidence-based guidelines or recommendations for treating and managing CML (5, 6). Implementation of the approved first generation TKIs (imatinib) and second generation TKIs (dasatinib, nilotinib, and bosutinib) comply with the recommendations provided in their guidelines (4-6).

CML has three clinical stages (chronic, accelerated, or blast) and CML patients are mostly diagnosed in the chronic phase. However, the progression might worsen for accelerated and blast phases in untreated patients or patients who develop resistance to treatment (7).

In the clinical management of CML in the chronic phase, imatinib is the most used treatment (8-12). Subsequently, the response of patients to the treatment is monitored hematologically (normalization of peripheral blood counts), cytogenetically (reduction in the number of Ph-positive meta-phases using bone marrow cytogenetics), and molecularly. Quantitative PCR is extensively used for molecular detection (decrease in the amount of BCR/ABL transcripts) of the treatment progression in accordance with the international scale (IS) (4-6).

It is possible to identify relapse and treatment responses at an early stage with an understanding of the molecular basis of CML. The molecular analysis of the BCR/ABL transcript levels is one of the most accurate ways to determine the stage of CML disease (11). According to the NCCN, there are three stages of molecular response; (i) the BCR/ABL IS value should be 10% or less at three and six months for early molecular response (EMR), (ii) the BCR/ABL IS value should be 0.1% or less for Major Molecular response (MMR), and (iii) the BCR/ABL IS value should be 0.01% or less for MR4.0 Deep Molecular Response (DMR) or the BCR/ABL IS value should be 0.0032% or less for MR4.5 DMR (5).

Many studies for understanding the importance of molecular response have shown that achieving molecular response is important in prognosis and progression-free survival. Hence, new molecular markers will be especially helpful in identifying individuals who are most at risk for disease progression or recurrence. The earliest sign that a patient has developed imatinib resistance and/or whether a BCR/ABL mutation has taken place may be the rise of the BCR/ABL levels (11). Following the first line TKI therapy, the importance of EMR has been proven by studies as a reliable indicator of progression-free survival (PFS) and overall survival (7, 11). Consequently, decisions can be made promptly regarding the therapeutic approaches that utilize molecular monitoring.

The rapid decline of the BCR/ABL transcript has important prognostic significance. However, a three month follow-up may be too long in terms of disease progression and treatment management for patients, who experience blast crisis (BC) after EMR failure during the first months of treatment. Therefore, it is highly important to develop different biomarkers for early response prediction (13-15).

Additionally, the BCR/ABL transcript level at diagnosis helps prognostic scoring systems for predicting EMR response, also current research reveals the existence of different biomarkers for this prediction. (14-16). A recent study investigated the gene expression signature (GES) of chronic phase CML patients who failed the EMR and found that some candidate genes (IGFBP2, PRSS57, and CPXM1) were expressed higher in patients who failed to achieve EMR when compared to patients who achieved EMR (15). These genes may be candidate markers that can be used to predict response to therapy at diagnosis. This study aimed to investigate the relationship between the PRSS57 gene expression at diagnosis and EMR in our CML patients.

MATERIALS and METHODS

Patient samples

Peripheral blood or bone marrow samples of 20 CML patients at diagnosis and after treatment were used with informed consent (2023/409) from Istanbul University, Istanbul Faculty of Medicine, Pediatric Hematology/Oncology Department and at Bezmialem Vakif University Faculty of Medicine Department of Internal Medicine, Division of Hematology. Patients ranged in age between 1-85 years, including seven children (mean age 11) and 13 adults (mean age 51). The gender distribution of these 20 patients was 11 (55%) female and 9 (45%) male (Table 1).

Table 1: Demographic characteristics of the patients

		Child	Adult	Total
Gender	Male	1	8	9
	Female	6	5	11
Median age (range)		11 (1-17)	51 (22-76)	37 (1-76)
Total		7	13	20

Molecular responses of the patients to the treatment were detected by quantitative real-time polymerase chain reaction (real-time qPCR) at three, six, and 12 months if the patients' follow-up data was available. These patients received imatinib (400mg/day) treatment as a first-line treatment.

RNA Isolation and cDNA Synthesis

Total RNAs were isolated by Trizol protocol from the blood samples of patients. Total RNA was quantified by Nanodrop, and cDNA was synthesized with a Thermo High-Capacity cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA). The cDNA

was synthesized from 500ng total RNA. The cDNA reaction mix was collected by adding 10x Buffer, dNTP (100mM), Random Primer (10X), RNase Inhibitors (50mM), and Reverse Transcriptase (50U/µL) in 20µL reaction volume. The cDNA synthesis was performed as follows; for pre-incubation 10 minutes at 25°C, for main incubation 120 minutes at 37°C, and for final incubation 5 minutes at 85°C.

Quantitative Real-Time PCR

The cDNA was amplified by RT-PCR on the Light Cycler 480 (Roche Diagnostic, Mannheim, Germany) for 50 cycles. The Ct-values and concentrations were then analyzed on the LC480 software (Roche Diagnostic, Mannheim, Germany). A final reaction volume of 20µL was done, which contained the SYBR Master mix (Roche Diagnostic, Mannheim, Germany), primers (10pmol/µl) (Table 2), cDNA (500ng), and RNAse-free water by the manufacturer's instructions. The cDNA synthesis was validated by the housekeeping gene GAPDH amplification. The BCR/ABL fusion gene was analyzed using qRT-PCR as previously described (17).

Table 2: Primer sequences used for qRT-PCR

Gene symbol	Primer sequences	PCR product size (bp)
PRSS57	Forward: 5'-TCACCACACACCCCGACTA-3' Reverse: 5'-CGGCAGCTCCTCAAAGTCAG-3'	194
GAPDH	Forward: 5'-AGAAGGCTGGGGCTCATTTG-3' Reverse: 5'-AGGGCCATCCAGAGTCTTC-3'	257

PRSS57: Serine protease 57, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase, bp: Base pair

Statistical analysis

Statistical analyses were performed with SPSS software (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) and GraphPad Prism 8.0 (GraphPad Software, Inc.). The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to test normality. Independent sample T-test for parametric data. The Kruskal-Wallis and Mann-Whitney U tests were utilized for non-parametric data. Spearman's tests were used for the correlation analysis and $p<0.05$ was statistically significant for all statistical analyses.

Ethical approval

Ethics committee approval for this study was obtained from the Local Ethics Committee of Istanbul University, Istanbul Faculty of Medicine (Date: 17.03.2023, No: 06).

RESULTS

In our study, the BCR/ABL IS ratio was determined from CML patients, who were followed for at least six months. The IS ratio of BCR/ABL did not fall below 10% in 6 patients in six months of follow-up, so we accepted an EMR failure. The remaining 14 patients were considered as EMR achieved (Table 3).

Table 3: Early molecular response status in patients

		Child	Adult	Total
Early molecular response: Failure	Male	1	1	2
	Female	2	2	4
Total		3	3	**6**
Early molecular response: Achieve	Male	-	7	7
	Female	4	3	7
Total		4	10	**14**

When the test of normality was performed, it found that the age and gender characteristics of patients showed normal distribution, while the PRSS57 gene concentration values and the BCR/ABL transcript values showed non-normal distribution.

The *PRSS57* gene expression analysis was normalized to the GAPDH housekeeping gene expression. Diagnosis samples for the EMR achieved and failure groups were examined in terms of *PRSS57* gene expression levels, it found that the EMR failure group had significantly higher gene expression levels (Figure 1). The *PRSS57* expression was approximately 4.47 times higher in the EMR failure group (p=0.002). The *PRSS57* gene expression was found to decrease in the post-treatment follow-up sample as compared to the diagnostic sample (p=0.000).

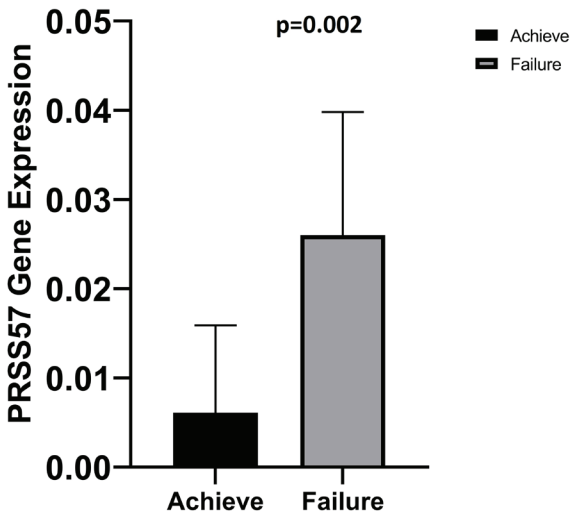

Figure 1: *PRSS57* gene expression levels in EMR achieve and failure patients at diagnosis (Achieve: EMR Achieved group, Failure: EMR Failure Group)
PRSS57: Serine protease 57

We analyzed the correlation between the *PRSS57* gene expression and the BCR/ABL IS value, and found that the *PRSS57* gene expression was moderately correlated with the BCR/ABL IS value (r=0.567, p=0.009) (Figure 2). We compared the *PRSS57* gene expression in terms of age and gender and we could not find any significant differences (p=0.125 and p=0.166 respectively).

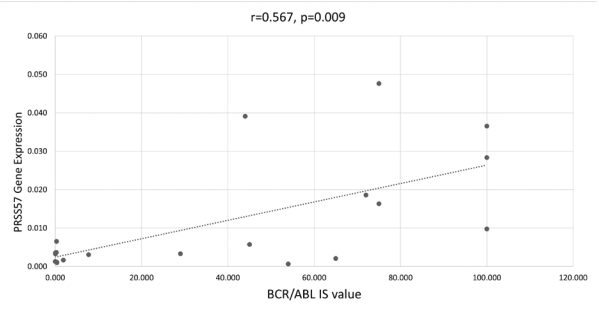

Figure 2: Correlation between *PRSS57* gene expression and BCR/ABL IS value
PRSS57: Serine protease 57

DISCUSSION

The guidelines determined many parameters (such as TKI treatment, post-treatment follow-up, and molecular response) that guide the clinical approach for patients with CML. Early prediction of disease prognosis is crucial for the treatment of the disease. In this context, showing the decrease in the BCR/ABL transcript level is an important parameter. Research has shown that when the BCR/ABL IS value is higher than 10% at three months, this is strong evidence for higher rates of disease progression (9). Observational studies report that the BCR/ABL ratio, which does not fall below 10% at three months post-treatment, carries a high risk of blast crisis progression and that the molecular response such as MMR-MR4.5 will occur at a low rate in 1-2 years. However, the prediction of treatment response at an early stage may change the prognosis of the disease with different treatment strategies (9). For these reasons, CML patients need different molecular markers to predict the prognosis of the disease at the time of diagnosis. This molecular marker may be a more accurate indicator than the BCR/ABL IS value, which can guide treatment at the time of diagnosis. In particular, the prognosis of the disease can be changed by the prediction of the patients at a high risk of EMR failure.

The therapeutic approaches used for leukemia may change as a result of the biological significance of the identified genes and their contribution to treatment resistance. Moreover, a diagnostic and prognostic biomarker found at the molecular level in research on hematological malignancies might allow for more precise evaluation. An early insight into treatment resistance will be provided by the evaluation of the achieve or failure status of the EMR. The study by Kok et al. analyzed the gene expression signature (GES) of chronic phase CML patients who failed EMR and determined that patients who failed EMR showed different gene signatures compared to those who achieved EMR (15). The results of this study showed that three genes (*IGFBP2, PRSS57*, and *CPXM1*) were expressed higher in patients who fail to achieve EMR when compared to patients who achieve EMR. Knowing the different gene signatures, whose prognostic value will be understood in the future, will improve the prediction of patients who fail EMR. While imatinib can be given to patients classified as low risk of EMR failure, patients with high risk of EMR failure can be identified at diagnosis and potentially recommended for stronger TKI therapy. Thus, the survival time in these patients could be extended.

The studies of Kok et al. and Harada et al. reported that one of the *PRSS57* genes show a different gene signature in patients who fail to achieve EMR when compared to those who achieve EMR. They found that the *PRSS57* gene expression levels were higher in patients who failed EMR (14, 15). This was determined by considering the IS ratio of the BCR/ABL for the prediction of EMR (15). Kok et al., and Harada et al. suggested that overexpression of the *PRSS57* gene is associated with poor prognosis (14, 15)

In this retrospective study, we analyzed the *PRSS57* gene expression both at the time of diagnosis and after treatment and then investigated its relationship with EMR. A significant positive correlation was found between EMR failure and the *PRSS57* gene expression at diagnosis. We found that the *PRSS57* gene expression was significantly higher in EMR failure patients than in EMR achieved patients. This study confirmed that the *PRSS57* gene is a biomarker that provides additional prognostic information. However, due to the small number of patients included in this study, it reveals the need for more comprehensive studies. Our results suggest that the *PRSS57* gene plays an important role in elucidating the pathogenesis of CML. In addition to providing positive results for clinical progress, this gene expression signature may also provide insight into the underlying resistance biology of CML.

Ethics Committee Approval: This study was approved by Istanbul University, Istanbul Faculty of Medicine (Date: 17.03.2023, No: 06).

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THE EFFECT OF LINEZOLID AGAINST VANCOMYCIN-RESISTANT ENTEROCOCCI BY VARIOUS METHODS

LİNEZOLİDİN VANKOMİSİNE DİRENÇLİ ENTEROKOK SUŞLARINA ETKİSİNİN ÇEŞİTLİ YÖNTEMLERLE ARAŞTIRILMASI

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ABSTRACT

Objectives: Enterococci are the causative agents of a variety of infections, particularly healthcare-associated infections. Linezolid is an important antibiotic in the treatment of infections caused by vancomycin-resistant enterococci. However, in recent years, an increasing rate of linezolid resistance has been reported in clinical enterococci strains. The aim of this study was to investigate the in vitro efficacy of linezolid against vancomycin-resistant *Enterococcus* (VRE) strains isolated from rectal swab samples of inpatients by disc diffusion, microdilution and E-test methods, and thus to evaluate the efficiency of linezolid against VRE strains by qualitative and quantitative methods.

Material and Methods: Fifty VRE strains were defined as enterococci by conventional methods. The efficiency of linezolid in strains was investigated by disk diffusion, E-test and microdilution methods. Species identification of enterococci strains was done with the GP24 Diagnostics kit.

Results: The identification of fifty enterococcal strains using the conventional methods revealed Gram-positive coccus, catalase-negative, bile growth-esculin hydrolysis positive, salt tolerance test (6.5% NaCl) positive, and L-pyrrolidonyl-β-naphthylamide (PYR) test positive. All strains were found to be susceptible to linezolid in disc diffusion, E-test and microdilution tests. In the microdilution test study, the MIC distrubution, MIC_{50} and MIC_{90} was detected as 1-2, 2 and 2 µg/mL, respectively. The MIC distribution, MIC_{50} and MIC_{90} values of linezolid by E-test were determined as 1-4, 2 and 3 µg/mL, respectively. In the study, 48 (96%) of 50 strains were identified as *Enterococcus casseliflavus* and 2 (4%) were *Enterococcus faecium*.

Conclusion: No linezolid resistance was detected in the study. This suggests that linezolid can be used safely in the treatment of VRE-induced infections. It will be important to conduct continuous and comprehensive studies on this subject and to monitor linezolid resistance surveillance.

Keywords: Vancomycin-resistant enterococci, linezolid, disc diffusion, microdilution, E-test

ÖZ

Amaç: Enterokoklar, özellikle sağlık hizmeti ile ilişkili enfeksiyonlar olmak üzere, çeşitli enfeksiyonların etkenidirler. Linezolid, vankomisine dirençli enterokokların sebep olduğu enfeksiyonların tedavisinde önemli bir antibiyotiktir. Fakat son yıllarda klinik enterokok suşlarında giderek artan oranlarda linezolid direnci rapor edilmektedir. Çalışmada, yatan hastaların rektal sürüntü örneklerinden izole edilen vankomisine dirençli enterokok (VRE) suşlarında linezolidin in vitro etkinliğinin disk difüzyon, E-test ve mikrodilüsyon yöntemleri ile araştırılması ve böylece linezolidin VRE suşlarına etkinliğinin kalitatif ve kantitatif yöntemler ile değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntemler: Elli VRE suşu konvansiyonel yöntemler ile enterokok cinsi olarak tanımlanmıştır. Suşlarda, linezolidin etkinliği disk difüzyon, E-test ve mikrodilüsyon metodları ile araştırılmıştır. Enterokok suşlarının tür tanımlaması GP24 Diagnostics kiti ile yapılmıştır.

Bulgular: Elli enterokok suşunun konvansiyonel yöntemlerle tanımlanmasında Gram-pozitif kok, katalaz negatif, safrada üreme-eskülin hidrolizi pozitif, tuz tolerans testi (%6,5 NaCl) pozitif ve L-pirolidonil-β-naftilamid (PYR) testi pozitif olarak belirlenmiştir. Disk difüzyon, E-test ve mikrodilüsyon testleri ile suşların tümü, linezolide duyarlı bulunmuştur. Mikrodilüsyon test çalışmasında linezolid MİK dağılımı, $MİK_{50}$ ve $MİK_{90}$ değerleri sırasıyla 1-2, 2 ve 2 µg/mL olarak belirlenmiştir. Linezolidin E-test yöntemiyle yapılan MİK araştırması sonucunda MİK dağılımı, $MİK_{50}$ ve $MİK_{90}$ değerleri sırasıyla 1-4, 2 ve 3 µg/mL olarak saptanmıştır. Çalışmada 50 suştan 48'i (%96) *Enterococcus casseliflavus* ve 2 suş (%4) *Enterococcus faecium* olarak tanımlanmıştır.

Sonuç: Çalışmada linezolid direnci saptanmamıştır. Bu da linezolidin VRE kaynaklı enfeksiyonların tedavisinde güvenle kullanılabileceği fikrini vermektedir. Bu konuda devamlı ve kapsamlı çalışmaların yapılması ve linezolid direnç sürveyansının izlenmesi önemli olacaktır.

Anahtar Kelimeler: Vankomisine dirençli enterokoklar, linezolid, disk difüzyon, mikrodilüsyon, E-test

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INTRODUCTION

Enterococci are important bacteria that live commensally in the bowel of humans and many other animals, including invertebrates. They are the first bacteria to colonize newborns and constitute an important part of the healthy adult gut microbiota. The most frequently isolated and clinically important species are *Enterococcus faecalis* and *Enterococcus faecium. Enterococcus casseliflavus* and *Enterococcus gallinarum* are prevalent in the human gut flora and are intrinsically resistant to vancomycin (1).

Although *Enterococci* do not have as wide a range of virulence factors as *Staphylococci* or *Streptococci*, they are important bacteria because they cause life-threatening diseases with antibiotic-resistant strains. There are two general features of virulence. One is the ability to form biofilms by adhering to tissues and the other is the ease with which antibiotic resistance can be developed. Clonal strains adapted to hospital conditions also have superior patient-to-patient transmission abilities (2).

Enterococci are one of the most common types of nosocomial infections. They frequently cause urinary tract infections, and this is usually associated with urinary catheterization or instrumentation. They also cause endocarditis and bacteremia. Pelvic, biliary, intra-abdominal, and wound infections are common. Meningitis may be caused by these bacteria, but only rarely (3).

The first vancomycin-resistant *Enterococcus* (VRE) strain was identified in 1988 in England (4). Since then, it has increasingly spread all over the world. The first antibiotic confirmed for the treatment of VRE infection was quinupristin/dalfopristin. Its use has been largely abandoned due to its effectiveness only against the *E. faecium* strain and its frequent side effects. Linezolid has the advantage that it penetrates well into various tissues (including CFS-Cerebrospinal fluid) and is available in oral form. It is mainly used in the treatment of infections caused by multidrug-resistant bacteria such as VRE, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae* (5). The first linezolid-resistant clinical isolate carrying the *cfr* gene was reported in 2005. It has been reported that the cause of linezolid resistance is related to the overuse of the drug (6).

The goal of this study was to research the efficacy of linezolid on vancomycin-resistant strains of the *Enterococcus* genus isolated from rectal swab samples of patients hospitalized in various clinics of our hospital by disc diffusion, E-test and microdilution methods.

MATERIAL and METHODS

This study was approved by the Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 23.09.2022, No: 17).

In the study, 50 different VRE strains isolated from rectal swab samples taken periodically from patients hospitalized in various wards of Istanbul University's Medical Faculty Hospital between 2022 and 2023 were used. All samples were cultured on Bile-Esculin Agar (BEA) (BD, BBL TM Bile Esculin Agar, USA) supplemented with vancomycin and incubated at 35°C for 24 hours. Cultures hydrolyzing esculin were identified at the *Enterococcus* genus level by conventional methods. Afterwords, the strains were stored in brain-heart infusion broth (Becton Dickonson, USA) storage medium with 20% glycerol and kept at -20°C until the study time (7).

During the study, each strain was seeded on Tryptic Soy Agar (TSA) (Oxoid, United Kingdom) and incubated at 35°C for 18-24 hours. The obtained pure cultures were investigated for growth in bile using the hydrolysis of esculin test, Gram stain, the catalase test, the salt tolerance test, and the L-pyrrolidonyl-β-naphthylamide (PYR) (PYR-Oxoid Biochemical Identification System) test for confirmation of the *Enterococcus* genus. The presence of growth and darkening in the bile-esculin agar medium, the presence of Gram-positive cocci morphology in the microscope examination, the negative catalase test, the in the salt tolerance medium turning from purple to yellow and the PYR test positive strains all indicated the presence of enterococci (7). A commercially available GP24 kit (Diagnostics, Slovak Republic) was used to identify strains at the species level. Identification of the strains was investigated in line with the manufacturer's recommendations and the results were evaluated with the IDmicro software program given. *Enterococcus faecalis* ATCC 29212 standard strain as quality control was studied with the GP24 kit and gave the results of *Enterococcus faecalis* with 100% accuracy in the IDmicro Software program. In addition, the hemolysis, the presence of β-lactamase enzyme, and the existence of high-level aminoglycoside resistance (HLAR) features of the strains were investigated (8).

Disc diffusion test was applied on all strains with vancomycin (30 µg) (Bioanalyse, Ankara, Turkey), teicoplanin (30 µg) (Bioanalyse, Ankara, Turkey) and linezolid (30 µg) (Bioanalyse, Ankara, Turkey) antibiotic discs. In addition, Minimum Inhibition Concentration (MIC) values were investigated for linezolid by both E-test (Bioanalyse, Turkey) and microdilution (Linezolid, Biosynth Carbosynth, United Kingdom) methods (8, 9). The studies were designed in line with the recommendations of the CLSI standard and the results were evaluated as to the same standard criteria. *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus aureus* ATCC 29213 standard strains were used as quality control strains in the studies (8, 10).

RESULTS

The distribution of patients by clinical units and gender is shown in Figure 1. Accordingly, 22 patients (44%) were in pediatrics, 20 (40%) in internal disease, 4 (8%) in anesthesia reanimation, 3 (6%) in general surgery, and 1 (2%) in neurology intensive care unit, respectively. The gender of the patients was determined as 27 (54%) male and 23 (46%) female. The number of patients by age was as follows: 22 (44%) aged 0-10, 1 (2%) aged 11-30, 6 (12%) aged 31-50, 15 (30%) aged 51-70 and 6 (12%) aged 71-90.

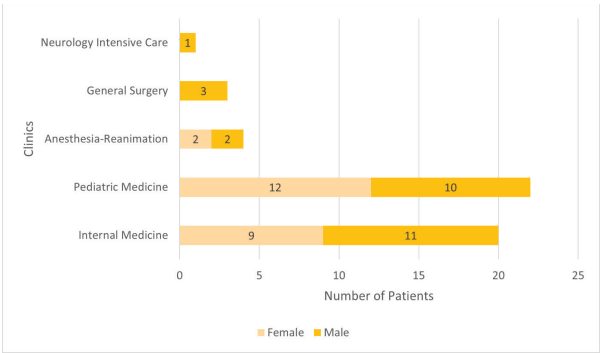

Figure 1: Distribution of 50 VRE strains by gender and clinical units from which they are isolated

In the identification of strains as *Enterococcus* genus by conventional methods, Gram stain, catalase, esculin hydrolysis, growth in bile, growth in 6.5% NaCl and PYR tests were applied. Accordingly, all the strains were catalase-negative, Gram-positive cocci in microscope image, esculin hydrolysis positive, bile growth positive and PYR test positive. All strains were defined as enterococci. The presence of β-laktamase enzyme was also investigated in all strains and it was not detected in any of the strains. All strains were determined to be resistant to vancomycin and teicoplanin (100%) by disc diffusion test. All strains were determined to be sensitive (100%) to linezolid by the same method.

High-level aminoglycoside resistance screening was also performed in strains. While 33 (67%) of 50 VRE strains were found to be HLAR positive, a high level of gentamycin resistance (HLGR) was found in 16 (32%) strains and a high level of streptomycin resistance (HLSR) was found in 1 (2%) strain.

The distribution of linezolid MIC values in the microdilution test study is shown in Figure 2. Accordingly, all strains were found to be susceptible (100%) to linezolid. The MIC distribution, MIC_{50} and MIC_{90} values of linezolid were determined as 1-2, 2 and 2 µg/mL, respectively.

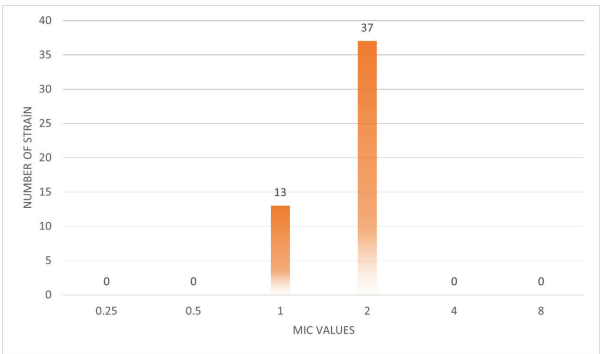

Figure 2: Distribution of linezolid MIC values obtained as a result of microdilution test

As a result of the investigation, the efficacy of linezolid against 50 VRE strains by E-test, the distribution of MIC values, MIC_{50} and MIC_{90} values were determined as 1-4, 2 and 3 µg/mL, respectively. All strains were found susceptible by this method. In addition, the E-test method was used to identify the effectiveness of vancomycin quantitatively, and the distribution of MIC values was determined as MIC distribution: 128 - > 256 µg/mL and $MIC_{50,90}$: > 256, 256 µg/mL, respectively.

Through the identification of *Enterococcus* strains using the GP24 Diagnostics kit, 48 (96%) of 50 VRE strains were determined as *Enterococcus casseliflavus* and 2 (4%) strains as *Enterococcus faecium.* In the study, *Enterococcus faecalis* ATCC 29212 strain was used as quality control strain and identified with 100% accuracy by the diagnostic kit.

DISCUSSION

Healthcare-associated infections are infections that patients encounter while receiving treatment and care for medical or surgical conditions (11). The primary source of healthcare-associated infections is through the contaminated hands of healthcare workers (12). There is growing concern that vancomycin-resistant enterococci are becoming increasingly resistant to the antibiotics used to treat VRE infections and that these antibiotics may be less effective (13).

Because the HLAR does not allow for synergistic treatments, strains with HLAR must be treated with alternative combinations of antibiotics (14). Schouten MA et al. investigated gentamicin resistance of 50 *E. gallinarum* and 21 *E. casseliflavus* strains and determined that 18% of *E. gallinarum* and 18.2% of *E. casseliflavus/flavescens* strains were highly resistant to gentamicin (15). In our study, all strains were screened for HLAR. It was determined in 31 of 48 *(E. casseliflavus)* strains. A high level of gentamicin resistance (HLGR) was determined in 16 strains, and a high level of streptomycin resistance (HLSR) in 1 strain. Additionally, 2 *E. faecium* strains were determined as HLAR.

In a study carried out at Marmara University Pendik Training and Research Hospital in Istanbul in 2021, rectal swab samples collected from patients hospitalized in all units were evaluated by performing VRE scanning. As a result of the study, in which 1710 samples were taken from 771 patients, VRE was detected in 8.1% (137/1710) of all samples. The highest positivity rate was found in intensive care patients (16).

Olearo et al. conducted a study in Germany and reported that the incidence of linezolid and vancomycin-resistant *Enterococcus faecium* (LVRE) is associated with antibiotic consumption. The researchers reported that the use of linezolid could be limited so that it may remain as a treatment alternative in VRE infections (17). According to another study conducted in Germany, the increasing prevalence of linezolid resistance among VRE strains was reported to be less than 1% in 2008, while it was reported to be greater than 9% in 2014 (18).

In a study conducted in Turkey in 2004, linezolid MIC values of 55 VRE strains were investigated using the E-test method. As a result of the study, MIC values of 55 strains were determined

in the range of 0.38-2.0 µg/mL (19). In another study, Aktaş G. et al. investigated linezolid MIC values by microdilution method of 100 VRE strains isolated from rectal swab samples of cases between 2006 and 2007. As a result of the study, linezolid MIC distribution and $MIC_{50,90}$ values were identified as 1-16 µg/mL, 4 µg/mL and 4 µg/mL, respectively, and 2 VRE strains were determined to be resistant to linezolid. MIC values of resistant strains were also investigated with the E-test method and were found to be 8 and 12 µg/mL. These two strains were identified as *E. faecium* (20).

In another study conducted in 2017, 79 *Enterococcus* spp. isolate was found susceptible to linezolid. For *E. faecalis* (69.6%) strains, the linezolid MIC range was found to be 0.25-2 µg/mL, MIC_{50} 0.75 µg/mL and MIC_{90} 1.5 µg/mL. For *E. faecium* (30.4%) strains, the linezolid MIC range was determined as 0.125-2 µg/mL, MIC_{50}: 0.5 µg/mL and MIC_{90}: 1 µg/mL. As a result of the study, attention was drawn to the importance of closely monitoring the changes by monitoring the MIC values of linezolid (21).

Comoglu et al. investigated the linezolid susceptibility of 20 VRE strains in Turkey. The latter study was conducted using disc diffusion and E-test methods. The MIC values of the strains were determined as 0.38-2 µg/mL. As a result of the study, linezolid resistance was not detected, and it was stated that linezolid is an important alternative in the treatment of VRE (22).

In our study, while the $MIC_{50,90}$ values of 50 VRE strains were found to be 2, 2 µg/mL, respectively, by the microdilution method, they were found to be sensitive as 2, 3 µg/mL by the E-test method. When the results of two different quantitative methods (microdilution and E-test) were evaluated, no significant difference was observed in terms of $MIC_{50,90}$ values, and it was determined that all methods, including the disc diffusion method, showed a high degree of parallelism.

A total of 97 enterococcal strains isolated from 67 patients in a university hospital in Brazil (2004) were examined by species identification, and it was determined that 34% of the strains were *E. faecium*, 33% *E. faecalis*, 23.7% *Enterococcus gallinarum* and 5.2% *Enterococcus casseliflavus* (23).

In a study published in 2006, 33 cases of non-faecalis and non-faecium enterococcal bacteremia were examined in a hospital in the USA, and it was determined that 10 of the patients were infected with *E. casseliflavus*, 8 with *E. mundtii*, 7 with *E. avium*, 5 with *E.durans* and 3 with *E. gallinarum*. As a result of the study, it was reported that bacteremia due to non-faecalis and non-faecium enterococci is a nosocomial infection. In addition, the importance of identifying all enterococci at the species level was emphasized in order to initiate appropriate infection control measures (24).

Species prevalence and antibacterial resistance among enterococci isolated in Tehran hospitals in Iran were investigated in 2009. Vancomycin, teicoplanin and linezolid antibiotics susceptibility of 200 enterococcal isolates were tested by disc diffusion and the agar dilution method. As a result of the study, 80% of 200 isolates were identified as *E. faecalis*, 11% as *E. faecium*, 6.5% as *E. casseliflavus*, 2% as *E. gallinarum* and 0.5% as *E. avium*. 2 *E. faecium*, 1 *E. gallinarum* and 1 *E. casseliflavus* strains were found resistant to linezolid. Linezolid MIC values for linezolid vancomycin resistant enterococci (LVRE) strains were between 16 and 32 µg/mL (25).

In a retrospective study conducted in Japan between 2005 and 2014, 410 cases with enterococcal bloodstream infections were studied. *Enterococcus casseliflavus* was detected in 37 (9%) of 410 cases. In the study, it was stated that *E. casseliflavus* was the third factor after *E. faecalis* and *E. faecium* in enterococcal bloodstream infection (26).

In a study conducted in a medical center in Taiwan in 2010 on infections caused by non-faecalis and non-faecium enterococci, 3017 enterococci isolated in blood cultures were examined and the most common species were identified as *E. casseliflavus, E. gallinarum*, *E. avium* and *E. hirae*, respectively. Infections caused by non-faecium non-faecalis enterococci were associated with patients with severely invasive diseases and immunocompromised patients (27).

In a retrospective study carried out in a hospital in the USA in 2015, *E. gallinarum* was found in 29 (60.4%) and *E. casseliflavus* in 19 (39.6%) of 48 patients hospitalized with the diagnosis of non-faecium non-faecalis VRE bloodstream infection (BSI). Generally, treatment with linezolid or daptomycin for vancomycin-resistant *E. casseliflavus* or *E. gallinarum* has been reported to produce better clinical outcomes compared to anti-enterococcal beta-lactam therapy (28).

In a prospective study conducted in India, 371 *Enterococcus* spp. isolates were determined by conventional biochemical tests and the VITEK 2 Compact identification system, and vancomycin resistance was investigated by PCR. As a result of the study, 239 *E. faecalis*, 114 *E. faecium*, 8 *E. avium*, 4 *E. durans*, 4 *E. casseliflavus* and 2 *E. gallinarum* were detected. Vancomycin resistance was detected in 14 *E. faecalis*, 4 *E. faecium*, 4 *E. casseliflavus* and 2 *E. gallinarum* strains, 2 linezolid resistant enterococci and 252 multidrug resistant enterococci (29).

In our study, 50 VRE strains isolated from different clinical care units were identified using the GP24 diagnostics species identification kit. Of the 50 strains, 48 (96%) were determined as *Enterococcus casseliflavus* and 2 strains (4%) were *Enterococcus faecium*. *Enterococcus faecalis* ATCC 29212 standard strain was used as the control strain and was identified as *E. faecalis* with 100% accuracy.

Surveillance follow-up should be performed to prevent healthcare-associated infections and to reduce the risk to patients, employees, and the environment. Infection control programs such as employee health, isolation, training of health personnel, infection prevention policies, and management should be established and implemented. Linezolid is an important antimicrobial agent in the treatment of VRE infections. Unneces-

sary use should be avoided so that resistance does not develop. In our study, the efficacy of linezolid on VRE strains was investigated and no linezolid resistance was found. This suggests that linezolid can safely be used in the treatment of VRE-induced infections. It will be important to carry out continuous and comprehensive studies on this subject and to monitor linezolid resistance surveillance.

Ethics Committee Approval: This study was approved by Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 23.09.2022, No: 17).

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

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HEALTH EDUCATION: TOWARDS THE AGE OF THE METAVERSE, HEALTH LITERACY GAME

SAĞLIK EĞİTİMİ: METAVERSE ÇAĞINA DOĞRU, SAĞLIK OKURYAZARLIĞI OYUNU

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ABSTRACT

Objective: The World Health Organization recommends people engage in settings where they can actively engage and take advantage of novel ideas and approaches in education to enhance their health literacy. Gamified systems are one of them. Also, The Metaverse concept has become widely popular, particularly amid the pandemic, as individuals resorted to virtual platforms for tasks such as work, education, shopping, and other pursuits. The opportunities presented by Metaverse are thrilling for both education and gamification, and they similarly apply to the improvement of health literacy. In this context, the present study was conducted to examine the effects of individuals with different demographic characteristics receiving education on health literacy in a game.

Material and Method: This study is a pre-test, post-test quasi-experimental study. Individuals over the age of 18 were included in the study with the convenience sampling method (n=199). A pre-test was given via Google Forms, and post-test data was obtained from a game.

Results: A statistically significant difference was detected between participants' post-test and pre-test scores ($p<0.001$). The findings revealed that the participant's level of education and age caused differences in the post-test scores ($p<0.001$). Finally, a statistically significant correlation was detected between body mass index (BMI) and post-test scores ($p<0.001$).

Conclusion: This study demonstrates that a game-based approach improves health literacy. The impact of the game varies across age groups and educational levels, indicating a need for customized strategies. A negative correlation between post-test scores and BMI suggests a potential link between health literacy and health outcomes.

Keywords: Gamification, health literacy, metaverse, health education

ÖZ

Amaç: Dünya Sağlık Örgütü, insanların sağlık okuryazarlıklarını artırmak için yeni fikirlerden ve yaklaşımlardan faydalanabilecekleri kapsayıcı eğitim ortamlarında aktif olarak yer almalarını önermektedir. Bu yaklaşımlara örnek olarak, oyunlaştırılmış sistemler gösterilebilir. Öte yandan, özellikle pandemi döneminde insanlar iş, eğitim, alışveriş ve diğer uğraşlar için sanal platformlara başvurduğundan, Metaverse kavramı geniş çapta popüler hale gelmiştir. Metaverse tarafından sunulan fırsatlar, eğitim ve oyunlaştırma için heyecan verici olmanın yanı sıra, sağlık okuryazarlığının geliştirilmesinde de benzer şekilde geçerlidir. Bu bağlamda, ilgili çalışma farklı demografik özelliklerdeki bireylerin sağlık okuryazarlığı konusunda eğitim aldıkları oyundaki etkilerini belirlemek için yapılmıştır.

Gereç ve Yöntem: Çalışma ön-test, son-test yarı deneysel bir çalışmadır. Çalışmaya 18 yaş üstü bireyler uygun örnekleme yöntemi ile dahil edilmiştir (n=199). Ön-test Google form aracılığıyla verilip son-test oyun içerisinde verilmiştir.

Bulgular: Araştırma katılımcılarının sağlık okuryazarlığı oyunu sonrasında post-test skorlarında ön-test skorlarına oranla istatistiksel olarak fark tespit edildi ($p<0,001$). Katılımcıların eğitim seviyelerinin ve yaşlarının son-test sonucu elde ettikleri skorlarda farklılıklara sebep olduğu bulundu ($p<0,001$). Son olarak da vücut kitle indeksi ile son-test skorları arasında istatiksel olarak anlamlı bir korelayson tespit edildi ($p<0,001$).

Sonuç: Bu çalışma, oyun temelli bir yaklaşımın sağlık okuryazarlığını geliştirmedeki etkinliğini göstermektedir, önemli ölçüde post-test gelişmeleri belirlenmiştir. Oyunun etkisi, yaş grupları ve eğitim seviyeleri arasında değişiklik gösterir, bu da özelleştirilmiş stratejilere ihtiyaç olduğunu gösterir. Post-test puanları ile BMI arasındaki negatif korelasyon, sağlık okuryazarlığı ile sağlık sonuçları arasında potansiyel bir bağlantıyı önermektedir.

Anahtar Kelimeler: Oyunlaştırma, sağlık okuryazarlığı, metaverse, sağlık eğitimi

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INTRODUCTION

Health education can improve communication between healthcare providers and recipients, promote correct resource utilization, and reduce health disparities, while also enhancing personal development, self-confidence, and social skills. Health literacy pertains to an individual's ability to understand grasp, utilize, and apply health-related information to make informed and suitable choices regarding their well-being. It encompasses having the necessary knowledge, competence, and social skills to navigate the healthcare decision-making process and make appropriate selections.

The World Health Organization (WHO) has defined health literacy as the cognitive and social skills that determine an individual's ability to access, understand, and use information in a way that contributes to maintaining good health. To increase the levels of health literacy in society, organizations, social associations, and individuals, the WHO has recommended specific actions, especially in the field of education (1). These encompass actions such as delivering crucial health information in early childhood, implementing rules in schools to foster positive behavior and attitudes toward health, engaging adult learners in health literacy education by igniting motivation and interest, utilizing multimedia resources that can captivate a broad audience, facilitating interactions and knowledge-sharing among individuals, and incorporating innovative approaches and techniques in health education. Adopting innovative and efficient learning tools, such as gamified systems, can also be beneficial for health literacy education and awareness.

Gamification is a method that employs digital game design strategies to enhance the effectiveness of the learning experience. It incorporates elements such as rewarding systems and competitive features commonly found in games. While there are various definitions of gamification, the concise definition proposed by Deterding and his team is widely acknowledged in academic literature. They define gamification as the integration of game elements into non-game contexts (2).

According to Sardi, Idri, and Fernández-Alemán gamified systems utilize core game elements like feedback, progression, social interaction, and incentives (3). These key features are further explained by Palmer, Lunceford, and Patton (4). Progress is based on completing achievements and missions with clear rules and visible stages, leading to an increase in gains and motivation for both expert and novice players. Reward and feedback are important indicators of success and failure that must be given promptly to motivate players. In contrast, socialization entails engaging with fellow users, which not only enhances communication but also boosts game competition and support, leading to a more fulfilling and enthusiastic gaming experience for players. Finally, the interface and user experience must be aesthetic, useful, and fun, and advancements in technology allow for the development of high-quality games through the internet, sensors, mobile devices, virtual reality, and augmented reality.

In the second half of 2010, gamification began to be used in various fields, particularly in industry and education. On the other hand, its adoption in the health sector has increased since 2014. Several scientific studies have been conducted on gamification in health, mainly in Canada, England, Finland, the Netherlands, Portugal, and the United States with very few studies in other countries (3).

Gamification is a technique that can enhance learners' motivation and focus during e-learning activities. By employing strategies that boost motivation, gamification can create valuable learning experiences that contribute to improved health literacy. A study conducted by Alahäivälä and Oinas-Kukkonen demonstrated the positive impact of "gamification in health behavior change support systems" (hBCSSs) for individuals with diverse health conditions (5). However, our research indicates a lack of serious games or gamified systems specifically designed for health literacy.

The literature contains many systematic reviews demonstrating that serious games and gamification elements can effectively induce changes in certain health behaviors by providing motivation, entertainment, and focus (6, 7). Johnson and his colleagues outlined seven potential advantages that can be derived from implementing gamification in the field of health, including the support of internal motivation, adaptation to mobile technologies and sensor-based data collection, the potential for widespread use, adaptability to various health domains, the ability to develop gamified systems, integration with existing activities, and the provision of positive experiences for users (8).

Davaris and colleagues conducted a literature review and came to similar conclusions (9). They found that digital health resources and gamification can enhance health literacy and support patients in preparing for surgery, as well as promote positive shifts in their perspectives regarding health issues. They suggest that gamified health care, which is a novel approach, has the potential to be a vital addition to patient-centered care.

The pandemic has accelerated the adoption of Metaverse platform applications, reigniting interest in gamification for online education. Furthermore, health literacy and gamification can facilitate the integration of personalized medicine and telemedicine applications supported by wearable technology. These advancements represent a significant shift in the current healthcare landscape, as highlighted by Bozbuğa (10). The concept of the "Metaverse" was initially coined by Neal Stephenson in his science fiction novel Snow Crash in 1992. In the book, Stephenson defines the Metaverse as a large virtual environment (11). Today, the term Metaverse generally refers to an online virtual world that enables users to connect and establish social and business relationships with a sense of immersion in a feeling of naturalness, both in online and offline contexts (12). The concept of the Metaverse has experienced a significant surge in popularity, particularly amid the pandemic, as individuals increasingly relied on online platforms for work, education, shopping, and various other activities (13). The Metaverse concept

is based on the idea of blending virtual and physical worlds to create unique interactions and experiences.

Schoenfeld claimed that using augmented reality in education can enhance students' analytical skills and performance (14). Furthermore, new models of Metaverse-powered distance education, such as 3D virtual campuses, can offer hybrid formal and informal educational opportunities (15). Some Metaverse-based virtual education sites have utilized gamification to teach about future social issues like climate change, low fertility, and aging, and even allowed officials to design a virtual smart city to address these issues (16).

Butt and colleagues argue that virtual reality, especially when combined with haptic technology and game-based learning, can revolutionize learning and retention (17). Considering the demand for more authentic distance learning settings in the aftermath of COVID-19, Metaverse platforms incorporating VR and AR technologies seem to be well-suited. While the Metaverse phenomenon may present potential problems, it is expected to become more prevalent, particularly in areas like education and health.

Metaverse can be used for laboratory applications, procedural skills development, and STEM education, and gamification can offer significant benefits by providing an immersive and engaging environment (18, 19). Metaverse is more flexible than traditional games and can provide global systems without user limits (20). The integration of gamification within the Metaverse has the potential to establish broader and enduring virtual environments that attract a larger user base, primarily focusing on fostering social interactions (21).

Metaverse has exciting opportunities in education and gamification, and the same applies to health literacy, which is the focus of this study. The Metaverse platform can offer users a more realistic and tangible experience, leading to possible gains in health literacy. The goal is to bring people a better life through the conveniences and opportunities offered by Metaverse environments while considering possible drawbacks.

Given all these, this study examines the gains of players with different demographic characteristics from a game designed for health literacy education. Additionally, it would be beneficial to look at the book chapter by the authors of the relevant study for a more comprehensive literature review (22).

MATERIAL and METHOD

Design and participants

A pre-test and post-test, quasi-experimental study was employed, as described by Creswell (23). The study examined the impact of the health literacy game on health literacy learning, with participants' age range, body mass index (BMI), and education level serving as independent variables. Convenience sampling was used due to the difficulty of obtaining random participants. Participants volunteered and had not previously played the health literacy game. A total of 225 participants were included in the data collection. 26 participants were excluded from the study for not having post-test scores.

Educational game

There were three different types of game versions prepared by using Unity. The only difference between the versions is that each one targets a specific age group: 18-25 for university, 25-65 for the workplace, and over 65 for home situations related to health literacy and media use. However, all versions have common situations, which aim to achieve the same learning objectives about health literacy. The learning material was reviewed for validity by Prof. Dr. Nilgün Bozbuğa. The game's instruction language is Turkish.

The intended learning outcomes for participants in the game are as follows: They can find information about the symptoms of commonly encountered diseases, know what to do in a medical emergency, understand at a basic level what the doctor says about the disease and its treatment, comprehend information given in the media about being healthier, find information about the treatment of mental health issues, and understand the importance of participating in physical activities, and so on.

Each version of the game is divided into six segments, modeling 3D environments of places people commonly encounter in their daily lives, such as homes, workplaces, schools, restaurants, markets, and hospitals. The game's scenario was designed to attract participants' attention to the learning materials by considering their age and background knowledge.

In the game, the player navigates these environments through the eyes of an avatar and encounters situations related to health literacy, prompting them to make choices based on these situations. Positive points are given for each correct decision and negative points for incorrect choices. Players also receive appropriate feedback for their decisions. The game records the players' total points on a health literacy certificate, along with the time spent playing the game, which is displayed in real-time. Another feature is that the game tracks the player's decisions during gameplay and presents them as a test at the end of the game for any incorrect decisions made, making the game personalized to each player.

Data collection and instruments

Before gathering and processing the data, ethical clearance was secured from the İstanbul University's Ethics Committee for Social and Human Sciences Research (Date: 24.04.2023, No: 1735456). 225 people participated in the study. Data from 199 people were taken into account. The research was carried out in two separate sessions; google Forms and Game. The research employed two data collection tools: (1) a pre-test administered through Google Form to assess participants' initial health literacy knowledge and (2) a post-test (corresponding to the pre-test) that evaluated students' understanding of health literacy through a game. Both tests featured 25 multiple-choice questions, with options ranging from two to four alternatives.

Statistical analyses

To address the research inquiries, a variety of statistical tests were performed. The data underwent a verification process to ensure that each participant had complete data for all four measurements, including age level, BMI, education level, pre-test score, and post-test score. Participants who were missing any of these data points were excluded from the study, resulting in the removal of twenty-six participants. IBM SPSS statistical software (Version 27) was utilized for all statistical analyses.

Initially, the data were checked for normality. The Kolmogorov-Smirnov test was employed to evaluate the normality of the quantitative data. The study utilized the Wilcoxon signed-rank test to analyze and compare the average scores of the participants' pre-test and post-test results. To compare data from three groups, the Kruskal-Wallis H test was employed for non-normally distributed variables. Spearman's correlation analysis was utilized to determine the level of correlation between quantitative variables, between non-normally distributed variables. A significance level of $p<0.05$ was accepted.

RESULTS

Differences between pre-test and post-test

Table 1 presents the demographic information of the participants included in the study. To examine whether the pre-test and post-test scores of the participants followed a normal distribution, the Kolmogorov-Smirnov test was employed. The results indicated that the data for both the pre-test and post-test scores in the current study ($p>0.05$) deviated from a normal distribution, as shown in Table 2.

Table 1: Demographic data of participants

	N (%)
Age	
18-25	59 (29.65)
25-65	60 (30.15)
65-65+	80 (40.20)
Education	
Primary Education	58 (29.14)
High School	77 (38.70)
University/Undergraduate	64 (32.16)
Body Mass Index	
Underweight (below 18.5)	2 (1.01)
Normal weight (18.5-24.9)	83 (41.70)
Pre-obesity (25.0-29.9)	65 (32.67)
Obesity class I (30.0-34.9)	34 (17.08)
Obesity class II (35.0-39.9)	12 (6.04)
Obesity class III (above 40.0)	3 (1.50)

N: Number

Table 2: Tests of normality (pre-test, post-test)

	Statistic	df	Sig.
Pre-test	.091	199	<.0001
Post-test	.155	199	<.0001

df: Degree of freedom, Sig.: Significance

The Wilcoxon signed-rank test was conducted to compare the means of the pre-test and post-test scores among the participants. The results of this test revealed that there was a statistically significant difference observed in the scores of participants between the pre-test and post-test phases who engaged in the health literacy game ($Z=-12.212$, $p<0.001$). Descriptive statistics of the pre-test and post-test scores of the participants are presented in Table 3.

Table 3: Descriptive statistics of pre-test and post-test

	N	Mean	SD	Minimum	Maximum
Pre-test	199	15.88	4.175	8	25
Post-test	199	22.30	2.249	18	25

N: Number, SD: Standard deviation

Post-test scores of groups formed according to educational levels.

To examine the normal distribution of the post-test scores among the groups, the Kolmogorov-Smirnov test was conducted. However, the results indicated that the post-test scores for all educational groups ($p>.05$) did not follow a normal distribution, as presented in Table 4 based on the analysis.

The Kruskal-Wallis H test was utilized to compare the average post-test scores among the different groups. The results of this test indicated a statistically significant difference among the post-test scores of the groups, $\chi2\ (2)=84.989$, $p<0.001$. The mean rank post-test score was 49.78 for the primary education group, 100.49 for the high school education group, and 144.93 for the university education group, as presented in Table 4.

Table 4: Tests of normality and ranks of post-test (group by educational level)

Education level	Statistic	df	Sig.	N	Mean Rank
Primary education	.191	58	<.0001	58	49.78
High school	.190	77	<.0001	77	100.49
University	.289	64	<.0001	64	144.93

df: Degree of freedom, Sig.: Significance, N: Number

Post-test scores of groups formed according to age levels.

To assess the normal distribution of the post-test scores among the groups, the Kolmogorov-Smirnov test was conducted. However, the results indicated that the data of post-test scores for all age groups ($p>.05$) did not demonstrate a normal distribution, as presented in Table 5 based on the analysis.

The Kruskal-Wallis H test was utilized to compare the average post-test scores among the different groups. The results of this test indicated a statistically significant difference among the post-test scores of the groups, $\chi2\ (2)=40.702$, $p<0.001$. The mean rank post-test score was 115.79 for the 18-25 age group, 125.87 for

the 25-65 age group, and 68.98 for the 65-65+ age group, as presented in Table 5.

Table 5: Tests of normality ranks of post-test (group by age level)

Age	Statistic	df	Sig.	N	Mean Rank
18-25	.204	59	<.0001	59	115.79
25-65	.213	60	<.0001	60	125.87
65-65+	.146	80	<.0001	80	68.96

df: Degree of freedom, Sig.: Significance, N: Number

Correlation between BMI and post-test scores

To examine whether the BMI scores and post-test scores of the participants followed a normal distribution, the Kolmogorov-Smirnov test was employed. The results indicated that the data for both the BMI scores and post-test scores in the current study ($p>0.05$) deviated from a normal distribution, as shown in Table 6.

Table 6: Tests of normality (post-test and BMI)

	Statistic	df	Sig.
Post-test	.155	199	<.0001
BMI	.074	199	.011

BMI: Body Mass Index, df: Degree of freedom, Sig.: Significance

The Spearman's rank-order correlation test was run to determine the relationship between participants' BMI scores and their post-test scores. There was a strong, negative correlation between participants' BMI scores and their post-test scores, which was statistically significant ($r_s(197)=-.306$, $p<0.001$), as presented in Table 7.

Table 7: Correlations between BMI and post-test

		Post-test	BMI
Post-test	Correlation Coefficient	1.000	-.306[a]
	Sig. (2-tailed)	.	<.001
	N	199	199
BMI	Correlation Coefficient	-.306[a]	1.000
	Sig. (2-tailed)	<.001	.
	N	199	199

BMI: Body Mass Index, Sig.: Significance, N: Number, [a]: Spearman's Rank-Order Correlation

DISCUSSION

In the study, we tested users before and after playing the game developed to enhance health literacy. Using a predefined treatment protocol, we evaluated users' levels of health literacy. We found a statistically significant difference between users' pre-test scores and post-test scores ($p<0.05$). These results indicate the potential of game-based approaches in improving health literacy. This finding is parallel with Alahäivälä and Oinas-Kukkonen's assertion that gamification positively contributes to making correct decisions about health (5). These findings may stem from some potential benefits of gamification as suggested by Johnson and colleagues (8). Firstly, the game provides users with interactive experiences while presenting health-related information. This interactive experience captures users' attention and encourages the learning process. Additionally, the competitive and enjoyable nature of the game may motivate users to spend more time and learn the information within the game more effectively. This research encompasses a study conducted to evaluate the influence of a game developed on health literacy. Davaris and colleagues also suggested in their 2021 literature review study that gamification would increase the level of health literacy (9). These results are supportive of their claims. It can also be seen as one of the potential actions to increase the level of health literacy in the community, an issue that Kickbusch and colleagues have emphasized (1).

In the study, we aimed to evaluate the effects of a game developed for health literacy by examining differences among young, middle-aged, and elderly individuals. When analyzing pre-test and post-test scores, we observed a significant difference among these three age groups. These results indicate that the impact of the game on health literacy levels may vary across different age groups. Older individuals scored lower on the post-test. This result supports the claim of the metric analysis conducted by Qi and colleagues on studies between the years 1995-2020 (24). One primary factor may be the variation in technology use and digital literacy levels among different age groups. The younger generation is generally more familiar with digital technologies and may engage with the game more easily, while older individuals may have limited digital skills and access to technology. These disparities can influence the effect of the game across age groups. However, it is important to note that the differences among age groups may also be associated with other factors. Health literacy can be influenced by factors such as experience, education, and the development of health awareness, which can vary with age.

In addition, we examined the differences among different groups based on educational levels to evaluate the impact of a game developed for health literacy. When analyzing pre-test and post-test scores, we observed a significant difference among the groups with different educational levels. These results indicate that educational level plays a role in health literacy levels and that the effect of the game may vary depending on the educational level. Likewise, in a survey study conducted by Frıis and colleagues in 2016 on 29,473 Danish citizens over the age of 25, they also concluded that health literacy was indirectly positively affected by the level of education (25). We can provide some explanations for the differences among groups with different educational levels. Individuals with higher educational levels often possess better reading, research, and information-processing skills. This can make it easier for them to adapt to the provided health literacy information and achieve the objectives of the game more effectively. On the other hand, individuals with lower educational levels may have limited access to information and a lower capacity for understan-

ding, which can reduce the impact of the game. In addition to educational level differences, it is important to consider other variables such as socioeconomic status, language skills, and cultural factors that can also influence health literacy.

Finally, we aimed to evaluate the impact of the health literacy game developed by examining the relationship between users' post-test scores and their BMI. The analysis revealed a negative correlation between users' post-test scores and their BMI, indicating that as BMI increased, their health literacy scores tended to decrease. Similarly, Toçi and his colleagues conducted a study in 2019, in which they examined the relationship between health literacy and BMI in Tirana, Albania (26). The study included 1154 participants aged 18 and above. According to the data they gathered, they found a strong negative correlation between health literacy and BMI. It could be attributed to several factors. Firstly, individuals with higher BMIs may be more prone to health-related issues and may have limited access to health information or face challenges in understanding and interpreting health-related content. This could result in lower health literacy scores. Conversely, individuals with lower BMIs may exhibit higher health literacy scores, potentially due to their proactive approach to maintaining a healthy lifestyle and seeking health-related knowledge. Other factors such as socioeconomic status, cultural influences, and education can also contribute to the relationship between BMI and health literacy.

However, this study has some limitations relating to its sampling and methodological approach. The findings should be prudently extrapolated to a broader participant population. It employed a convenience sampling technique. Therefore, to enhance the applicability of these findings, it is advisable to repeat the present study using a genuinely experimental design. On the other hand, conducting further subgroup analyses can provide a better understanding of specific differences within the age groups. For instance, the impact of factors such as gender, educational level, health status, socioeconomic status, or access to technology on the differences among age groups could be explored. Finally, conducting the same research with a professional game development team to create a more realistic and technically comprehensive game could provide more diverse and profound findings.

CONCLUSION

In conclusion, this study provides compelling evidence for the effectiveness of a game-based approach in enhancing health literacy. The significant improvement in post-test scores compared to pre-test scores underscores the potential of gamification in health literacy education. The study also highlights the differential impact of the game across various age groups and educational levels, suggesting the need for tailored strategies in promoting health literacy. Furthermore, the observed negative correlation between users' post-test scores and their BMI provides an intriguing avenue for future research, potentially linking health literacy to health outcomes. As we enter the Metaverse era, the development and presence of health literacy games in this environment will greatly contribute to individuals' educational development in health. Particularly, their existence in other health-related topics can significantly enhance individuals' understanding and knowledge about health. However, it is crucial to acknowledge that this study is not without limitations, including its sampling method and the need for a more comprehensive game development process. Future research should aim to address these limitations and further explore the potential of gamification in health education. Ultimately, this study adds to the expanding body of evidence that endorses the utilization of innovative, technology-driven approaches in health education and promotion.

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VOLUMETRIC ANALYSIS OF OSTEOMAS OF THE SPHENOID SINUS USING CONE BEAM COMPUTED TOMOGRAPHY

KONİK IŞINLI BİLGİSAYARLI TOMOGRAFİ KULLANILARAK SFENOİD SINÜS OSTEOMLARININ HACİMSEL ANALİZİ

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ABSTRACT

Objectives: Osteomas are benign bone tumors characterized by slow growth, well-defined borders, and often asymptomatic presentation, frequently incidentally identified through radiography. Sphenoid sinus osteomas are particularly rare occurrences within the paranasal sinuses. This study aims to investigate the incidence and volumetric characteristics of sphenoid sinus osteomas.

Material and Methods: This retrospective analysis involved the examination of cone-beam computed tomography (CBCT) images obtained from patients referred to the Department of Oral and Maxillofacial Radiology for various complaints. Two radiologists independently reviewed the images, recording instances of sphenoid sinus osteomas, their volumes, and patient demographics, which were subsequently subjected to statistical analysis.

Results: A total of 1466 tomography images (821 females, 645 males) were assessed. Among these, 23 osteomas were identified within the sphenoid sinuses of 17 patients (eight females, nine males). The mean volume of these osteomas was 183.59 mm^3±168.56 (with a range of 9.98 to 552.80). No statistically significant difference in sphenoid osteoma volumes between males and females was observed (Mann-Whitney U test, U=30.000, p=0.564, z=-0.577).

Conclusion: Consistent with existing literature, the incidence of sphenoid sinus osteomas in this study was found to be 1.15%. While asymptomatic osteomas detected via radiography typically do not warrant surgical intervention, regular radiographic follow-up is recommended to monitor for potential complications.

Keywords: Cone-beam computed tomography, osteoma, sphenoid sinus

ÖZ

Amaç: Osteomlar, yavaş büyüme, iyi tanımlanmış sınırlar ve sıklıkla asemptomatik görünüm ile karakterize, sıklıkla tesadüfen radyografi ile tanımlanan iyi huylu kemik tümörleridir. Sfenoid sinüs osteomu paranazal sinüste görülen en nadir osteomlardır. Bu çalışmanın amacı sfenoid sinüs osteomlarının sıklığını ve volümetrik analizini araştırmaktır.

Gereç ve Yöntemler: Bu retrospektif analiz, Ağız Diş ve Çene Radyolojisi Bölümü'ne çeşitli şikayetler nedeniyle başvuran hastalardan elde edilen konik ışınlı bilgisayarlı tomografi (KIBT) görüntülerinin incelenmesini içeriyordu. İki radyolog görüntüleri bağımsız olarak inceledi, sfenoid sinüs osteomlarının örneklerini, hacimlerini ve hasta demografik özelliklerini kaydetti ve bunlar daha sonra istatistiksel analize tabi tutuldu.

Bulgular: 1466 (821 kadın, 645 erkek) hastanın tomografi görüntüleri değerlendirildi. 17 hastanın (Sekiz kadın, dokuz erkek) sfenoid sinüslerinde toplam 23 osteom saptandı. Ortalama osteoma hacmi 183,59 mm^3±168,56 (min. 9,98, maks. 552,80) idi. Erkek ve kadın sfenoid osteoma hacimleri arasında anlamlı fark bulunamadı (Mann-Whitney U testi, U=30,000, p=0,564, z=-0,577).

Sonuç: Literatüre uygun olarak bu çalışmada sfenoid sinüste osteom görülme sıklığı %1,15 olarak bulundu. Radyografide asemptomatik saptanan osteomlarda cerrahi tedavi önerilmemekle birlikte olası komplikasyon riskine karşı periyodik radyografik muayene ile takip edilmelidir.

Anahtar Kelimeler: Konik ışınlı bilgisayarlı tomografi, osteoma, sfenoid sinüs

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INTRODUCTION

Osteomas are generally benign, slow-growing bone tumors often discovered incidentally due to their asymptomatic nature. They are commonly found in the paranasal sinuses (PNS) and the mandible within the head and neck region (1-3). Osteomas can be composed of cancellous bone, cortical bone, or a combination of both (4, 5). While Veiga's pioneering report in 1586 marked the initial removal of a frontal sinus tumor, subsequent literature began featuring cases of osteomas (6). Although osteomas are the prevailing benign PNS tumors, they frequently remain symptomless, emerging only as chance findings in radiographic assessments (7, 8). Sinus osteomas initiate from the sinus walls and develop toward the sinus cavity.

Among the PNS, osteomas predominantly appear in the frontal sinus, followed by the ethmoid, maxillary, and sphenoid sinuses in decreasing order of prevalence (3, 7, 9). The literature predominantly consists of case reports detailing sphenoid sinus osteomas, while scant resources address the frequency of these occurrences. This study endeavors to assess the incidence and volumes of sphenoid sinus osteomas using cone beam computed tomography (CBCT) scans.

MATERIALS and METHODS

Ethical approval for this study was granted by the Clinical Research Ethics Committee of Istanbul University, Faculty of Dentistry (Date: 30.04.2021, No: 2021/24). CBCT images from patients attending our clinic between December 2015 and October 2019 were reviewed, and only images capturing the sphenoid sinus within the field of view (FOV) were included.

A total of 1466 patients (821 females and 641 males) aged 18 to 94 were retrospectively evaluated using CBCT images. Patients with prior pathologies or surgical procedures in the evaluation site, syndromic conditions, and images marred by image-quality impairing artifacts were excluded. CBCT images, acquired via a Scanora® 3Dx CBCT device (Soredex, Tuusula, Finland), were analyzed using OnDemand 3D™ software (Cybermed, California, USA) on a medical monitor. Images with a FOV size of 14x16 and a slice thickness of 0.2 mm were evaluated.

Two- and five-year dentomaxillofacial specialists assessed and measured the variables. A senior oral and maxillofacial radiologist with over 30 years of experience made the final determinations. The CBCT images were reviewed in axial, sagittal, and coronal sections. Osteoma presence within the sphenoid sinus, patients' age and gender details, and volumetric measurements were recorded (Figure 1-3). The ITK-SNAP (Penn Image Computing and Science Laboratory) software was employed for osteoma volume measurements.

Statistical analysis entailed using the chi-square test for categorical variable ratios, while the Mann-Whitney U test gauged the correlation between sphenoid osteoma volume and gender. Intra- and inter-examiner reliability was evaluated using Kappa tests. A significance level of $p<0.05$ was set.

RESULTS

This study encompassed 1466 patients (821 females, 645 males) with an average age of 42.88±17.704 (range: 18 to 94). Sphenoid osteomas were identified in 17 patients (1.15%) with an average age of 32.29±12.922 (Range: 20 to 67). The distribution of the patients by gender is outlined in Table 1. No statistically significant difference emerged between osteoma presence and gender ($p>0.05$).

While single osteomas were observed in 13 patients, four patients presented multiple osteomas (3 osteomas in 2 patients, 2 osteomas in 2 patients). Among these four patients, two were female, and two were male.

The average osteoma volume across the 17 patients was 183.59 mm^3±168.56 (range: 9.98 to 552.80, median: 120.10, range: 543). The volumetric analysis based on gender is displayed in

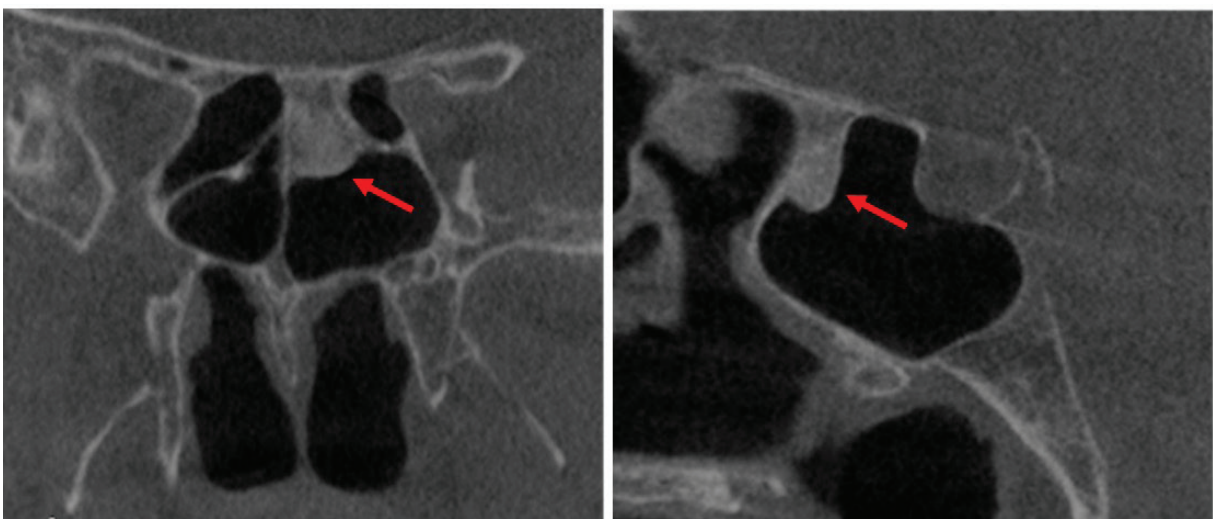

Figure 1: Osteoma located on the left sphenoid sinus

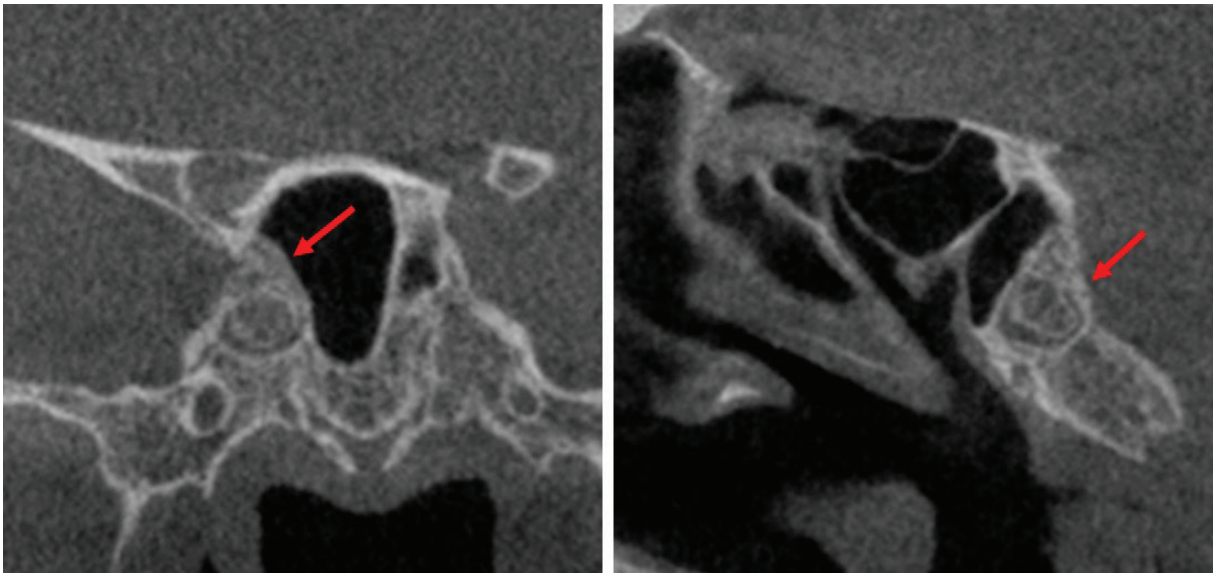

Figure 2: Osteoma located on the right sphenoid sinus base

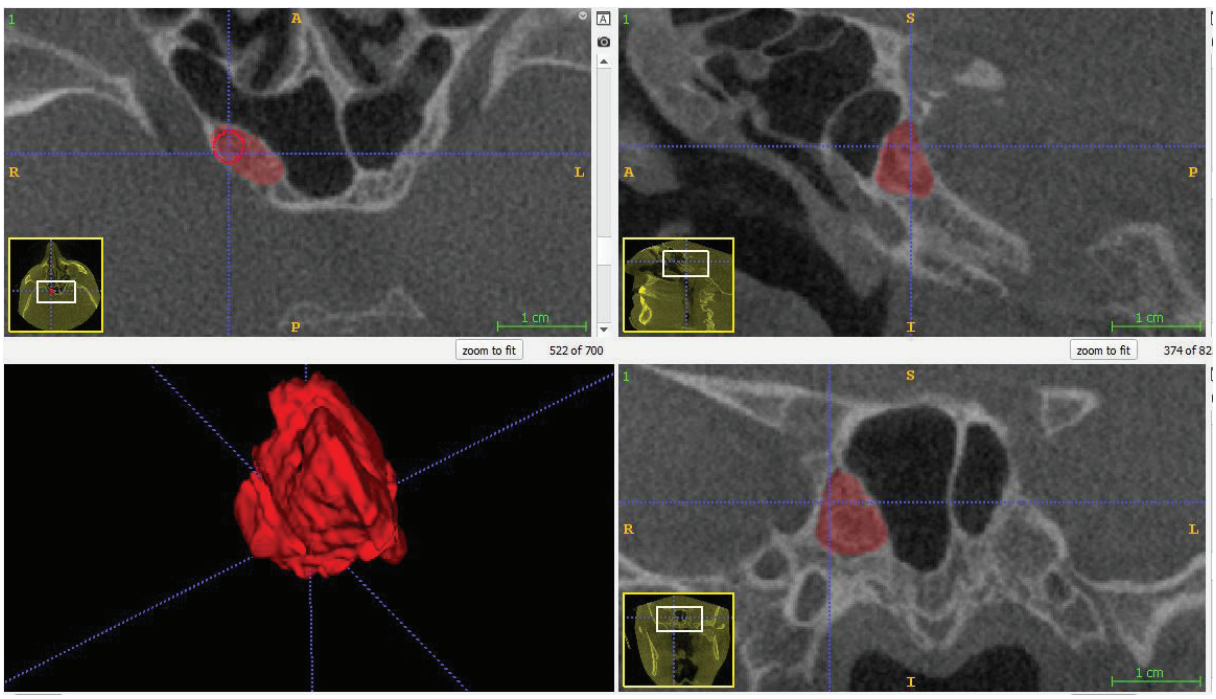

Figure 3: Volumetric measurements of the osteoma in Figure 2

Table 2. Mann-Whitney U test results indicated no significant disparity in sphenoid osteoma volumes between males and females (U=30.000, p=0.564, z=-0.577).

Intra- and inter-observer agreement showed strong concordance ranging from 81% to 90%.

DISCUSSION

The present study unveils a 1.15% occurrence rate for sphenoid sinus osteomas. Additionally, CBCT images of the 17 patients exhibiting radiologically diagnosed sphenoid osteomas were scrutinized. The detected sphenoid osteoma frequency aligns with reported PNS osteoma prevalence figures; however, specific investigations into sphenoid sinus osteomas remain limited.

The literature reports PNS osteoma prevalence ranging from 0.01% to 3%, signifying their rare nature (1, 3, 9, 10). The exact etiology behind PNS osteomas remains elusive, though they are speculated to result from craniofacial trauma, abnormal embryonic tissue growth, chronic nasal and PNS inflammation, calcification of polyps, metaplasia, calcium metabolism alterations, and heredity (1, 2, 7, 11).

Compact osteomas, also referred to as “ivory” osteomas, consist of fully developed lamellar bone characterized by minimal

Table 1: Distribution of osteoma according to gender.

Presence of osteoma	Gender		Total	p value
	Female	Male		
Absent	813	636	1449	0.616*
Present	8	9	17	
Total	821	645	1466	

*Yates Chi Square Test

Table 2: Volumetric analysis of sphenoid sinus osteomas according to gender.

Volume (mm^3)	Mean±SD [Min-Max]	*p
Male (n=9)	163.96±165.64 [9.99-552.80]	
Female (n=8)	205.67±180.38 [32.02-509.00]	0.564
Total (n=17)	183.59±168.56 [9.99-552.80]	

*Mann-Whitney U test

marrow spaces and occasional haversian canals, lacking any fibrous structure. Computed Tomography (CT) stands as the preferred imaging technique for investigating osteomas. It effectively identifies their "ivory-like" appearance, facilitating differentiation from other bone disorders and supporting presurgical assessments (12-14).

Over the past two decades, CBCT has gained significant traction in diagnosing and planning treatment for craniofacial osteomas. CBCT offers the advantages of requiring lower radiation exposure compared to conventional multi-detector CT scans (MDCT), ensuring precise spatial resolution, and enabling multiplanar reconstructions for comprehensive pre- and post-treatment evaluations (14-19).

While PNS osteomas can emerge at any age, they are more common within the 4th to 6th decades and in males (male-to-female ratio of 1.3:1 to 3.1:1) (1, 6, 7, 10). In our study, 10 out of the 17 patients with sphenoid osteomas were within their third decade. This age divergence might be attributed to the potential detection of even small osteomas using thin CBCT sectioning.

Due to their slow growth patterns, only around 10% of osteomas produce clinical symptoms (11). Among these symptoms, facial pain and headaches rank prominently. Headache frequency varies between 52% and 100% across various osteoma series (6-9). Osteomas can also lead to severe complications like facial deformity, anosmia, diplopia, proptosis, and even vision loss due to pressure exerted on the optic nerve (11, 20).

While osteomas can present as isolated occurrences, they may also be symptomatic components of Gardner syndrome, an autosomal dominant hereditary disorder characterized by osteomas, intestinal polyposis, epidermal cysts, and fibromatosis. Typically, multiple and appearing in the jaw, skull, and long bones, these osteomas can also manifest within the PNS (1, 21, 22). In this study, 23.52% of osteoma patients displayed multiple osteomas within the sphenoid sinus. Notably, secondary mucocele development has been linked to PNS osteomas. Although slow-growing and often asymptomatic, osteomas can lead to aggressive behavior when concurrent with mucoceles (23, 24).

Radiologically, osteomas present as well-defined, hyperdense masses that are generally round or oval and homogenous (3, 9). Differential diagnoses encompass other bone tumors, fibrous dysplasia, and ossifying fibroma (2, 3). Computed tomography is the diagnostic gold standard, particularly in surgical decision-making. Magnetic resonance imaging aids in differential diagnosis, especially for cases involving intracranial or intraorbital soft tissue expansion (4, 9). The volumetric analysis of osteomas is clinically significant, and CBCT proves valuable for preoperative assessment (25).

While the treatment approach for PNS osteomas is disputed, asymptomatic small osteomas, particularly in older patients, are typically monitored clinically and radiographically over time. Surgical intervention is advised if certain criteria are met, such as rapid growth (>1 mm/year), size exceeding 50% of the sinus volume, extension into intracranial or intraorbital structures, symptomatic manifestations, bone erosion and facial deformity, chronic sinusitis and mucocele induction (9-11, 26). Surgical objectives center on removing the lesion without harming adjacent structures. Surgical strategies hinge on osteoma location, size, and extent, alongside surgeon expertise (2, 9, 27). Asymptomatic and small osteomas generally do not warrant surgical intervention, yet regular imaging follow-up at 1 to 2 year intervals is recommended to mitigate potential complications (2, 3).

CONCLUSION

The infrequently encountered sphenoid sinus osteoma holds critical importance in surgical decision-making, with CBCT serving as a vital detection tool. The study reveals a 1.15% osteoma incidence within the sphenoid sinus via CBCT. Utilizing CBCT for volumetric analysis aids in precise surgical planning and improved postoperative clinical-radiological monitoring. The research highlights radiological attributes tied to osteoma volume and underscores the value of CBCT volumetric analysis in crafting treatment strategies for critically affected patients.

Ethics Committee Approval: This study was approved by İstanbul University, Faculty of Dentistry (Date: 30.04.2021, No: 2021/24).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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THE FEAR OF ARTIFICIAL INTELLIGENCE: DENTISTS AND THE ANXIETY OF THE UNKNOWN

YAPAY ZEKA KORKUSU: DİŞ HEKİMLERİ VE BİLİNMEYENİN KAYGISI

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ABSTRACT

Objective: Artificial Intelligence (AI) has the potential to improve patient care and treatment outcomes; however, it also raises concerns about job security, ethical issues, and the impact on the quality of care provided. It is important to investigate the attitudes and concerns of dental professionals towards AI to develop effective strategies for its implementation that ensure patient safety and quality of care while also addressing the concerns of dental professionals. This study aimed to explore the levels of AI anxiety (AIA) experienced by dentists and to investigate the influence of various factors.

Materials and Methods: Data were collected online from 328 dentists (116 males, and 212 females) regarding their age, sex, marital status, field of specialization, and years of professional experience. The levels of AIA among the participants were assessed using the Artificial Intelligence Anxiety Scale (AIAS).

Results: The Dentists participated in the survey, revealing a moderate level of AIA (65.60±28.55). The AIA levels were significantly higher in females compared to males ($p<0.05$). Prosthodontists exhibited the highest levels of AIA (75.63±34.86), whereas restorative dentists showed the lowest levels (44.63±12.50). AIA did not show any significant correlations with age or length of work in the profession ($p>0.05$). There were correlations between AIA and all sub-dimensions, as well as among the sub-dimensions themselves ($p<0.01$).

Conclusion: Although dentists experience moderate levels of anxiety toward AI, they must acquire the knowledge and skills required to effectively utilize this innovative technology for their benefit.

Keywords: Anxiety, artificial intelligence, artificial intelligence anxiety, dentists.

ÖZ

Amaç: Diş hekimliğinde Yapay Zeka (YZ), hasta bakımını ve tedavi sonuçlarını iyileştirme potansiyeline sahiptir, ancak aynı zamanda iş güvenliği, etik sorunlar ve sağlanan bakımın kalitesi üzerindeki etkisi hakkında endişeler doğurur. Bu nedenle, diş hekimlerinin endişelerini ele alırken aynı zamanda hasta güvenliğini ve bakım kalitesini sağlayan etkili stratejiler geliştirmek için diş hekimlerinin YZ'ye yönelik tutumlarını ve endişelerini araştırmak önemlidir. Bu çalışmanın amacı, çeşitli seçilmiş faktörlerin etkisini araştırırken aynı zamanda diş hekimlerinin yaşadığı YZ kaygı (YZK) düzeylerini araştırmaktı.

Gereç ve Yöntem Bu çalışma için diş hekimlerinden yaş, cinsiyet, medeni durum, uzmanlık alanı ve mesleki deneyim yılına ilişkin veriler çevrimiçi olarak toplanmıştır. Katılımcıların YZK düzeyleri Yapay Zeka Kaygı Ölçeği (YZKÖ) kullanılarak değerlendirildi.

Bulgular: Ankete 116 erkek ve 212 kadın olmak üzere 328 diş hekimi katılmıştır ve orta düzeyde YZK (65,60±28,55) ortaya çıkmıştır. YZK düzeyleri kadınlarda erkeklerden anlamlı olarak yüksekti. ($p<0,05$). Protez uzmanları en yüksek YZK seviyelerini (75,63±34,86) sergilerken, restoratif diş hekimleri en düşük seviyeleri (44,63±12,50) gösterdi. YZK, yaş veya meslekte çalışma süresi ile anlamlı bir ilişki göstermedi ($p>0,05$). YZK ile tüm alt boyutlar arasında ve alt boyutların kendi aralarında da korelasyon vardı ($p<0,01$). Cronbach's Alpha tüm maddeler için 0,96 idi.

Sonuç: Diş hekimleri yapay zekaya karşı orta düzeyde kaygı yaşasalar da, inovatif teknolojiyi kendi yararlarına etkili bir şekilde kullanmak için gerekli bilgi ve becerileri edinmeleri çok önemlidir.

Anahtar Kelimeler: Kaygı, yapay zeka, yapay zeka kaygısı, diş hekimleri.

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INTRODUCTION

Artificial intelligence (AI) is advancing rapidly, and its impact on dentistry is uncertain. Dentists are anxious about the potential impact of AI on their profession, as AI can automate many of the tasks that dentists perform, such as diagnosing dental diseases and performing dental procedures. AI could lead to a decrease in the cost of dental care, as it could be a more cost-effective and efficient alternative (1). Additionally, AI can be used to analyze large amounts of data and to identify patterns that may not be visible to dentists (2,3). Thus, AI can lead to more accurate diagnoses and treatments, resulting in improved patient outcomes (4,5). However, AI is unlikely to completely replace dentists, as AI cannot provide the same level of care and empathy that a dentist can (6).

Dentists are increasingly turning to AI to help improve their practice (7-10). However, with this new technology comes a variety of anxieties that can affect dentists' comfort levels. This can be caused by several factors, including lack of training, unfamiliarity with new technology, and fear of the unknown. This fear can lead to a reluctance to adopt AI technology, which can hurt patient care. Without embracing new technologies, dentists may be unable to provide the best possible care to their patients. In addition, they may miss out on opportunities to improve efficiency and reduce costs.

Dentists may experience various anxieties related to AI, including fear of the unknown, uncertainty about the benefits of AI in their practice, fear of job loss due to AI, potential mistakes when using AI, and concerns about the security of their data. The negative implications of AI anxiety (AIA) can extend to both dentists and their patients, making it imperative to explore the underlying reasons for this anxiety. Hence, this study aimed to evaluate AIA levels among dentists and investigate the potential influence of various contributing factors.

MATERIALS and METHODS

The conformance of the study to the ethical guidelines of the Helsinki Declaration was approved by the Non-Interventional Clinical Research Ethics Committee of Biruni University (Date: 27.05.2022, No:2022/70-13). Based on the mean and standard deviation of the AIA level (76.30±27.87) in a previous study a minimum required sample size of 326 to achieve a power of 95% and a significance level (α) of 5% using the software R program (Version 4.1.3) (11).

This online descriptive study was carried out among dentists practicing in the province of Istanbul. Before participation, all potential participants were informed about the study and provided with online informed consent. By proceeding to and completing the online survey, participants implicitly gave their consent. The survey was designed using a Google Form and utilized a forced-choice format to minimize missing data. From June to July 2022, the survey was distributed electronically via email and WhatsApp. To ensure confidentiality, all participants were anonymized, and no personal information was collected. The socio-demographic data on age, sex, marital status, field of specialization (i.e., general practitioner/dental specialties), and years of experience in the profession were collected from the participating dentists (n=328). The dentists' level of artificial intelligence anxiety was assessed using the AIA Scale (AIAS).

Artificial Intelligence Anxiety Scale

The AIAS was developed by Wang and Wang and adapted into Turkish by Terzi (12,13). The Cronbach's alpha reliability coefficient was 0.96 for the complete scale, which indicates that the tool is reliable and valid. The 7-point Likert type (strongly disagree-strongly agree) scale consists of 21 items and four sub-dimensions: learning, job replacement, sociotechnical blindness, and AI configuration. The Learning sub-dimension (L) assesses anxiety levels when learning about AI applications in one's career. The Job Replacement sub-dimension (J) measures the anxiety levels experienced by individuals who could potentially face job loss due to AI. The Sociotechnical Blindness sub-dimension (S) evaluates anxiety levels in those who do not fully understand that AI requires human cooperation and social institutions. The AI Configuration sub-dimension (C) measures anxiety levels in those who find AI techniques/products scary and intimidating. Scores on the scale range from 21 (lowest) to 147 (highest). Permission was obtained from the author, who adapted the scale into Turkish via e-mail.

Statistics

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc., version 17, Chicago, IL, USA). The normality of data distribution was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Differences between two groups were analyzed using the Mann-Whitney U test, while differences between more than two groups were compared using the Kruskal Wallis test, followed by pairwise comparisons between every two groups using Bonferroni correction. The relationships between variables were analyzed using Pearson's correlation test. A p-value less than 0.05 was considered statistically significant.

RESULTS

All 328 dentists who were invited to participate completed the survey without missing any data. Of the respondents, 35.36% (n=116) were male and 64.64% (n=212) were female. The mean age of the participants was 29.79±7.64, and the mean length of work in the profession was 5.98±7.50 years. Most respondents (n=221, 67.38%) were single, while 30.79% (n=101) were married, and 1.83% (n=6) were either divorced or widowed. Of the participants, 66.77% (n=219) were general dentists, while the remaining 33.23% (n=109) were specialists in various fields, including endodontics (n=7), oral and maxillofacial radiology (n=5), oral and maxillofacial surgery (n=10), orthodontics (n=20), pediatric dentistry (n=14), periodontics (n=37), prosthodontics (n=8), and restorative dentistry (n=8).

Table 1 displays the mean scores for each item and sub-dimension of the questionnaire. Among the 21 items, the item with the lowest mean score (2.24±1.54) was "Taking a

Table 1: The mean scores of items and subdimensions in the questionnaire

Subdimension	Item	Mean±SD	Mean±SD
L	L1	2.63±1.63	2.57±1.39
	L2	2.52±1.62	
	L3	2.50±1.62	
	L4	2.36±1.52	
	L5	2.44±1.60	
	L6	2.24±1.54	
	L7	2.34±1.61	
J	L8	3.54±2.00	3.30±1.68
	J1	3.15±1.78	
	J2	3.41±1.96	
	J3	3.37±1.98	
	J4	3.45±1.97	
	J5	2.98±1.85	
	J6	3.45±1.91	
S	S1	4.42±1.97	3.88±1.65
	S2	3.75±1.80	
	S3	4.03±1.87	
	S4	3.32±1.90	
C	C1	3.28±1.92	3.23±1.81
	C2	3.29±1.89	
	C3	3.13±1.90	
Total			3.12±1.35

AI: Artificial intelligence, SD: Standard deviation, L: Learning subdimension; J: Job replacement subdimension, S: Sociotechnical blindness subdimension; C: AI configuration subdimension, AIA: Artificial intelligence anxiety

class about the development of AI techniques/products makes me anxious," while the item with the highest mean score (4.42±1.90) was "I am afraid that an AI technique/product may be misused."

The dentists in the study had a moderate level of AIA, with a mean score of 65.60±28.55. The AIA levels in females were found to be statistically significantly higher in females than males (p<0.05). However, there were no significant differences in the AIA levels based on marital status (p>0.05). Prosthodontists had the highest AIA levels (75.63±34.86), while restorative dentists had the lowest (44.63±12.50). AIA levels in general practitioners were significantly higher than those in restorative dentists and periodontists (p=0.01, p=0.002, respectively). Similarly, pediatric dentists had significantly higher AIA levels than restorative dentists and periodontists (p=0.019 and p=0.034, respectively) (Table 2).

Correlations

Table 3 presents the correlations among the sub-dimensions of the AIAS. Strong and moderate correlations were found between all sub-dimensions (p<0.01). Moreover, the AIA level exhibited significant and strong correlations with all sub-dimensions,

Table 2: Artificial intelligence anxiety levels according to gender, marital status, and specialty

		n	AIA (Mean±SD)
Gender	Male	116	59.62±26.99
	Female	212	68.86±28.92
	p=0.04 (Mann Whitney U-test)		
Marital Status	Single	221	58.67±32.54
	Married	101	54.26±29.14
	Other	6	81.83±24.96
	p=0.529 (Kruskal-Wallis test)		
Specialty	General Practioner*,#	219	68.92±28.86
	Endodontist	7	62.00±21.16
	Oral and Maxillofacial Radiologist	5	60.60±23.44
	Oral and Maxillofacial Surgeon	10	53.20±26.24
	Orthodontist	20	59.25±25.62
	Pediatric Dentist*,#	14	71.14±29.38
	Periodontist	37	54.37±26.98
	Prosthodontist	8	75.62±34.86
	Restorative Dentist	8	44.62±12.50
	p=0.014 (Kruskal-Wallis test)		

n: number, AIA: Artificial intelligence anxiety, SD: Standard deviation, *compared to restorative dentist (Bonferroni correction), # compared to periodontist (Bonferroni correction).

including learning, job replacement, sociotechnical blindness, and AI configuration (p<0.01, with correlation coefficients of 0.827, 0.908, 0.844, and 0.840, respectively).

AIA did not show any significant correlations with either age or length of work in the profession (p>0.05). No statistically significant correlations were observed between any of the subdimensions and age (p>0.05). A weak and negative correlation was determined between the learning sub-dimension and length of work in the profession (p<0.05; r=-0.124). There was a strong correlation between length of work in the profession and age (p<0.01; r=0.966) (Table 3).

The Cronbach's alpha was 0.96 for all items, 0.942 for learning sub-dimension, 0.941 for job replacement sub-dimension, 0.899 for sociotechnical blindness, and 0.950 for AI configuration, indicating excellent reliability.

DISCUSSION

The present study is the first study that has revealed that dentists have moderate anxiety against AI. While a lot of studies have focused on AI anxiety in various occupational groups including health workers, none of them deal with dentists (11,13-15). Therefore, there is no data available to compare the results of our research with those of. Nevertheless, the results of the present study are consistent with the findings of other studies that have analyzed different populations.

Table 3: Correlations among AIA, AIAS' Sub-Dimensions, length of work in the profession, and age

	Mean±SD	AIA	Learning	Job replacement	Sociotechnical blindness	AI configuration	Length of work in the profession	Age
AIA	65.60±28.55	1	0.827**	0.908**	0.844**	0.840**	-0.028	-0.008
Learning	20.57±11.13	0.827**	1	0.609**	0.493**	0.563**	-0.124*	-0.103
Job replacement	19.81±10.10	0.908**	0.609**	1	0.779**	0.716**	-0.007	0.016
Sociotechnical blindness	15.52±6.61	0.844**	0.493**	0.779**	1	0.759**	0.063	0.073
AI configuration	9.69±5.45	0.840**	0.563**	0.716**	0.759**	1	0.046	0.053
Length of work in the profession	5.98±7.50	-0.028	-0.124*	-0.007	0.063	0.046	1	0.966**
Age	29.79±7.64	-0.008	-0.103	0.016	0.073	0.053	0.966**	1

AI: Artificial intelligence, AIA: Artificial intelligence anxiety, AIAS: Artificial intelligence anxiety scale, SD: Standard Deviation. *Correlation significant was at p<0.05 level (2-tailed), ** Correlation significant was at p<0.001 (2-tailed).

In a study conducted on family physicians in Turkey, the AIA was reported to be moderate (76.30±27.87), which is in line with our findings (11). The item L6 had the lowest average in both studies, while the item S1 had the highest. The sociotechnical blindness sub-dimension expresses anxiety arising from the inability to accept that AI is a system and always and only works with people and social institutions (16). Our findings suggest that like the general population, dentists perceive AI technology as a self-sufficient and advanced autonomous entity (13). Learning about AI can be instrumental in fostering more favorable attitudes towards this technology. Our study's low levels of anxiety regarding the learning sub-dimension are promising findings, consistent with those reported by Baser et al (11). This suggests that dentists may possess a general sense of competence and adaptability when it comes to learning and adopting new technologies and innovations, including AI. However, it is important to note that the low anxiety levels observed in the learning sub-dimension do not necessarily imply that dentists do not require education or training on AI. Dentists may still require education and training on the use of AI in dentistry to effectively integrate it into their practice and to provide high-quality care to their patients. Furthermore, factors such as age, experience, and familiarity with AI may influence the low anxiety levels noted in the learning sub-dimension.

In our study, no significant differences were found between sociodemographic variables, age and years of work experience, and AIA. These results are in line with those of previous studies (11,14,17). In a study evaluating the AIA of internal medicine nurses, no difference was found among four different age groups in terms of AIA (17). Thus, age does not seem to be a confounding factor regarding AIA according to the available literature. Since young people are more familiar with AI and use AI tools in daily life, AI concerns can be expected to be low (14,18). The present study was conducted on relatively young dentists who are part of the modern generations growing up with technology in their hands. This may be one of the reasons why the AIA levels were not high in our study. Future studies may need to explore other sociodemographic and contextual factors that may impact dentists' attitudes and perceptions related to AI.

This study also examined the differences in AIA levels based on sex and found that females had higher levels. However, available data on the impact of sex on AIA are inconclusive, and there is no consensus on this matter. Like our study, Terzi reported higher AIA levels in females (13). A possible explanation for this might be that males generally exhibit more positive attitudes towards AI technologies and show a greater interest in technological developments compared to females (14,18-20). On the other hand, there are also studies showing that there is no difference between the sexes in terms of AIA (11,14,17). One possible explanation is related to differences in gender roles and stereotypes, which may impact how males and females perceive and interact with technology. Females may have different expectations and experiences related to the use of technology compared to males, which may impact their attitudes and anxiety related to the use of AI in dentistry. For example, females may be socialized to be more cautious or risk-averse in new situations, which could contribute to higher levels of anxiety related to the use of new technologies like AI.

In our study, we observed correlations between AIA and all the sub-dimensions, as well as between the sub-dimensions themselves. This finding highlights the complex and multifaceted nature of AI anxiety in dentistry. Dentists may experience anxiety related to various aspects of AI, and these concerns may be interrelated and influence each other.

The finding that prosthodontists had the highest AIA levels could be attributed to several factors. The use of technology, including AI, is becoming increasingly common in prosthodontics, and prosthodontists may have more exposure to AI and its applications in their specialty compared to other dental specialists

(21,22). Restorative dentists, on the other hand, had the lowest AIA levels in this study, which may indicate a lack of awareness or interest in the potential impact of AI in their field. This could be due to several factors, such as a lack of exposure to AI technology, a perception that AI is not relevant to their practice, or a lack of understanding of the potential benefits and drawbacks of AI. Although the replacement of oral radiologists by AI remains a topic of speculation and ongoing research, the practitioners involved in this study did not exhibit a significant level of anxiety toward AI. However, the study's findings may be influenced by its small sample size of five participants. To ensure more representative results, further research with a larger sample is necessary.

The finding that AIA levels were significantly higher in general practitioners compared to restorative dentists and periodontists (with p-values of 0.01 and 0.002, respectively) warrants further discussion. One possible explanation for this could be that general practitioners are likely to encounter a wider range of dental problems, which may require them to use a broader range of AI technologies. As a result, they may feel more pressure to keep up with the latest AI advancements, leading to increased levels of anxiety. Another factor that may contribute to this finding is the level of training and experience in working with AI technologies. General practitioners may have had less exposure to AI during their training, making them less familiar and less comfortable with its use. In contrast, restorative dentists and periodontists may have received more specialized training in using AI technologies, making them more confident in their ability to use them effectively. Overall, this finding highlights the need for targeted interventions to address AIA-related anxiety, particularly among general practitioners, who may be more vulnerable to this issue. Such interventions could include increased training and education on AI technologies such as lectures, seminars, scientific meetings, and workshops, as well as initiatives to reduce the anxiety associated with their use. While basic AI courses are accessible in various health domains, they are frequently one-time events. Nevertheless, such training has been demonstrated efficacy in mitigating AI-related concerns (23,24). Despite prior calls for curriculum adjustments to accommodate AI in healthcare including dentistry, the literature lacks an evidence-based methodology to substantiate these suggestions (23,25,26). Prospective, comprehensive, and longitudinal research is imperative to address this lack of evidence.

The limitations of this study need to be acknowledged. First, the present study was conducted in a relatively small group of participants, although the sample size met the adequacy criterion. Yet, these findings cannot be extrapolated to all dentists. Secondly, the data from our study could potentially have been influenced by the subjective opinions and perceptions of the participants. This study did not focus on those factors that may affect AIA. The results therefore should be interpreted with caution. Lastly, while the AIAS was originally developed to measure public anxiety towards AI, its usefulness as an instrument for assessing AI-related anxiety exclusively among dentists may be limited due to its lack of specificity, relevance, and validation within the dental profession. Dentists could benefit from a customized assessment tool that addresses their distinctive concerns and anxieties related to AI in dentistry. Further research investigating the confounding factors of anxiety and attitudes towards AI and evaluating the effectiveness of continuing education sessions to eliminate these factors should be conducted with more dentists. The findings from such investigations can inform strategies to address dentists' concerns and facilitate the adoption of AI in dental practice.

CONCLUSION

Overall, it is important to recognize and address the anxieties that dentists may experience related to AI in dentistry, as these anxieties may impact their willingness and ability to use AI systems in their practice. By addressing these anxieties and promoting the benefits of AI in dentistry, we can support the adoption and successful integration of these technologies into dental practice, leading to improved patient outcomes and more efficient and effective dental care.

Ethics Committee Approval: This study was approved by Biruni University Non-Interventional Clinical Research Ethics Committee (Date: 25.05.2022, 2022/70-13).

Informed Consent: Before participation, all potential participants were informed about the study and provided with online informed consent.

Peer Review: Externally peer-reviewed.

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DETERMINATION OF MELOXICAM IN TABLETS BY THIRD DERIVATIVE UV SPECTROPHOTOMETRIC METHOD

ÜÇÜNCÜ TÜREV UV SPEKTROFOTOMETRİ İLE TABLETLERDE MELOKSİKAM TAYİNİ

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ABSTRACT

Objectives: Meloxicam (MEL) is a selective cyclooxygenase inhibitor of enolic acid class drugs with analgesic and antipyretic effects. In this study, an easy, selective, fast, and sensitive third-order derivative spectrophotometric method was developed and validated for the determination of MEL in tablet formulation.

Material and Methods: The absorption of MEL in a solution of methanol and 1 M sodium hydroxide (1:1, v/v) mixture was measured using the peak-to-zero method. This solution was found to be the most suitable for determining the drug by third-order derivative spectrometry. The wavelength at which the maximum absorption was achieved in the measurements was 341nm. The developed method has been validated in accordance with the International Conference on Harmonization guidelines (ICH).

Results: The linear working range was 1.0 - 14.0 µg/mL. The limit of detection (LOD) and limit of quantification (LOQ) values were 0.22 and 0.75 µg/mL, respectively. The method was validated for linearity, accuracy, precision, recovery, and stability. The developed method was performed for the quantification of MEL in tablets, and the recovery percentage was found to be between 97.50% and 98.12%.

Conclusion: The results show that the method is easy, simple, inexpensive, and fast compared to other published methods, in addition to being accurate and sensitive. The proposed method can be used as a very convenient alternative for the determination of MEL in pharmaceutical formulations in routine analysis in quality control.

Keywords: Third-derivative spectrophotometry, determination, meloxicam, tablets

ÖZ

Amaç: Meloksikam (MEL), analjezik ve antipiretik etkileri olan seçici bir siklooksijenaz inhibitörü enolik asit sınıfı bir ilaçtır. Bu çalışmada, tablet formülasyonunda meloksikam tayini için kolay, seçici, hızlı ve hassas bir üçüncü dereceden türev spektrofotometrik yöntem geliştirilmiş ve valide edilmiştir.

Gereç ve Yöntemler: Üçüncü türev spektrometrisi ile ilaç tayini için en uygun bulunan metanol-1 M sodyum hidroksit (1:1, v/v) çözeltisinde meloksikamın absorpsiyonu pik-sıfır yöntemi ile okundu. Ölçümlerde maksimum absorpsiyonun elde edildiği dalga boyu 341 nm idi. Geliştirilen yöntem, Uluslararası Uyumlaştırma Kılavuzuna (ICH) uygun olarak valide edilmiştir.

Bulgular: Doğrusal çalışma aralığı 1,0 - 14,0 µg/mL idi. Gözlenebilme sınırı (LOD) ve tayin sınırı (LOQ) değerleri sırasıyla 0,22 ve 0,75 µg/mL idi. Yöntem, doğrusallık, doğruluk, kesinlik, geri kazanım ve kararlılık açısından doğrulandı. Geliştirilen yöntem tabletlerde meloksikam miktar tayinine uygulanmış ve geri kazanım yüzdesi %97,50 ile %98,12 arasında bulunmuştur.

Sonuç: Sonuçlar, yöntemin doğru ve duyarlı olmasının yanı sıra, yayınlanmış diğer yöntemlere göre kolay, basit, ucuz ve hızlı olduğunu göstermektedir. Önerilen yöntem, kalite kontrolde rutin analizlerde farmasötik formülasyonlarda meloksikam tayini için çok uygun bir alternatif olarak kullanılabilir.

Anahtar Kelimeler: Üçüncü türev spektrofotometrisi, tayin, meloksikam, tablet

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INTRODUCTION

Meloxicam (MEL, 4-hydroxy-2-methyl-*N*-(5-methyl-1,3-thiazol-2-yl)-1,1-dioxo-1λ⁶,2-benzothiazine-3-carboxamide) (Figure 1), a non-steroidal anti-inflammatory, has analgesic and antipyretic properties. MEL is used in the treatment of calcification, joint pain and deformity, progressive rheumatism, acute musculoskeletal pain, symptoms of acute gouty arthritis, and relief of postoperative swelling (1-2). It is also widely used for dysmenorrhea, low back pain, postoperative analgesia, and pain related to dental interventions. MEL acts by inhibiting cyclooxygenase (COX-1 and COX-2). As COX-2 does not inhibit myocardial prostacyclin like specific products, MEL does not cause hypertension and edema (3).

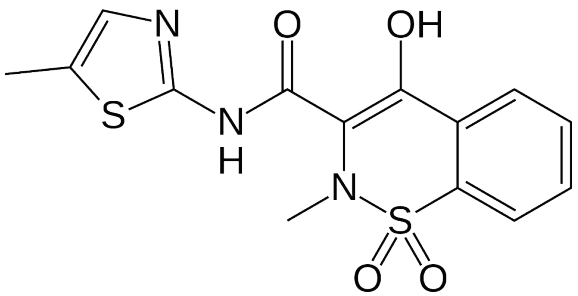

Figure 1: Chemical structure of meloxicam

Based on the literature review, some electrochemical methods (4-6) for the determination of MEL in pharmaceutical forms and several high-performance liquid chromatography (HPLC) methods for its determination in plasma (7-9) and pharmaceutical preparations alone (11-13) and simultaneously with other anti-inflammatory drugs (14-18) are available. Also, in addition to a spectrofluorometric method, there are many spectrophotometric methods based on measuring the absorbance directly in different solutions, measuring the absorbance after complexation or derivatization and chemometric measurement (19-30). Among these spectrophotometric studies, two first-order derivative spectrometry studies (31-33) and simultaneous second-order derivative spectrometry studies for MEL determination seem to be registered so far (33).

In quantitative analysis, derivative spectrophotometry provides quite an advantage over conventional absorption spectra in cases of spectral similarities, the overlap of analyte absorption bands, and broad absorption bands. In addition, derivative spectrophotometry is commonly used in drug analysis in the presence of impurities to eliminate background absorbance errors in cases of overlapping fuzzy matrices, to eliminate effects such as beam scattering, and to increase band resolution (34).

In this study, MEL was determined and validated for the first time by the third-order derivative spectroscopic method, which is much more sensitive, easier, faster, and more accurate than many existing methods. The suggested method was implemented for MEL determination in tablets with high recovery.

MATERIAL and METHODS

Apparatus

Spectrophotometric measurements were taken with an ultraviolet-visible (UV-Vis) absorption spectrophotometer (Shimadzu, UV-160 A, Japan), and 1.0 cm quartz cells were used. Spectra were acquired at a scanning range of 200-600 nm, a scanning speed of 1500 nm/min, a slit width of 2 nm, and a derivation interval (Δλ) of 2.8 nm for third-order derivative (^{3}D, d^3 A / $d\lambda^3$) spectra.

Reagents and solutions

MEL and its tablet (Melox®) were obtained from the Abdi Ibrahim Pharmaceutical Company (Istanbul). Sodium hydroxide (NaOH), hydrochloric acid (HCl, 37%), methanol, acetonitrile, and ethanol chemicals from Merck were all analytical grades. Ultra-pure water obtained from the Elga Purelab Option water purification device (Lane End, UK) was used in the analysis.

An amount of 2.0 mg of MEL was weighed exactly, dissolved in methanol :1 M NaOH (1:1, v/v), and made up to 100 mL (stock solution, 20 µg/mL). It was used by making various dilutions in the analysis. Stock solutions were kept refrigerated, and we worked with a freshly prepared solution every week.

Calibration curve

For the calibration curve, 0.5, 2, 4, 5, 6, and 7 mL of the stock solution containing 20 µg/mL MEL (equivalent to 1, 4, 8, 10, 12, and 14 µg/mL, respectively) were transferred into 10 mL flasks and completed to volumes with the selected methanol:1M NaOH (1:1 v/v) mixture solution. The UV spectra of these solutions were taken against the blank solution (methanol:1M NaOH, 1:1 v/v) in the 200-600 nm range and operated to obtain its third-order derivative (^{3}D). The peak absorption ($d^3A/d\lambda^3$) was measured by the peak-to-zero technique (*height* of *peak* from *zero*) at 341 nm. The calibration curve was established by drawing the third derivative absorbance versus the concentration of MEL, and the regression analysis was performed. The calibration curve was created by replicating at least six separate analyzes.

Determination in tablets

Ten tablets containing 7.5 mg of MEL, trade name Melox®, were weighed one by one, and the average tablet weight was determined and ground into powder in a mortar. An amount of tablet powder equivalent to the weight of one tablet was precisely weighed. It was transferred to a 100.0 mL flask and kept in an ultrasonic bath for 60.0 min with 70.0 mL of a methanol:1 M NaOH (1:1, v/v) mixture. It was then completed to its volume and filtered through blue banded filter paper. One mL of tablet solution was taken, and after completion to 10.0 mL (7.5 µg/mL) with the same solution, it was worked out as in the section on the calibration curve study.

RESULTS

Appropriate solvent and wavelength selection

Solvents, wavelengths, and derivative spectrophotometry scans

Table 1: Spectral parameters of MEL in different solvents

Tested solution	MEL µg/mL	^{0}D, λ nm	Absorbance	^{3}D, λ nm	Absorbance
Methanol	10	366	0.358	345	0.685
1 M NaOH	10	360	0.690	341	0.991
MeOH:1M HCl (1:1, v/v)	10	343	0.408	323	0.742
Acetonitrile	10	365	0.298	348	0.603
MeOH:1M NaOH (1:1, v/v)	10	360	0.822	341	1.285
MeOH:water (1:1, v/v)	10	363	0.254	341	0.570

MeOH: Methanol; NaOH: Sodium hydroxide; HCl: Hydrochloric acid

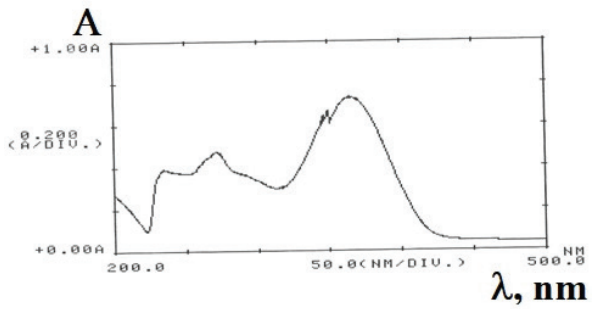

Figure 2: The zero-order spectrum of meloxicam at 10.0 µg/mL in methanol:1 M sodium hydroxide (1:1, v/v)

were investigated to obtain the most appropriate conditions for the method. For MEL determination, firstly the spectra between the zero-order and fourth derivatives were taken. Considering the high absorption response proportional to the concentration and a well-separated peak, the 3rd derivative spectrometry method was determined to be the most appropriate, so studies were continued with this method. To determine the appropriate solvent for which the MEL gives the highest absorbance, the third derivative (^{3}D) absorption values of MEL at 10.0 µg/mL concentration were recorded in methanol, 1 M NaOH, methanol:1 M HCl (1:1, v/v), acetonitrile, methanol:1 M NaOH (1:1, v/v), and methanol: water (1:1, v/v). Under these conditions, the highest absorbance value was obtained in methanol:1 M NaOH (1:1, v /v) solvents. The absorption and wavelength (λ) values of MEL obtained by a zero-order (direct, ^{0}D) and ^{3}D spectrophotometric methods in the tested solvent systems are summarized in Table 1. The maximum absorbance wavelength recorded in the spectrum with the peak-to-zero technique was 341 nm. The ^{0}D and ^{3}D spectra of the drug recorded in the selected solution are given in Figures 2 and 3.

Method Validation

For the validation of the developed method, the following parameters were examined in accordance with the recommendations of the International Council of Harmonization (35).

Linearity and sensitivity

From the calibration curve obtained by plotting the third-order derivative absorbance values read against the concentrations of the MEL solutions, the dynamic linear range of the MEL was

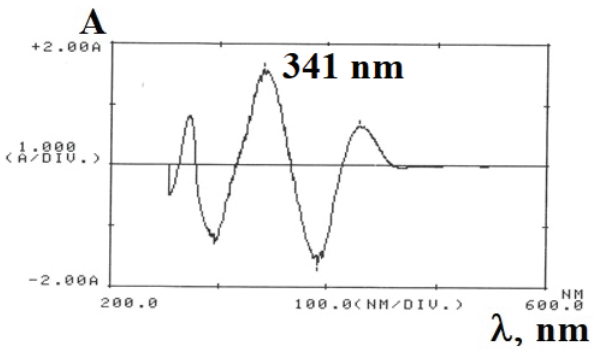

Figure 3: The third-order derivative spectrum of meloxicam at a concentration of 14.0 µg/mL in methanol:1 M sodium hydroxide (1:1, v/v)

Table 2: Analytical figures of merit for the presented method

Parameters	Standard solution
^{3}D (nm)	341
Beer's law range (µg/mL)	1.0-14.0
Regression equation (n= 6)[a]	y=0.1107C + 0.0135
Slope ± SD	0.1107±0.0017
Intercept ± SD	0.0135±0.016
LOD (µg/mL)	0.22
LOQ (µg/mL)	0.75
Correlation coefficient, R^2	0.9998

ay= aC + b (where C is the concentration of the drug in µg/mL. y is absorbance, a is slope, and b is intercept). aAverage of six determinations for six concentration levels. SD: Standard deviation, LOD: The limit of detection, LOQ: Limit of quantification.

determined to be between 1.0 and 14.0 µg/mL. The regression equation corresponding to this curve was calculated as y ($d^3A/d\lambda^3$) = 0.1107C(µg/mL) + 0.0135 (Figure 4). The correlation coefficient (R^2) value of this equation is 0.9998, indicating perfect linearity (Table 2).

The LOD and LOQ values were calculated with the following formulas: LOD = 3 x SD/m and LOQ = 10 x SD/m. Here, SD is the standard deviation of the y-intercept of the calibration line, and m is the slope of the calibration line. The LOD and LOQ values

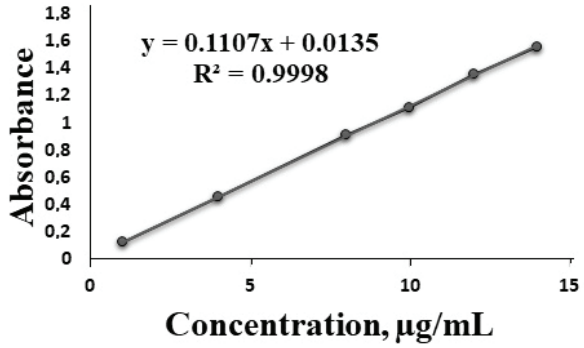

Figure 4: Calibration curve of meloxicam

calculated according to the given equations were found to be 0.22 and 0.75 µg/mL for MEL, respectively.

Precision study

To ascertain the intraday and interday precision, MEL solutions prepared daily at two different concentrations (2.0 and 12.0 µg/mL) were taken and studied on the same day and on six different days (n= 6), as described in the "Calibration Curve" section.

In the intraday repeatability study, the standard deviation (SD) and percent recovery values ranged from 0.033 to 0.074 and 97.5% to 100.93%, respectively. *Relative standard deviation* (RSD%) was found to be between 0.61% and 1.69%. In the interday reproducibility study, SD and % recoveries values were found to be between 0.041-0.071 and 97.65%-99.59%, respectively. The RSD% was between 0.60% and 2.12%, indicating excellent precision (Table 3).

Table 3: Intraday and interday analysis of MEL (n=6)

	Intraday		Interday	
Concentration (µg/mL)	**Recovery[a] (%) ± SD[b]**	**RSD[b] (%)**	**Recovery[a] (%) ± SD[b]**	**RSD[b] (%)**
2.0	97.50±0.03	1.69	97.65±0.04	2.12
12.0	100.93±0.07	0.61	99.59±0.07	0.60
Mean	99.22±0.20	1.15	98.62±0.06	1.36

[a]Mean of five determinations (n=5), [b]SD is the standard deviation and RSD% is the relative standard deviation.

Table 4: The accuracy of the method by standard addition method (n=6)

Taken tablet amount (µg/mL)	Added standard MEL amount (µg/mL)	Total found amount (µg/mL)	Recovery%	SD[a]	CV[b](%)
1.0	1.0	1.97	98.32	0.047	2.39
5.0	5.0	9.84	98.43	0.050	0.51
13.0	5.0	17.46	97.01	0.164	0.94

[a]SD is the standard deviation, [b]CV % is the coefficient of variation.

Accuracy studies

The accuracy of the study was assessed with the standard addition technique by adding standard MEL solution (at 1.0 and 5.0 µg/mL) to the tablet solution (at 1.0, 5.0, and 13.0 µg/mL) and analyzing at three different concentration levels in the calibration curve range. The results represent the average of six separate analyses. The percent recovery was calculated by the equation [% = [[(Ct-Cu)/Ca]x100]: where Ct = total concentration of MEL found; Cu = MEL concentration of tablet solution; and Ca= added standard solution. The recovery % of the drug varies between 97.01% and 98.43%. RSD% values were between 0.51% and 2.43% (Table 4). The high recovery rate indicates the accuracy of the method, and the MEL is unaffected by any additives used in the tablet formulation.

Stability studies

For determination of the stability of MEL in bulk, the solutions at 10.0 µg/mL were kept at 4 °C and room temperature for 1, 2, 4, 6, and 24h and then analyzed. Recovery results of the drug showed no significant difference within 24 hours. In the analysis results given in Table 5, the mean SD and RSD% values were 0.06 and 0.55% for room temperature holding, and 0.17 and 1.66% for 4°C storage, respectively. Recovery percentages were found to be 100.86% and 99.32% for the bulk solution of MEL

Table 5: Stability results for MEL at different conditions

		Room temperature	+4 °C
Duration (hour)	**Concentration Taken µg/mL**	**Concentration Found µg/mL**	**Concentration Found µg/mL**
1	10.0	10.14	10.18
2	10.0	10.14	9.96
4	10.0	10.09	9.91
6	10.0	10.05	9.87
24	10.0	10.01	9.74
Mean values		10.09	9.93
SD[a]		0.06	0.17
RSD%[b]		0.55	1.66
Recovery%		100.86	99.32

[a]SD is the standard deviation, RSD%[b] is percentage relative standard deviation

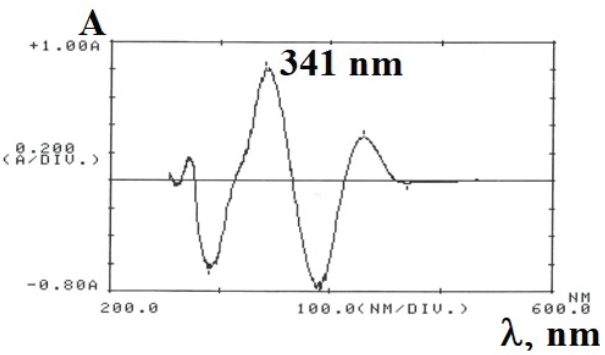

Figure 5: The third-order derivative spectrum of a tablet solution containing 7.5 µg/mL meloxicam, taken under selected conditions

at room temperature and at 4˚C, respectively. These results can indicate that the MEL is stable in the chosen solvent of analysis (methanol: 0.1 M NaOH, 1:1 v/v) at room temperature and refrigerator and is also resistant to sunlight and in moderate alkaline conditions.

Determination of MEL in tablets

To see the feasibility of the developed and validated method, MEL determination was carried out on tablet samples (Figure 5). The determination of tablet content was calculated by putting the absorbance values in the regression equation prepared with the standard MEL solution, and then their recovery was found. As a result of at least 6 separate analyses, the average recovery of MEL in tablets was found to be 97.50%. The SD and RSD% were 0.14 and 1.93%, respectively.

DISCUSSION

In this study, a series of preliminary experiments were conducted to determine the most suitable conditions for the determination and validation of MEL drug in tablets by a new third-order derivative spectrophotometric method.

Compared to previously reported methods in terms of LOD, the current method was found to be significantly more sensitive to reported derivative spectrometry studies, most chemically pretreated UV-visible spectrophotometric methods, ^{0}D

Table 6: Comparison of the statistical performances of the proposed method with published spectrophotometric methods of MEL

Methods	Analysis medium	λ (nm)	LOD/LOQ, µg/mL	Linearity, µg/mL	Ref.
^{3}D-*UV*	Methanol:1 M NaOH (1:1 v/v)	341	0.22 /0.75	1.0 - 14.0	Proposed
^{0}D-UV ^{1}D-*UV* ^{2}D-*UV*	0.1 M NaOH	270.0 339.6 315.6	1.30/3.50 1.0/3.50 1.20/3.80	4.0 - 14.0	33
^{0}D-UV ^{1}D-*UV* ^{2}D-*UV* UV-vis	0.1 M NaOH Ethanolic solution:0.1 M HCl Borax: phosphate buffer pH 8.0 safranin T:borax-phosphate pH 8.0	339.9-384.7 322-368 343.2-385.6 518	0.11/2.0 0.07/1.0 0.1/1.0 0.33/4.0	2.0-10.0 1.0-10.0 1.0-10.0 4.0-12.0	19
^{1}D-*UV* TLC:densitometric	0.1N NaOH	338 365	Not given	5-20 2-10	31
^{0}D-UV UV-AUC ^{1}D-*UV*	0.1N NaOH	269 253-279 275	Not given	5-30 5-30 50-300	32
^{0}D-UV	0.1 M NaOH	365	0.12/0.38	2.0- 12.0	20
^{0}D-UV	Methanol:0.1M HCl	346.0	0.13/0.41	5.0-150	21
^{0}D-UV	Etanol	365	1.28/2.0	2.0 -18.0	22
UV-vis- Flow-injection (UV)	Fe (III) [2Meloxicam/Fe (III)]: methanolic solution 0.1 M NaOH	570 362	0.47/-1.51 0.72 /2.52 0.04/0.13	2.0-200 5.00-250 0.5-20	23
Direct flow injection (UV) Indirect flow Injection (UV)	Diazotized procaine Benzylpenicillin:alkaline MEL:p-methylaminophenol sulfate	492 656	2.73/4.21 5.26/ 9.62	5-80 15-225	24
UV-vis	Acetonitrile: methanol (50:50): 1% aluminium chloride	375	0.68/ 2.25	5-30	25
^{0}D-UV UV-vis Hydrotropic (UV)	0.1M NaOH 0.1M NaOH:5% ferric chloride % Trisodium citrate in water	269 476 269	0.038 / 0.11 0.33/ 0.94 0.038/0.11	5- 30 50- 250 5-30	26
UV-vis	Sodium nitroprusside:Hydroxylamine HCl:sodium carbonate Ferric chloride:1,10-Phenanthroline	363 343	0.16/ 0.23 0.49/0.71	4-20 10-50	28
UV-vis	Phosphate buffer (pH=7.5)	350	0.88/2.9	3.5-19.6	30
UV-vis ^{0}D-UV	Methanol:Ferric Ammonium sulfate: 0.1 N NaOH	396 354	Not given	5-30 3-12	*29*

LOD/LOQ: The limit of detection/ limit of quantification, NaOH: Sodium hydroxide, UV-vis: ultraviolet- visible spectrophotometer, ^{0}D-UV: Zero order derivative ultraviolet absorption, MEL: Meloxicam, TLC: Thin layer chromatography

methods and flow spectrophotometric methods (Table 6) (19, 22-26, 28, 30, 32). As given in Table 6, LOD and LOQ values were not given in some studies with a linear working range at high concentrations. When the determinations were compared in terms of linear ranges, it was found that our developed method was mostly more sensitive and/or had a wider range than almost all of them. Furthermore, a comparison of the LOD and linear range values of the developed method with those obtained by some published HPLC methods, which are much more expensive, time-consuming, and require greater solvent consumption, revealed that the developed method is fairly sensitive (11-16). Moreover, the absence of any additives in the absorbance of the tablet solution because of the solvent and possible tablet additional ingredients proves the selectiveness of the method and contributes to high recovery (Figures 3 and 5).

CONCLUSION

In this study, a new selective, stable, accurate, and simple third-order derivative spectrophotometric method using a peak-to-zero measurement technique was developed for the determination of MEL in bulk and tablets. The new method was more sensitive than some reported spectrophotometric and HPLC-UV methods when compared with the detected LOD value of 0.22 µg/mL and the wide linear range of 1.0-14.0 µg/mL. The current method is very quick and cheap compared to complex and expensive advanced HPLC and HPLC/MS methods that are not available in every analytical laboratory. The developed method offers significant advantages due to its easy and fast sample preparation, which requires no processing and uses a low amount of non-destructive solvents. Its practicality and precision make it a preferable choice for routine analysis of MEL in both pure and tablet forms, compared to the reported methods.

Ethics Committee Approval: Ethics committee approval is not required since our study is a quantification of the drug in tablet formulation and is not a clinical study.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- Z.A., F.A.; Data Acquisition- F.A., Z.A.; Data Analysis/Interpretation- F.A., Z.A.; Drafting Manuscript- Z.A., F.A.; Critical Revision of Manuscript- Z.A., F.A.; Final Approval and Accountability- Z.A., F.A.; Material and Technical Support- Z.A., F.A.; Supervision- Z.A.

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

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Aims and Scope

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Journal of Advanced Research in Health Sciences (JARHS) aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of medicine. The journal publishes original experimental and clinical research articles, reports of rare cases, reviews that contain sufficient amount of source data conveying the experiences of experts in a particular field, and letters to the editors as well as brief reports on a recently established method or technique or preliminary results of original studies related to all disciplines of medicine from all countries.

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