E-ISSN: 2587-0262

NKMJ

NAMIK KEMAL MEDICAL JOURNAL

Volume: 11
Issue: 1
March 2023

ORIGINAL ARTICLES

galenos yayınevi

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Online Publication Date: March 2023
E-ISSN: 2587-0262
International scientific journal published quarterly.

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Ozkan G, Ulusoy S, Guvercin B, Menteşe A, Karahan SC, Yavuz A. A new player in chronic kidney disease mineral and bone disorder: tenascin-C. Int J Artif Organs. 2015;38:481-7.

For books:

West JB: Respiratory Physiology (2nd ed). Baltimore: Williams and Wilkins, 1974; 72-5.

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For online articles:

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For the citations from the thesis:

Kulu A. Evaluation of Quality of Life After Surgical Interventions Applied to Patients with Bladder Tumors, Trakya University, Institute of Health Sciences, Department of Nursing. Master Thesis. 2010; Edirne.

For congress papers:

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NAMIK KEMAL MEDICAL JOURNAL

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ORIGINAL ARTICLES

ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.74745
Nam Kem Med J 2023;11(1):1-11

Comparative Analysis of the Mutation and Expression Profile of the Cytoprotective NRF2/KEAP1/P62/SQSTM1 Signaling Pathway in Different Glioma Subtypes: An *In Silico* Study

Farklı Glioma Alt Tiplerinde Sitoprotektif NRF2/KEAP1/P62/ SQSTM1 Sinyal Yolunun Mutasyon ve Ekspresyon Profilinin Karşılaştırmalı Analizi: Bir *In Silico* Çalışma

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ABSTRACT

Aim: The NRF2/KEAP1/p62/SQSTM1 pathway is the master regulator of antioxidant enzymes and detoxification proteins, both of which play a critical role in redox homeostasis. It shows that the this structurally active pathway has a crucial role in cancer as it inhibits tumorigenesis and metastatic processes and it induces pro-survival genes that promote chemoresistance. The relationship between the molecular mechanisms causing the pathway to malfunction and the development of brain tumors has yet to be fully clarified. The aim of this study is to analyze the genetic changes and expression level differences of the NRF2/KEAP1 pathway comparatively in low-grade gliomas (LGG) and glioblastoma multiforme (GBM) pathology.

Materials and Methods: Gene expression profiles and DNA sequences of GBM (n=591) and LGG (n=511) patients and healthy tissue were downloaded from the TCGA database. Not only were gene expression and mutation patterns determined in this study, but also the impacts of genes on survival were assessed. PolyPhen-2 and SNAP tools were used to estimate the pathogenic properties of the changes identified.

Results: A total of 16 mutations and gene amplification were identified in the *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP1* for both cancer groups, and the mutation carrying frequency was 4.6%. IDH1 p.R132H and NRF2 p.S164* mutation association was determined in 1 patient with LGG. *KEAP1*, *NRF2*, and *HMOX1* expression levels for both LGG and GBM subtypes were determined to be high in patient samples compared to healthy samples ($p<0.05$).

Conclusion: By targeting the NRF2/KEAP1/p62/SQSTM1 pathway anomalies, new therapeutic approaches can be provided in the treatment of glioma, particularly for chemotherapy sensitivity.

Keywords: NRF2/KEAP1/p62/SQSTM1 signaling pathway, glioma, mutation, gene expression, oxidative stress

ÖZ

Amaç: NRF2/KEAP1/p62/SQSTM1 sinyalizasyon yolağı redoks homeostazında önemli rol oynayan antioksidan enzimlerin ve detoksifikasyon proteinlerinin ana düzenleyicisidir. Yapısal olarak aktif NRF2/KEAP1 yolağının, tümörigenezi ve metastatik süreçleri inhibe ettiği ve kemorezistansı teşvik eden hayatta kalma yanlısı genleri indüklediği için kanserde çok önemli bir role sahip olduğunu göstermektedir. Yolağın fonksiyonunun bozulduğu moleküler mekanizmalar ile beyin tümörleri gelişimi arasındaki ilişki tam olarak aydınlatılamamıştır. Bu çalışmanın amacı NRF2/KEAP1 yolağının genetik değişikliklerini ve ifade seviyesi farklılıklarını düşük dereceli glioma (LGG) ve glioblastoma multiform (GBM) patolojisinde karşılaştırmalı olarak analiz etmektir.

Gereç ve Yöntem: GBM ve LGG hastalarına ve sağlıklı doku örneklerine ait gen ekspresyon profilleri ve DNA dizileri, kanser genom atlas veri tabanından indirildi. *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1* ve *MOAP1* genlerinin mutasyon ve ifade profilleri kapsamlı olarak analiz edildi. Çalışmada

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Received: 31.05.2022 **Accepted:** 06.01.2023

Presented in: The study was presented as abstract in 14th National Congress of Medical Genetics Digital Congress 20-22 November 2020.

sadece gen ekspresyonu ve mutasyon paternlerinin tespiti değil, aynı zamanda hedef genlerin sağkalım üzerine olan etkileri de belirlendi. Ayrıca belirlenen değişikliklerin hastalık yapıcı patojenik özellikleri tahmini için PolyPhen-2 ve SNAP araçları kullanıldı.

Bulgular: Her iki kanser grubu için *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1* ve *MOAP1* genlerinde toplam 16 (12 missense mutasyon, 1 nonsense mutasyon, 1 delesyon, 2 translokasyon) mutasyon ve gen amplifikasyonu belirlendi ve mutasyon taşıma sıklığı %4,6 idi. LGG'li 1 hastada IDH1 R132H ve NRF2 S164* mutasyon birlikteliği belirlendi. LGG ve GBM alt tiplerinin her ikisi için de *KEAP1*, *NRF2* ve *HMOX1* gen ekspresyon seviyeleri, hasta örneklerinde sağlıklı örneklere göre yüksek olarak belirlendi (p<0,05).

Sonuç: NRF2/KEAP1/p62/SQSTM1 sinyalizasyon yolağı anomalilerinin hedeflenmesi ile glioma tedavisinde özellikle kemoterapi duyarlılığı için yeni terapötik yaklaşımlar sağlanabilir.

Anahtar Kelimeler: NRF2/KEAP1/p62/SQSTM1 yolağı, glioma, mutasyon, gen ekspresyonu, oksidatif stress

INTRODUCTION

Brain tissue has limited antioxidant capacity and is highly indefensible to oxidative stress due to its higher energy requirement and higher content of lipids and iron, the auto-oxidation properties of neurotransmitters in an environment where free oxygen radicals are concentrated, and oxidized neurotransmitters also have the potential to cause more production of reactive oxygen species (ROS)[1-3]. This situation causes morphological and functional changes in the brain, as well as cognitive dysfunction and retardation. The NRF2/KEAP1/p62/SQSTM1 pathway responsible for the activation of the NRF2 transcription factor, which controls the transactivation of more than 500 cytoprotective genes, is a significant cellular component in protecting cells and tissues from electrophilic, oxidative, and xenobiotic stress[4-6]. NRF2 is bound to the KEAP1 in the cytoplasm under normal circumstances. As cells are subjected to oxidative stress, it has been found that NRF2 detaches from KEAP1, to which it is attached in the cytoplasm, and moves into the nucleus, where it binds to its target gene and stimulates transcription[7,8]. In addition to the KEAP1-dependent regulation of NRF2, various alternative KEAP1-independent mechanisms contribute to the regulation of NRF2. These include the cellular NRF2 protein and binding of the disrupting protein p62/SQSTM1 to KEAP1, which inhibits the interaction of NRF2 and KEAP1 causing an increase in its activity[7-9]. Abnormal NRF2/KEAP1 pathway causes the development of treatment resistance and provides cancer cells with a growth advantage due to the constitutive expression of cytoprotective genes[3,5-8]. Malignant cells are known to take advantage of their increased NRF2 pathway activity. This condition was first discovered in lung cancer and then in a variety of other types of cancer, such as ovarian, pancreatic, liver, pediatric leukemia, and bladder cancers[6-9].

Gliomas are the most malignant and aggressive form of brain tumors, and account for the majority of brain cancer-related deaths. Gliomas are the most common primary intracranial tumor, representing 81% of malignant brain tumors[10,11]. Recent data support that the concept "ROS is an indispensable participant in fostering proliferation, survival, and migration" in various cancer cell types including glioblastoma cells[2,3,11]. Overproduction of ROS is known to play a role in promoting these changes[2,3,10,12]. The goal of this study is to predict the functional effects of pathogenic mutations and expression level profiles in the NRF2/KEAP1/p62/SQSTM1 pathway genes in glioma subtypes, as well as to clarify the effects of these pathway elements on glioma pathogenesis and progression.

MATERIALS AND METHODS

Data Collection

The GBM and LGG data sets were obtained from the cbioPortal database and the demographic, clinical, and genetic data for our patient group are summarized in Table 1.

Mutation Profile Analysis

The cBio Cancer Genomics Portal (http://cbioportal.org) is an open-access bioinformatics tool that uses data from The Cancer Genome Atlas (TCGA) to provide mutation data[13]. In order to comprehensively examine the mutations found in KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 in GBM and LGG patient samples, GBM (n=591), LGG (n=511), it was selected as the cancer type of interest from the web interface. For this purpose, comprehensive mutation profile analyses were carried out through cBioportal using the features provided by the interface of the genes of interest.

In Silico Analysis of Mutation Impact

The probable pathogenicity of the mutations found in the KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 was determined using scores from the PolyPhen-2, SNAP, and the COSMIC databases. The PolyPhen-2 estimates the probability of the missense mutation damaging the protein and provides the user with this result (probably damaging, possibly damaging, benign or unknown) with a score[14]. The SNAP is an online tool that distinguishes between impact and neutral variants/non-synonym SNPs by considering various sequence and variant properties[15]. Furthermore, the detected mutations were scanned in the COSMIC database and their pathogenicity scores were determined[16]. Besides, evolutionary conservation analyses of the detected mutant amino acids were evaluated

Table 1. Demographic, clinical and genetic data of patients with GBM and LGG

Characteristic	GBM n=591 (%)	LGG n=511 (%)
Gender		
Male/Female/NA	175/122/294	285/226
Diagnosis age, years	59.6 (range, 21-89)	43 (range,19-87)
Race category		
White	252 (43.1)	474 (92.7)
Black or African American	31 (5.3)	21 (4.1)
Asian	4 (0.7)	7 (1.3)
NA	297 (50.2)	9 (1.7)
Sample type		
Primary	584 (98.8)	511 (100)
Recurrence	7 (1.2)	-
Overall survival status		
Living	103 (17.4)	388 (75.9)
Deceased	478 (80.8)	125 (24.4)
NA	10 (1.7)	-
Radiation therapy		
Yes	236 (39.9)	296 (57.9)
No	41 (6.9)	183 (35.8)
NA	314 (53.1)	34 (6.6)
Tumor disease anatomy		
CNS	-	511 (100)
Brain	298 (50.4)	-
NA	293 (49.5)	-
Tumor subtypes		
Glioblastoma multiforme	591 (100)	-
Astrocytoma	-	192 (37.5)
Oligodendroglioma	-	189 (36.9)
Oligoastrocytoma	-	130 (25.4)
Neoplasm histologic grade		
Grade I		-
Grade II		248
Grade III		263
Grade IV		-
NA		-
Genetic abnormalities	**Case (frequency %)**	
IDH1 mutation	25 (6.3)	394 (76.8)
IDH2 mutation	1 (0.3)	21 (3.5)
NFE2L2 mutation	1 (0.3)	1 (0.2)
SQSTM1 mutation	3 (0.8)	2 (0.4)
HMOX1 mutation	2 (0.5)	-
MOAP1 mutation	2 (0.5)	1 (0.2)
SQSTM1-NTRK2 fusion	-	1 (0.2)
KEAP1 amplification	7 (1.2)	12 (2.4)
NFE2L2 deep deletion	1 (0.2)	1 (0.2)
SQSTM1 amplification	1 (0.2)	2 (0.4)
SQSTM1 deep deletion	1 (0.2)	2 (0.4)
HMOX1 amplification	2 (0.5)	4 (0.8)
HMOX1 deep deletion	1 (0.3)	3 (0.6)
TXNRD2-HMOX1 fusion	1 (0.2)	-
MOAP1 deep deletion	1 (0.2)	-
1p status		
Gained	41 (6.9)	12 (2.34)
Lost	8 (1.3)	173 (33.8)
Not called	477 (80.7)	307 (60)
NA	65 (10.9)	19 (37.2)
19q status		
Gained	144 (24.3)	19 (3.7)
Lost	22 (3.7)	185 (36.2)
Not called	313 (52.9)	233 (45.5)
NA	112 (18.9)	74 (14.4)

NA: Not applicable, CNS: Central nervous system, LGG: Lower grade glioma, GBM: Glioblastoma multiforme

among different species via "Multiple sequence alignment" tool in the PolyPhen-2.

Gene Expression and Survival Analyses

GEPIA is an interactive tool developed to provide customizable analyses such as differential expression analysis in tumor or normal tissues, profiling according to cancer types or pathological stages, survival analysis, similar gene detection, and correlation analysis[17]. The profiles of KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 expressions were analyzed in box plot graphs created by the GEPIA using the data of the samples of GBM (n=163), LGG (n=518) obtained from TCGA data, and of 207 healthy tissues. Survival analyses (overall/disease free) of genes according to varying gene expression levels were conducted using GEPIA. Overall survival (OS) and disease free survival (DFS) analyses based on Log-rank test with 95% confidence interval were performed to create survival plots.

Statistical Analysis

All statistical analyses that were used in the evaluation of the study data were performed on the GEPIA database. The one-way ANOVA test was used to measure differential expression. GEPIA performs the analysis of OS or DFS, also called relapse-free survival, based on gene expression. GEPIA uses Log-rank test, the Mantel-Cox test, for hypothesis test. To compare low and high expression groups, the log-rank test was used. In all tests, the statistically significant value was accepted as $p<0.05$.

RESULTS

Results of Mutation Profile Analysis

The cBioPortal interface was used to analyze the genome sequencing data of 1106 patients in order to identify genetic changes in the KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 in GBM and LGG patient samples. Of GBM and LGG patients, 4.6% were found to have at least one genetic change in the KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1. A total of 16 genetic changes (12 missense, 1 nonsense, 1 deletion, 2 translocations) were detected in all study genes (Table 2). When the frequency of changes among the analyzed genes was examined, it was determined that KEAP1 was the gene with more mutations among the patient groups (1.7%), and NRF2 was determined to be the gene with less mutation (0.4%) (Figure 1). While no nucleotide changes in KEAP1 were detected, amplification of the gene was found in both glioma subtypes. In GBM and LGG patient samples, the localization of mutations detected on the domains of proteins belonging to the study genes is shown in Figure 2 as a representation.

Results of Mutation Profile Analysis in LGG Patients

The frequency of carrying LGG genetic anomalies was determined to be 6.1%. Six mutations (NRF2, p.S164*; SQSTM1, p.R107Q and p.A2V; MOAP1, p.K164N and p.R204T) were detected in the LGG group. The nonsense p.S164* mutation found in the *NRF2* gene was thought to cause early termination of the NRF2 polypeptide at the 164. amino acid, resulting in the formation of a truncated protein, according to our findings. There was one patient in Astrocytoma subtype with the coexistence of NRF2 p.S164*and the IDH1 p.R132H missense mutation characteristic for glioma.

SQSTM1, p.R107Q and p.A2V missense mutations were identified in two different patients with Astrocytoma subtype SQSTM1. It was determined that the patient carrying the p.A2V mutation also carried the IDH1 p.R132H mutation. The p62/SQSTM1 t (5;9) (q35;q21) fusion gene with NTRK2, which is a tyrosine kinase responsible for neural development, was identified in a patient of the Oligodendroglioma subtype. Apoptosis modulator 1 (MOAP-1), a BH3-like protein that plays a key role in apoptosis, was identified with p.K164N and p.R204T mutations on the PNMA domain in two different patients in Astrocytoma subtype carrying IDH1 p.R132H missense mutation.

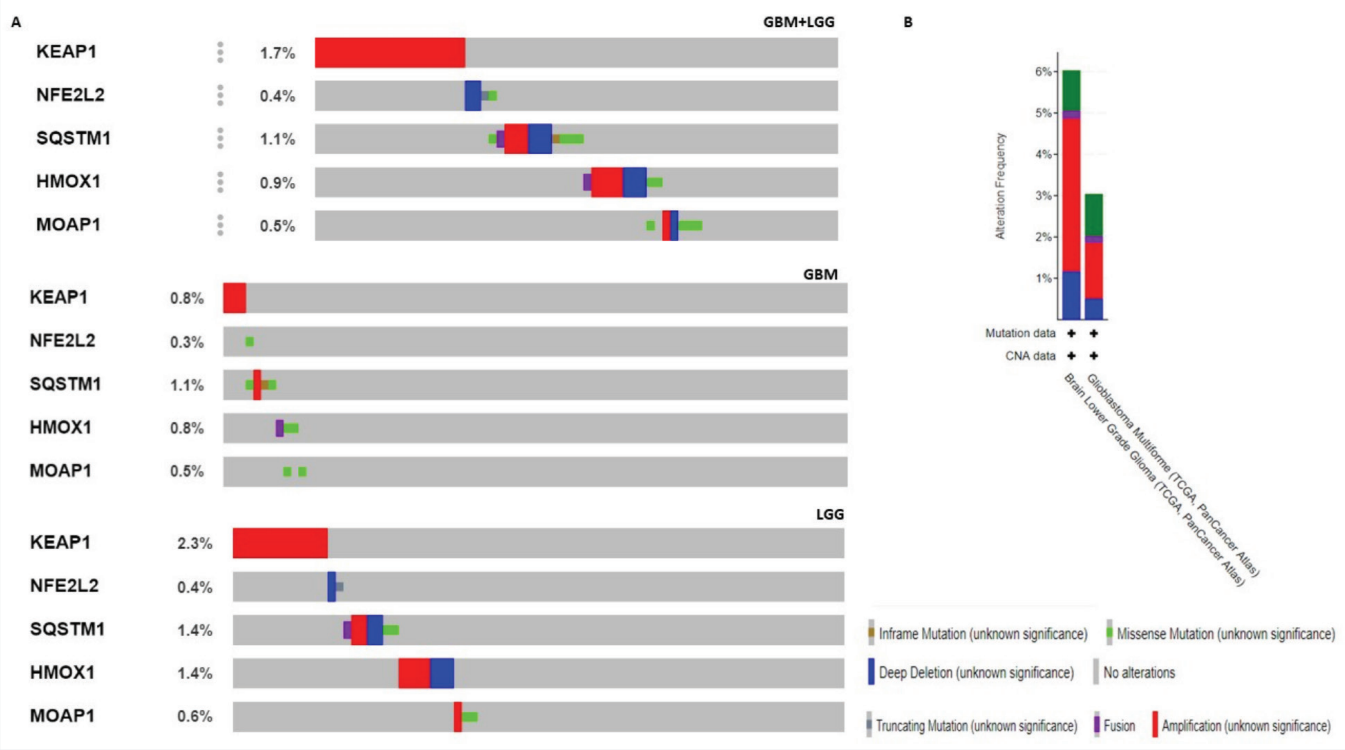

Figure 1. Distribution of mutations in *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP-1* genes in GBM and LGG cancer patient group (A, B)

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas

Results of Mutation Profile Analysis in GBM Patients

The carrying prevalence of GBM genetic abnormalities was detected to be 3,5%. Ten mutations *NRF2*, p.E564K; p62/SQSTM1 p.R96Q; p.E280del, p.F193L; p.R183P, *HMOX-1*, p.A194T; p.F33L, *TXNRD2-HMOX1* fusion gene; *MOAP-1*, p.P45L; p.A111V were detected in the GBM group. The frequency of carrying mutation with co-occurrence in NRF2-p62/SQSTM1; HMOX1-MOAP1 was statistically significant for the GBM patient group (p=0.037 and p=0.055, respectively). Mutations identified on the p62/SQSTM1 were on the PBI, LIR, and TBS domains. The p.R183P mutation was on the TBS Domain. The mutation p.F193L in the same domain and p.R96Q in the PBI domain was identified in a 23-year-old female patient carrying the IDH2 p.G383V and p.K251N missense mutations. It was also determined that same patient had NRF2 on the DNA binding domain p.E564K missense mutation. SOSTM1 p.E280del frame shift deletion was on the LIR domain. In addition, the fusion gene t(5;9)(q35;q21) was identified with p62/SQSTM1 and NTRK2, a tyrosine kinase responsible for neural development. HMOX1 mutations were identified only in the GBM patient group. All missense mutations detected in the HMOX-1 were located on the Heme oxygenease Domain. Two missense mutations (p.P45L and p.A111V) in the MOAP1 were identified on the PNMA domain.

Results of *In Silico* Analysis of Mutation Impact

According to the analysis results of PolyPhen-2, SNAP Database, and COSMIC programs, it is determined that among the mutations detected in our study, especially the pathogenic scores of the NRF2 p.E564K, SQSTM1 p.R96Q, and HMOX1 p.F33L mutations may be pathogenic due to the fact that they are close to 1 and "affected" and they are predicted to have disease-causing properties. Especially in Table 2, it is seen that all of the mutations found in the GBM patient group are pathogenic. As a result of multiple sequence alignment analysis, it was discovered that 10 of the 12 mutations detected modified their amino acids located at the critical point that was preserved among different species. In addition, p.E564K, p.S164*; SQSTM1 p.R96Q, p.R183P, and p.R107Q;

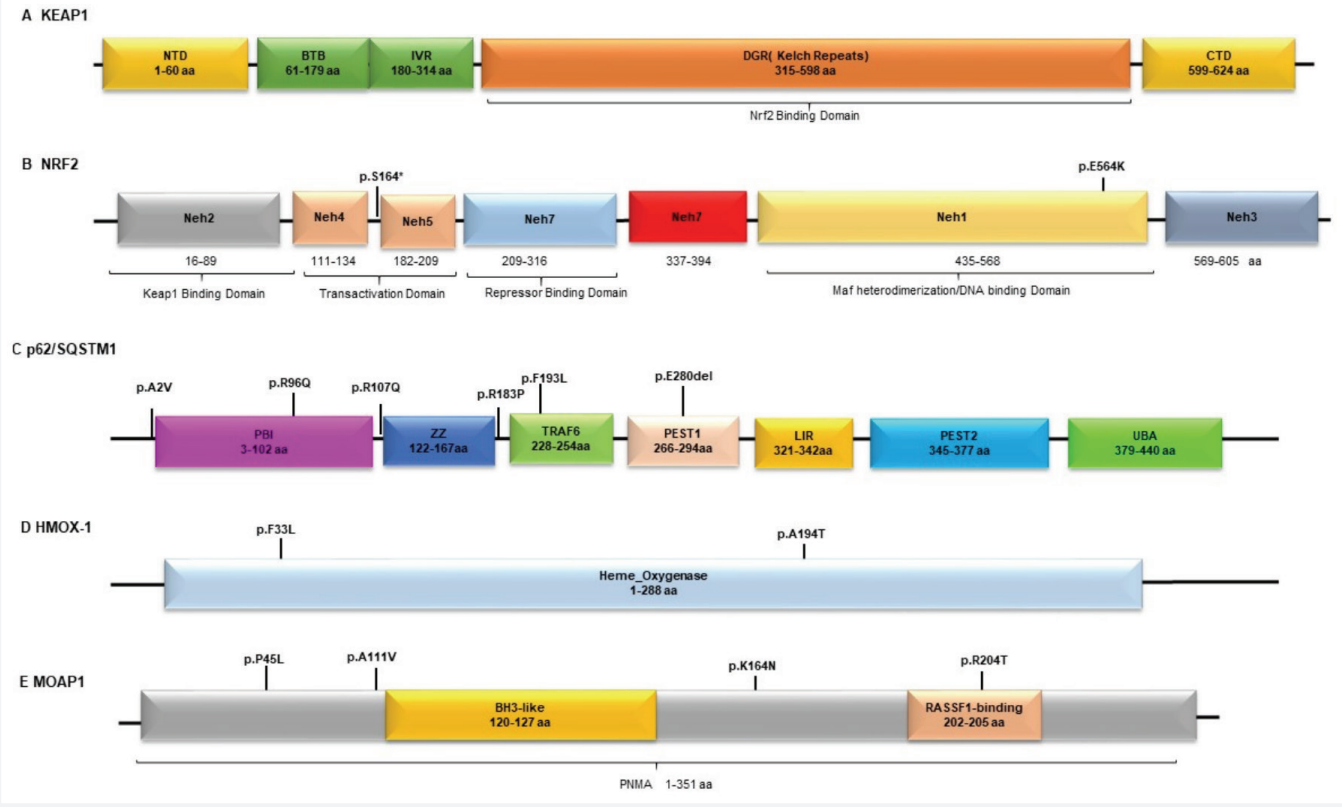

Figure 2. Schematic representation of domain architecture of the KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 proteins and mutations detected in patients with GBM and LGG (A) Human Keap1 is a polypeptide comprising 624 amino acids. (B) Human NRF2 is a polypeptide comprising 605 amino acids, which contains seven Neh domains. (C) Human p62/SQSTM1 is a polypeptide comprising 440 amino acids. (D) Human HMOX-1 is a polypeptide comprising 288 amino acids. (E) Human MOAP1 is a polypeptide comprising 351 amino acids

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas

Table 2. Mutations of the *NRF2*, *p62/SQSTM1*, *HMOX-1* and *MOAP1* genes in LGG and GBM patients

No	Gene	Nt alteration	Rs number	Alteration type	Localization	AA position	Coexistence with IDH1/2 mutation	Cancer type	Tumor type	Clinical significance		
										PolyPhen-2 (score)	SNAP (score)	COSMIC prediction
C-1	*NRF2*	c.1687G>A	COSV67960917	Missense mutation	Neh1 DNA binding Domain	p.E564K	p.G383V p.K251N	GBM	Primary	Probably Damaging (1.00)	Effect (51)	Pathogenic (0.94)
C-2	*NRF2*	c.491C>G	COSV67962580	Nonsense mutation	Neh5 Domain	p.S164*	p.R132H	LGG	Primary	NA	NA	Pathogenic (0.91)
C-3	*SQSTM1*	c.287G>A	COSV62435109	Missense mutation	PBI Domain	p.R96Q	p.G383V p.K251N	GBM	Primary	Probably Damaging (1.00)	Effect (60)	Pathogenic (0.99)
C-4	*SQSTM1*	-	-	Deletion	LIR Domain	p.E280del	-	GBM	Primary	NA	NA	UNK
C-5	*SQSTM1*	c.579C>T	COSV100657686	Missense mutation	TBS Domain	p.F193L	p.G383V p.K251N	GBM	Primary	Benign (0.12)	Effect (30)	Neutral (0.02)
C-6	*SQSTM1*	c.548G>C	COSV100657714	Missense mutation	TBS Domain	p.R183P	-	GBM	Primary	Benign (0.22)	Effect (38)	Pathogenic (0.97)
C-7	*SQSTM1*	c.320G>A	COSV62434138	Missense mutation	-	p.R107Q	-	LGG	Primary	Possibly Damaging (0.65)	Neutral (-10)	Pathogenic (0.90)
C-8	*SQSTM1*	c.5C>T	COSV62435509	Missense mutation	PBI Domain	p.A2V	p.R132H	LGG	Primary	Benign (0.33)	Neutral (-94)	UNK
C-9	*SQSTM1*	t(5;9)(q35;q21)	-	Fusion gene	-	SQSTM1-NTRK2	-	LGG	Primary	NA	NA	UNK
C-10	*HMOX1*	c.580G>A	COSV53340335	Missense mutation	Heme oxygenase Domain	p.A194T	p.R132H	GBM	Primary	Benign (0.02)	Neutral (-83)	Pathogenic (0.76)
C-11	*HMOX1*	c.99C>A	COSV99330761	Missense mutation	Heme oxygenase Domain	p.F33L	-	GBM	Primary	Possibly Damaging (0.86)	Effect (30)	Pathogenic (0.96)
C-12	*HMOX1*	-	-	Fusion gene	Heme oxygenase Domain	TXNRD2-HMOX1	-	GBM	Primary	NA	NA	UNK
C-13	*MOAP1*	c.134C>T	COSV52092948	Missense mutation	PNMA Domain	p.P45L	-	GBM	Primary	Probably Damaging (0.98)	Effect (66)	Neutral (0.03)
C-14	*MOAP1*	c.332C>G	COSV52092285	Missense mutation	PNMA Domain	p.A111V	-	GBM	Primary	Possibly Damaging (0.61)	Neutral (-83)	Neutral (0.05)
C-15	*MOAP1*	c.492G>C	COSV99365609	Missense mutation	PNMA Domain	p.K164N	p.R132H	LGG	Primary	Probably Damaging (0.99)	Effect (17)	Neutral (0.46)
C-16	*MOAP1*	c.614G>A	COSV52093035	Missense mutation	PNMA Domain	p.R204T	p.R132H	LGG	Primary	Probably Damaging (0.96)	Effect (55)	Neutral (e 0.04)

UNK: Unknown, NA: Not available, SNP: Single nucleotide polymorphism, C: Change, UTR: Untranslated region, LGG: Lower grade glioma; GBM: Glioblastoma multiforme

HMOX1 p.A194T, p.F33L mutations found in NRF2 are available as somatic mutations in the COSMIC database, and they are specifically noted for different types of solid cancers.

Gene Expression and Survival Analysis Results

The gene expression profiles of KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 were determined as a result of the comparison of GBM (n=163), LGG (n=518) patients with respect to the healthy group. According to the analysis results, KEAP1, NRF2, and HMOX1 expression levels were determined to be statistically significant in patient samples compared to healthy samples and higher in both cancer groups, while MOAP1 expression was found to be lower in patient samples compared to the healthy group ($p<0.005$) (Figure 3). The p value for NRF2 in the LGG group was found to be significant based on the OS analysis findings. Those with low levels of NRF2 expression were found to have a statistically significant longer OS time than those with high levels ($p=0.00027$). However, according to our DFS analysis results, high NRF2 expression in LGG patient group was statistically significant compared to individuals with low NRF2 expression ($p=0.0011$). Individuals with low gene expression of the HMOX1 have a statistically significant longer OS period than those with high gene expression ($p=0.025$) in the LGG patient group. Individuals with low levels of MOAP1 expression have longer OS than those with high levels of expression ($p=0.008$, Figure 4). Individuals with low gene expression of the SQSTM1 have a statistically significant longer DFS period than those with high gene expression in the GBM patient group ($p=0.0043$, Figure 5). Besides, individuals with high levels of expression in all other genes and both cancer subtypes, except for high levels of HMOX1 expression, have long OS. As a result of comparing the m-RNA levels of individuals with and without mutations for each gene, no statistical difference was found between the groups, the results are presented in Figure 5.

DISCUSSION

In our study, the mutation profiles and gene expression patterns of the *KEAP1*, *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP1*, which are the key actors in the cytoprotective

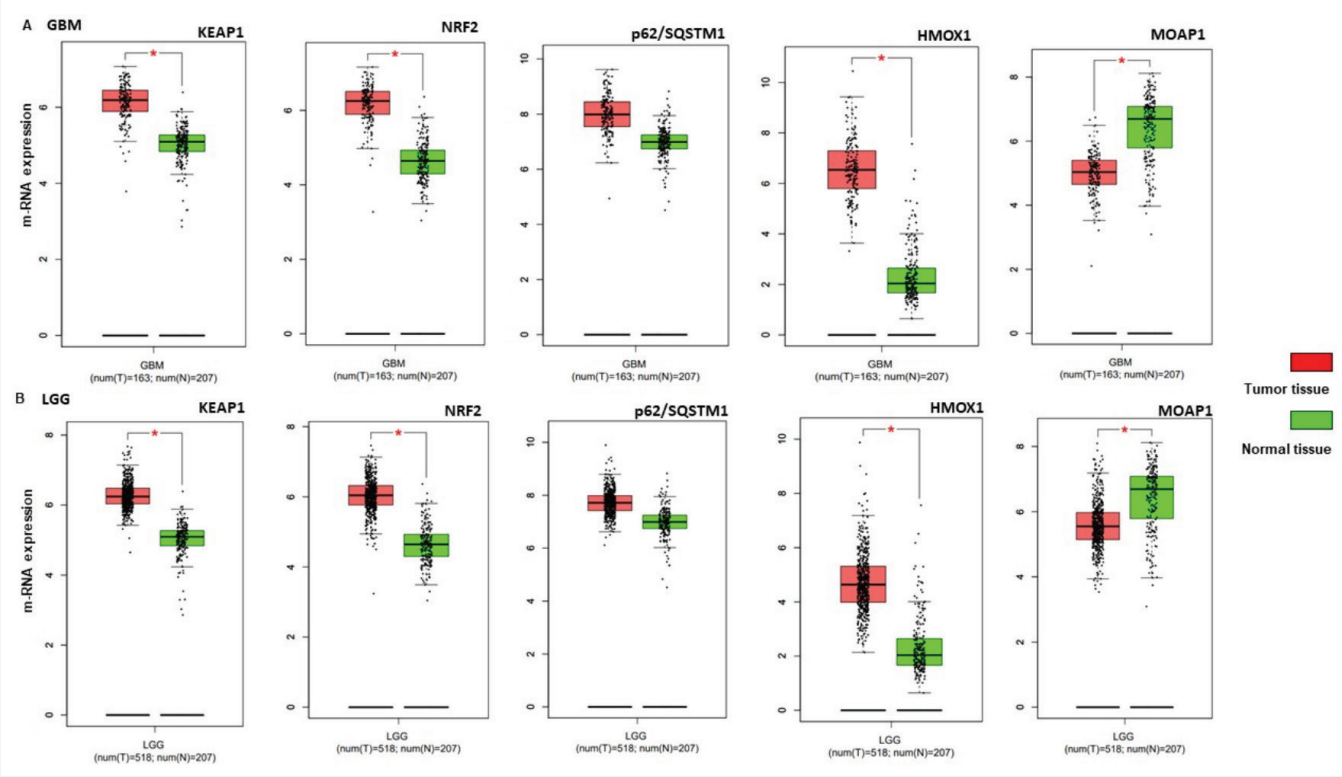

Figure 3. Comparative analysis of the tissue-specific differential expression of *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP1* genes in brain tissues using GEPIA in GBM (A) and LGG (B) patients. The m-RNA expression of *NRF2*, *p62/SQSTM1*, *HMOX-1*, and *MOAP1* genes between tumor and normal tissues. TPM (Transcripts Per Million) were used to measure gene expression levels. The expression data are first log2 (TPM+1) transformed for differential analysis and the log2FC is defined as median (Tumor) – median (Normal). Genes with higher [log2FC] values and lower q values than pre-set thresholds are considered differentially expressed genes. Statistically significant value was considered as $p<0.05$. Log2(TPM + 1) was used for log-scale

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas

pathway, were evaluated and compared in GBM and LGG patient samples. Primarily, among the genome sequencing results of a total of 1102 GBM and LGG patients that are available in TGCA data sets, the mutation profiles of the genes of interest KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 have been comprehensively analyzed. In our study, 12 of the 16 mutations detected in the NRF2/KEAP1 signaling pathway in GBM and LGG patients were found to be missense mutations, 1 was non-sense mutation, 1 was deletion, and 2 were translocations. It was determined that patient groups carried 6.1% mutations for LGG and 3.5% for GBM, and the gene carrying the most genetic abnormalities was KEAP1 1.7% for both tumor types. Mutations have been detected in study genes, particularly in sequences encoding important domains of the genes. It was determined that the NRF2 p.E564K, p62/SQSTM1 p.R96Q, and HMOX1 p.F33L missense mutations that we detected in our study affected the critical amino acids conserved in the evolutionary process according to the results of the inter-species evolutionary analysis. According to the results of the evolutionary analysis, it is thought that the missense mutations in question may be effective in the development of glioma due to their effect on amino acids that have been conserved throughout the evolutionary process and their domain regions, and their possible pathogenic properties obtained as a result of functional pathogenic effect analyses and the possibility of altering the expression of antioxidant response genes.

There are seven highly conserved domains known as NRF2-ECH homology domains[6,8,9,18]. The p.E564K mutation that we defined in NRF2 is located on the Maf heterodimerization/DNA binding domain/Neh1 of the protein. Neh1 contains a well-preserved CNC-bZIP region, which is required for DNA binding and heterodimer formation with NRF2 dimerization partners, sMaf proteins, as well as a nuclear localization signal required for NRF2 nuclear translocation. Furthermore, Neh1 is able to interact with UbcM2, the E2 ubiquitin-conjugation enzyme, to regulate NRF2 protein stability[5,6,8,9,18]. Considering that NRF2 is a transcription factor, the p.E564K

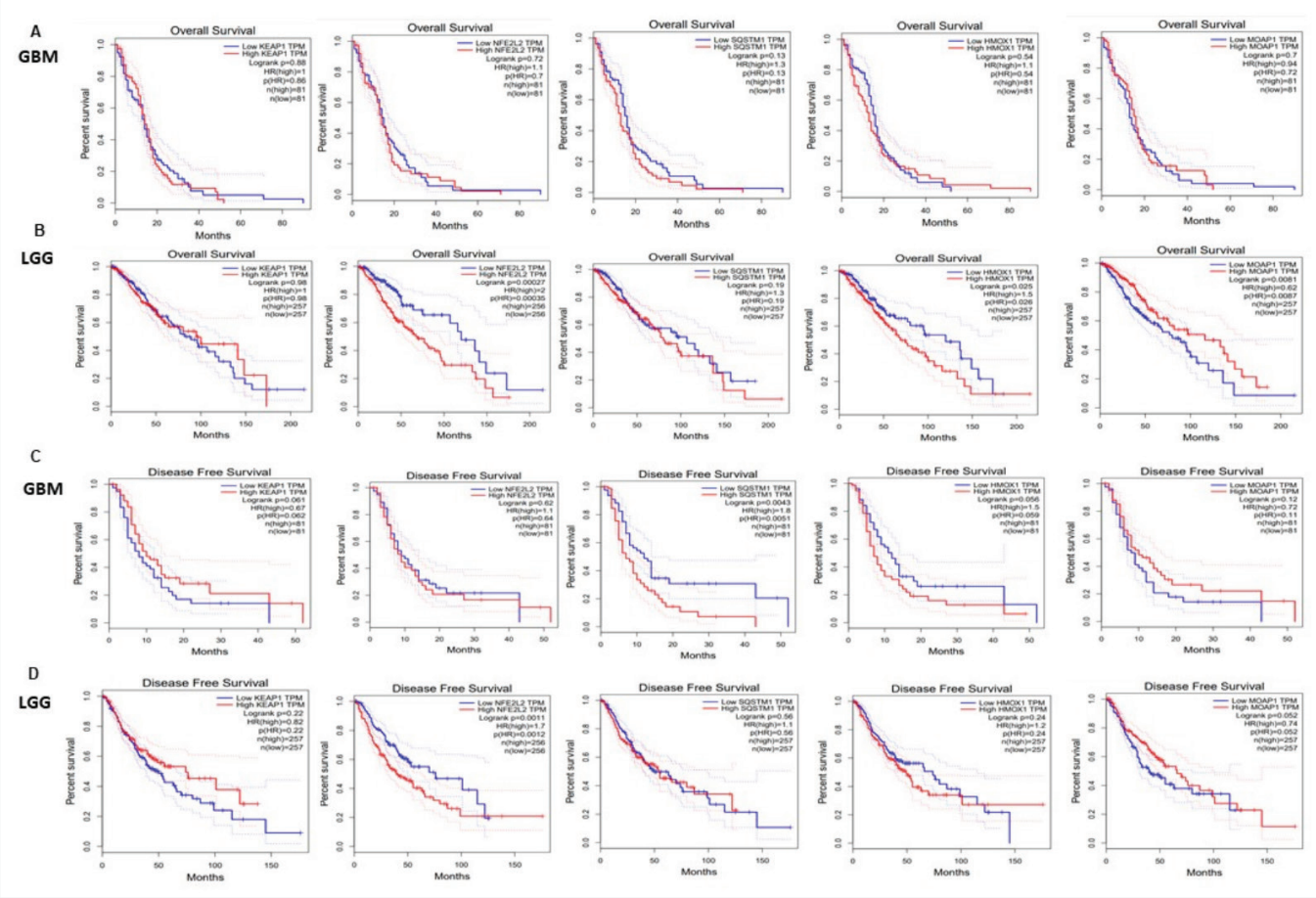

Figure 4. Kaplan–Meier analysis of OS (A, B) and DFS (C, D) of the GBM and LGG patients according to different NRF2, p62/SQSTM1, HMOX-1, and MOAP1 levels

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas, DFS: Disease free survival, OS: Overall survival

mutation is in a position to affect the activation/constitutive expression of the genes responsible for detoxification and may pose a potential problem for the localization of the protein as the same mutation is located on the domain containing NLS signaling. The NRF2 p.S164* nonsense mutation is truncating mutation and is expected to cause the formation of the stop codon as early as the 164. codon before NRF2 protein synthesis is complete. IDH1 is involved in energy metabolism with the production of NADPH in the cytoplasm and peroxisomes by catalyzing the conversion of isocitrate to alpha-ketoglutarata (α-KG). Numerous studies have reported that the IDH1 mutation can cause a decline in NADPH and the accumulation of ROS in cells[19,20]. However, the relationship between the biological significance of IDH1 mutations and cellular redox homeostasis is not completely known. In particular, it has been determined that cells overexpressing IDH1 p.R132H are more sensitive to the chemotherapeutic temozolomide and expression of NRF2 in these cells is significantly decreased[20]. NRF2 is known to be associated with treatment resistance and poor prognosis in glioma[3,4,20]. In our study, there are 2 individuals carrying both IDH1; NRF2 (p.R132H; p.S164*) mutations in LGG subtype and mutations in GBM subtype (p.G383V; p.K251N; p.E564K). When it was examined in the patients by the cBioPortal, it was determined that the NRF2 expression level decreased at the cellular level. The association of p.R132H mutation in LGG and NRF2 p.S164* mutation, which causes early termination of the polypeptide, may cause chemotherapy sensitivity.

The most well-known mechanism of the noncanonical pathway is NRF2 activation through the p62/SQSTM1 protein. p62/SQSTM1, a multi-domain and multi-functional protein, protects cells from stress by autophagy pathway and NRF2 activation. p62/SQSTM1, which encodes the p62 protein, is an adapter protein involved in a variety of fundamental cellular processes, including OS response, apoptosis, and cell differentiation[4,21]. p62/SQSTM1 facilitates the degradation of unwanted molecules by fixing ubiquitin proteins to the autophagosome membrane. Furthermore, p62/SQSTM1 acts as a signaling hub for multiple pathways associated with neurodegeneration and is seen as a potential therapeutic target in the treatment of neurodegenerative diseases[4,12,21]. The p.R96Q mutation, shown in Figure 2, affects the PB1 domain, which is a modulator for protein-protein interactions. It is located in the ZZ-type zincfinger domain, which is also involved in p.F193L and p.R183P protein-protein interactions. p.E280del is also on the PEST domain, and the frameshift is in a position to cause mutation, leading to nonfunctional polypeptide production. It is known in the literature that full-length p62/SQSTM1 regulates NRF2 activation through a positive feedback loop[4,12,21].

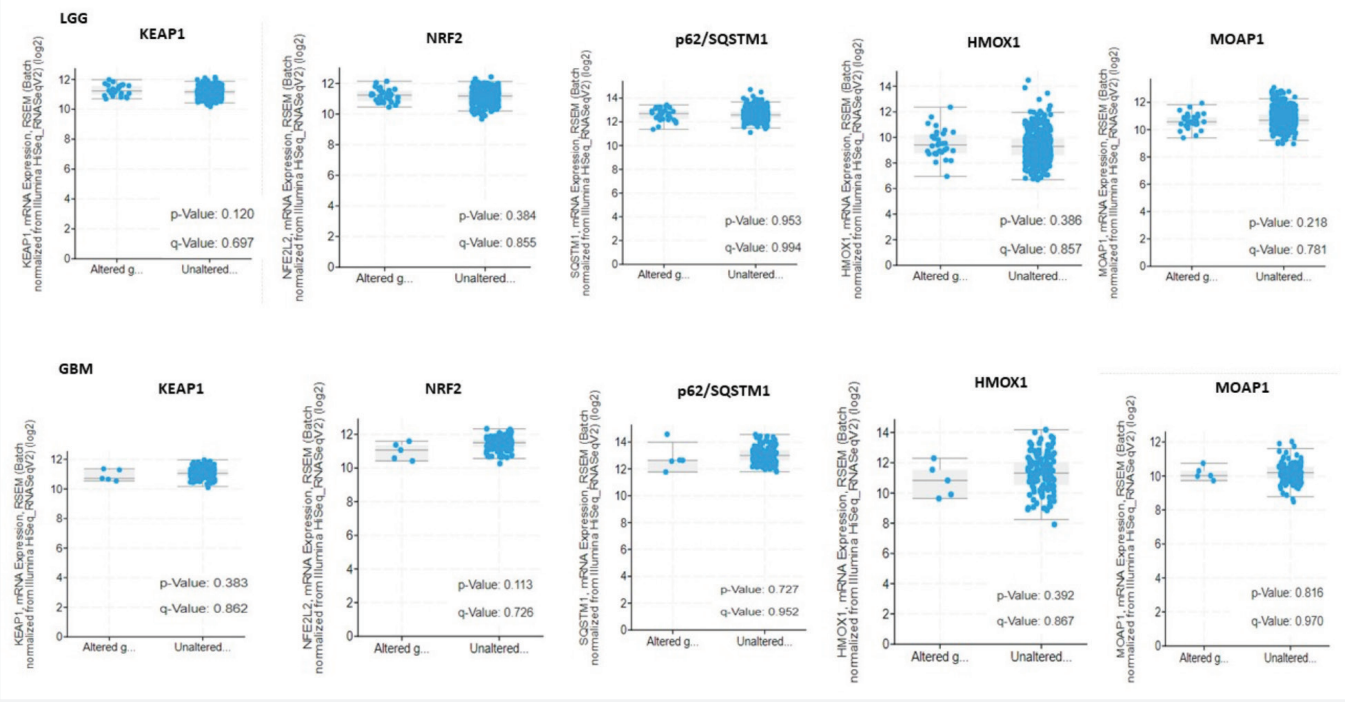

Figure 5. Mutational status for each gene is shown and the fold change indicates expression levels in the altered groups normalized to the expression levels in the unaltered groups (A) LGG (B) GBM

GBM: Glioblastoma multiforme, LGG: Low-grade gliomas

Gene fusions including NTRK1, 2, and 3 cause structural activation or overexpression of TRK receptors, potentially causing oncogenesis[22]. In the p62/SQSTM1-NTRK2 fusion protein, we detected in our study, SQSTM1, a multifunctional signal adapter involved in autophagy, fused to the 16-20 exons of NTRK2 with exon 1-5, resulting in the formation of a reading frame, which links the aminoterminal part of p62/SQSTM1 to the kinase domain of the TrkB. This, in turn, can lead to uncontrolled activation of p62/SQSTM1[4,12,21,22]. It has only been recently reported in the literature that Bax binding protein MOAP1 regulates the p62/SQSTM1-KEAP1-NRF2 signal via p62 degradation. MOAP1 interacts with the PB1-ZZ domains of p62 and disintegrates the p62 by interfering with its own oligomerization and liquid-liquid phase separation[23]. Since the p.R96Q mutation is located on the PBI domain, it may have the property to affect the MOAP1-p62/SQSTM1 interaction. HMOX-1, which expression is regulated by the NRF2 protein, is considered a cytoprotective agent and its modulation of expression activity levels offers therapeutic potential. HMOX-1 has been recognized as an antioxidant, anti-inflammatory, anti-apoptotic factor and is known to form one of the defense mechanisms against tissue damage caused by OS. In human glioma tumors, HMOX-1 is known to be overexpressed in comparison to normal brain tissues and during tumor progression, but the molecular mechanisms underlying how HMOX-1 affects glioblastoma tumor progression remain unclear[24,25]. The p.F33L and p.A96T mutations detected in our study are in the Heme oxygenease domain. We determined that p.F33L mutation was preserved among species throughout the evolutionary process. Pölönen et al.[12] reported that NRF2/p62 activation could be a prognostic marker for the mesenchymal subtype of GBM. We think that the missense mutations (C-1, C-3, C-4, C-5, and C-6) in the NRF2 and p62 in the GBM subgroup in present study may be responsible for the development of the mesenchymal subtype.

Study Limitations

We have performed comprehensive molecular profile analyses of the genes responsible for glioma pathogenesis. We are mindful that our study has certain limitations. This is because this study was carried out with a limited experimental design using bioinformatics tools. Therefore, a wet laboratory study and a larger sample group are needed to clarify the effect of KEAP1, NRF2, p62/SQSTM1, HMOX-1, and MOAP1 on glioma pathogenesis.

CONCLUSION

Gene expression analyses were also performed according to healthy patient samples in two tumor groups formed from the same patient population. As a result of our analysis of gene expression profiles, this pathway was found to be upregulated in both gliomas when compared to healthy samples of NRF2, KEAP1, and HMOX-1. The expression level of the MOAP1 is statistically significantly lower than the healthy sample group ($p<0.05$). However, we did not detect any significant changes in the level of expression of SQSTM1. Our study has results that can be useful in the development of new therapeutic approaches by determining the molecular differences and expression profiles between GBM and LGG. This study is important in terms of understanding the frequency and molecular features of the NRF2/KEAP1/p62/SQSTM pathway mutations detected in gliomas.

Acknowledgements

The data used in our study were obtained from public database the TCGA Research Network: https://www.cancer.gov/tcga. We thank the TCGA and GEPIA databases for the availability of the data.

Ethics

Ethics Committee Approval and Informed Consent: It is not required as it is analyzed *in silico* for bioinformatics.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: D.F.A., Design: D.F.A., Data Collection or Processing: D.F.A., S.H.A., Analysis or Interpretation: D.F.A., S.H.A., Literature Search: D.F.A., S.H.A., Writing: D.F.A.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.61587
Nam Kem Med J 2023;11(1):12-16

Predictive Value of Serum Calprotectin Level in Response to Treatment, a New Inflammatory Marker in Patients with Breast Cancer Requesting Neoadjuvant Treatment

Neoadjuvan Tedavi Alan Meme Kanserli Hastalarda Yeni Bir Enflamatuvar Belirteç Olan Serum Kalprotektin Düzeyinin Tedaviye Yanıtta Prediktif Değeri

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ABSTRACT

Aim: There is a close relationship between inflammation and cancer. Calprotectin is a protein released during inflammation. The aim of this study is to investigate the relationship between breast cancer and calprotectin levels in breast cancer patients receiving neoadjuvant therapy the predictive role of calprotectin in response to treatment.

Materials and Methods: In our prospective study, a patient group with 69 breast cancer patients and a control group with 20 patients were formed. Calprotectin was studied from the blood tests taken from the whole sample. Patient data were obtained from the electronic record system. In our study, statistical evaluations were made using a package program called IBM Statistical Package for the Social Sciences Statistics 24.

Results: Eighty-nine patients (69 cancer, 20 controls) were included in the study. The median age of breast cancer patients was 48 [minimum (min): 24-maximum (max): 73], the control group was 44.5 (min: 19-max: 68) and the ages of the two groups were similar (p=0.599). Mean calprotectin levels in breast cancer patients were 28.63±30.5, median 16.5 (min: 6.7-max: 160.7). The mean in the control group was 16.09±6.1 (min: 8.7-max: 27.4) and there was no statistical difference between the 2 groups (p=0.072). A statistically significant difference was found in terms of calprotectin values according to Ki67 classes (Z=-20.043; p=0.041). Calprotectin values of those with Ki67 class >20 were statistically significantly higher than those with ≤20. Parameters that could predict complete chemotherapy response were evaluated with logistic regression analysis. There was no correlation between calprotectin level and complete response. There was a positive correlation between age increase and complete response.

Conclusion: There was no significant difference between serum calprotectin levels of the patient and control groups, but calprotectin level was found to be associated with Ki67 level. There was no relationship between calprotectin and chemotherapy response. Studies with larger sample numbers may make a significant difference.

Keywords: Breast cancer, serum calprotectin, inflammation, complete response

ÖZ

Amaç: Enflamasyon ile kanser arasındaki yakın ilişki vardır. Kalprotektin enflamasyon sırasında salınan bir proteindir. Bu çalışma ile neoadjuvan tedavi alan meme kanserli hastalarda kalprotektin seviyesi ile meme kanseri ilişkisi ve tedavi yanıtı için kalprotektinin prediktif rolünün araştırılması amaçlanmıştır.

Gereç ve Yöntem: Prospektif bir araştırma olarak dizayn edilen çalışmamızda 69 meme kanseri tanılı hasta ile hasta grubu ve 20 hasta ile kontrol grubu oluşturuldu. Örneklemin tamamından alınan kan tetkiklerinden kalprotektin çalışıldı. Hasta verileri elektronik kayıt sisteminden elde edildi. Çalışmamızda istatistiksel değerlendirmeler IBM Statistical Package for the Social Sciences Statistics 24 adlı paket program kullanılarak yapıldı.

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Received: 22.08.2022 **Accepted:** 09.01.2023

Bulgular: Çalışmaya 89 hasta (69 kanser, 20 kontrol) dahil edildi. Meme kanseri hastalarının median yaşı 48 [minimum (min): 24-maksimum (maks): 73], kontrol grubunda 44,5 (min: 19-maks: 68) olarak saptandı ve iki grubun yaşları benzerdi (p=0,599). Meme kanseri hastalarında kalprotektin düzeylerinin ortalaması 28,63±30,5, median 16,5 (min: 6,7-maks: 160,7) saptandı. Kontrol grubunda ortalama 16,09±6,1 (min: 8,7-maks: 27,4) olarak görüldü ve 2 grup arasında istatistiksel fark yoktu (p=0,072). Ki67 sınıflarına göre kalprotektin değerleri açısından istatistiksel olarak anlamlı farklılık tespit edildi (Z=-20,043; p=0,041). Ki67 sınıfı >20 olanların kalprotektin değerleri, ≤20 olanlara göre istatistiksel olarak anlamlı düzeyde daha yüksekti. Kemoterapi tam yanıtını ön görebilecek parametreler lojistik regresyon analizi ile değerlendirildi. Kalprotektin düzeyi ile tam yanıt arasında ilişki yoktu. Yaş artışı ile tam yanıt arasında pozitif bir ilişki vardı.

Sonuç: Hasta ve kontrol grubunun serum kalprotektin düzeyleri arasında anlamlı fark saptanmadı fakat kalprotektin seviyesi Ki67 düzeyi ile ilişkili bulundu. Bu önemli ilişkisine rağmen kalprotektin ile kemoterapi yanıtı arasında ilişki yoktu. Daha büyük örneklem sayıları ile yapılacak çalışmalar anlamlı bir fark oluşturabilir.

Anahtar Kelimeler: Meme kanseri, serum kalprotektin, enflamasyon, tam yanıt

INTRODUCTION

Breast cancer was reported to be the second most frequently diagnosed cancer in 2018, and it is the most common cause of cancer-related death in women[1]. Neoadjuvant therapy, which is an important modality in the treatment of breast cancer, is defined as all systemic treatments applied to the breast tumor before surgical operations[2]. Although discussions continue, it has been found that patients with a complete response to neoadjuvant chemotherapy, especially in HER2-positive and triple-negative breast cancers, experience better results, have a longer disease-free survival, and have a higher overall survival compared to those without any response[3,4].

Calprotectin is a heterodimeric calcium-binding protein consisting of S100A8 and S100A9 subunits from the family of S100 proteins[5]. Calprotectins are expressed in a wide variety of cell types, but are particularly abundant in myeloid cells such as macrophages in the early differentiation stage, neutrophils, monocytes, and keratinocytes. After being released from activated granulocytes, S100A8/S100A9 binds to cell surface receptors that trigger signaling pathways associated with inflammatory processes, with a cytokine-like behavior pattern, and plays a critical role in numerous cellular processes such as cell cycle progression, cell survival, proliferation, differentiation and cell migration[6,7].

In this study, it was aimed to compare the serum calprotectin levels of patients with breast cancer planned to receive various neoadjuvant treatments and the serum calprotectin levels of patients without breast cancer, to try to determine the cut-off value for calprotectin if found to be associated with the diagnosis, to investigate the relationship between the calprotectin level and the response to the treatment in the breast cancer group, and to evaluate the relationship between calprotectin and other blood parameters, already known to be related to the inflammatory processes.

MATERIALS AND METHODS

In this study, which is planned to be a specialty thesis in medicine, the screening model, one of the quantitative research models, and the relational screening model, one of the sub-types of survey models, were used.

Selection and Definition of Cases

Our study included 89 patients who were admitted to Tekirdağ Namık Kemal University Hospital, Department of Internal Medicine Diseases, Unit of Medical Oncology and Department of General Surgery, Breast Outpatient Clinic between 15.03.2019 and 19.10.2020. While the "patient arm" of our study was formed with 69 patients diagnosed with breast cancer as a result of their application to the relevant polyclinic and planned to receive neoadjuvant treatment in the multidisciplinary council of our hospital, the "control group" of our study was also formed with 20 patients who applied to the same polyclinics with the suspicion of breast cancer but were not found to have malignant pathology. Patients over the age of 18 years, who gave the consent form prepared for participation in the study, were included in the study. Those who were pregnant, who did not sign the voluntary consent form, who had additional malignancies, who had a diagnosis or sign of infection during sampling and evaluation, and who had hematological or rheumatological disorders were excluded from the study. Patient data were obtained from the hospital archive and the hospital electronic recording system.

Calprotectin Measurement

After the decision was made to include the patients in the study and their written and verbal consents were obtained, 5 mL of venous blood sample was taken to study the calprotectin levels of the patient in addition to the routine blood tests. These tubes were centrifuged at 2000 G for 20 minutes and at room temperature. After centrifugation, the supernatant was aliquoted into two microtubes as it helped to partition the separator gel in the tube. These serum samples, which were taken into microtubes, were labeled and stored at -20 °C to be stored until the time of batch analysis. Analysis of serum calprotectin levels from samples was performed with a commercially produced kit based on the enzyme-linked immunosorbent analysis method (Bioassay Technology

Laboratory, Cat. No: E4010Hu). It was ensured that the person performing the experiments was blinded in terms of the knowledge of the groups in which the studied blood samples were included. After all samples were collected, they were analyzed with the same kit. Evaluation was made by following all the instructions and experimental processes specified in the commercial kit.

Statistical Analysis

In our study, statistical evaluations were made using a package program called Statistical Package for the Social Sciences (SPSS) (IBM SPSS Statistics 24). Categorical measurements were summarized as numbers and percentages, and continuous measurements as median and standard deviation. Comparison between two qualitative clinical variables was analyzed with the "Mann-Whitney U test", "independent sample-t test" and "Kruskal-Wallis H test". The "Fisher's exact" and "Pearson-χ^2" crosstabs were used according to expected value levels in the examination of the relationships between two qualitative variables. The relationship of calprotectin level with treatment response was evaluated with ROC curve and ROC-AUC analysis. Logistic regression model was used in predictive factor analysis for chemotherapy response.

RESULTS

Eighty-nine patients (69 cancer, 20 control) were included in the study. The median age was 48 [minimum (min): 24-maximum (max): 73] years in patients with breast cancer and 44.5 (min: 19-max: 68) years in the control group, and the ages of the two groups were similar (p=0.599). Of the cancer patients, 33% were ER negative, 46% were PR negative, and 54% were HER2 negative (Table 1).

In breast cancer patients, the mean calprotectin level was found to be 28.63±30.5, the median level was 16.5 (min: 6.7-max: 160.7). In the control group, the mean calprotectin level was detected to be 16.09±6.1 (min: 8.7-max: 27.4) and there was no statistical difference between the two groups (p=0.072). A statistically significant difference was found in terms of calprotectin values according to Ki67 categories (Z=-20.043; p=0.041). Calprotectin values of those with Ki67 level >20 were statistically significantly higher than those with ≤20.

Calprotectin level, which predicted complete response, was not diagnostically predictive of complete response when analyzed with ROC-curve (p=0.587, AUC: 0.453) (Figure 1).

Parameters that could predict complete chemotherapy response were evaluated with logistic regression analysis. There was no correlation between calprotectin level and complete response [Odds ratio (OR): 1.049 95% confidence interval (CI): 0.982-1.120, p=0.153]. There was a positive correlation between increasing age and complete response (OR: 1.092,

Table 1. Clinical and pathological characteristics of the patients included in the study

	Cancer (n=69)	Control (n=20)
Age		
<45	30 (43%)	12 (60)
≥45	39 (57%)	8 (40%)
Estrogen		
Negative	23 (33%)	
Positive	46 (67%)	
Progesterone		
Negative	32 (46%)	
Positive	37 (54%)	
Ki67		
<20	26 (38%)	
≥20	43 (62%)	
HER2		
Negative	37 (54%)	
Positive	32 (46%)	
Grade		
1	12 (17%)	
2	30 (43%)	
3	27 (39%)	
Tumor type		
Invasive ductal	59 (85.5%)	
Other	10 (14.5%)	

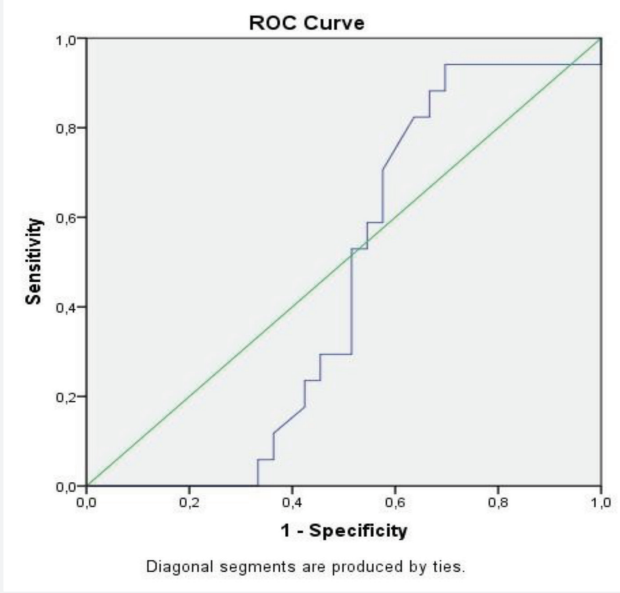

Figure 1. Cut-off determination of calprotectin level, which can predict complete response, with ROC-curve

95% CI: 1.022-1.168, p=0.010). Estrogen negative (OR: 0.284 95% CI: 0.082-0.984, p=0.047) and progesterone negative (OR: 0.238 95% CI: 0.067-0.841, p=0.026) were more likely to have complete responses (Table 2).

DISCUSSION

In our study, the value of serum calprotectin level in the diagnosis of breast cancer and response to treatment was investigated. In our analyses, we found that the calprotectin level was higher in those with high Ki67. Although there was a numerical difference in calprotectin levels between the included patient and control groups, no statistically significant difference was found, whereas pre-treatment calprotectin level could not predict treatment response. Age, estrogen receptor and progesterone receptor were predictive for complete response to neoadjuvant chemotherapy.

In the study of Khorrami et al.[8] investigating the value of calprotectin for the diagnosis of breast cancer, it was reported that serum calprotectin level could be a valuable marker in the diagnosis of cancer patients. According to the study of Gunaldi et al.[9], which was published in 2015 and examined the diagnostic importance of S100A9 and S100A12 proteins for breast cancer, the S100A9 protein, one of the 2 sub-units of the calprotectin protein, could not distinguish breast cancer from the control group. In our study, calprotectin levels were similar in cancer and control groups, and there was no statistical difference between the two groups. These differences between the studies may be related to the histological types of the patients included. While Gunaldi et al.'s[9] study included different histological types as in our study, Khorrami et al.'s[8] study consisted of only invasive ductal types. Future studies involving homogeneous patients will clarify this situation.

There was no significant difference between the serum calprotectin levels of the patient and control groups. However, while the mean serum calprotectin value was 28.63±30.46 in the patient group, it was found as 16.09±6.12 in the control group. The mean serum calprotectin value was found to be 40.03±1.54 for the control group consisting of 30 healthy volunteers in the study of Zaki et al.[10], who used exactly the same serum calprotectin kit we used in our study with all procedures. Although statistical comparison analysis was not performed for the control group in our study, we think that it was relatively high. However, unlike our study, the rate of male volunteers was 66.7% and the mean age was 32.30±11.43 years in the control group of this study, which was quite different in design from our study in terms of sample distribution[10]. In the current publication of Shaik et al.[11] in 2021, it was reported that even though benign breast disease was diagnosed, there was a significant increase in inflammatory markers in the benign diagnosis periods of patients with a high risk of breast cancer afterwards. It also comes to mind that this may be the reason why the inflammatory markers, including the calprotectin value, of the patients in the control group of our study were similar to the patient group because in the design of our study, clinical follow-up of the patients diagnosed as benign was not carried out to determine whether these pathologies converted to malignancy within a specified period.

Table 2. Logistic regression analysis of variables to predict complete response after neoadjuvant chemotherapy

		Univariate analysis	
Variable	**Category**	**OR (95% CI)**	**p**
Calprotectin	Continuous	1.049 (0.982-1.120)	0.153
Age	Continuous	1.092 (1.022-1.168)	**0.010**
Ki67	≤20/>20	0.392 (0.113-1.364)	0.141
NLR	Continuous	0.635 (0.336-1.200)	0.162
Estrogen	-/+	0.284 (0.082-0.984)	**0.047**
Progesterone	-/+	0.238 (0.067-0.841)	**0.026**
HER2	-/+	1.714 (0.525-5.603)	0.372

Statistically significant p values were marked in bold.
NLR: Neutrophil/lymphocyte ratio, CI: Confidence interval, OR: Odds ratio

Analyses were made in order to evaluate the patient and control groups in our study in terms of our main variable, and in these analyses, a difference was determined in terms of age. The age of our patient group was higher than that of the control group. Due to this difference, when the change in serum calprotectin value with age variable is examined from the literature, it is seen that many studies indicate that age is not an effective parameter. In the study of Oosterwijk et al.[12] in 2020, in which they examined variables that might be determinants of serum calprotectin levels in patients with diabetes mellitus, the age variable was determined as an insignificant parameter in this respect (r=-0.035 for age, p=0.051). In addition, in a study by Zaki et al.[10] in 2019, evaluating the relationship between clinical severity and serum calprotectin levels in psoriasis patients, the age variable was not found to be associated with serum calprotectin levels (r=0.214; p=0.136).

In our study, when two groups were formed according to the 20 cut-off value of Ki67 level, serum calprotectin value was found to be significantly higher in those with Ki67 above 20. Ki67 is one of the most well-known predictors of chemotherapy response[13,14]. Ki67 is a parameter that is generally thought to be expressed less than 3% in healthy breasts and has been used in routine pathology reports in the last 10 years[15]. At the same time, the number of studies reporting that Ki67 is prognostic has been increasing recently[16,17]. However, in our treatment response analysis in this study, Ki67 did not show predictive properties for treatment response. Nevertheless, the results of our study are promising for serum calprotectin values in response to treatments or in follow-up, since it is known that there is a significant correlation between the increased percent

expression of Ki67 value and the clinical aggressiveness of the tumor.

Current oncology guidelines report that luminal breast cancer subtypes have a worse response to chemotherapy[18]. Chou et al.'s[19] study in 2019 and Verdial et al.'s[20] study in 2022 evaluated the predictors of response to neoadjuvant chemotherapy in locally advanced breast cancers, and young age was determined to predict complete response. In our study, age and hormone receptors were found to be predictive for treatment response. Hormone receptor positive patients and older patients gave worse response to the treatment.

Study Limitations

There were some limitations regarding our study. The most important limitation was the small number of patients included in the study and the single center design. In addition, the heterogeneity of the included breast cancer patient groups was our other limitation. However, it is important that this study is the first to our knowledge to evaluate calprotectin for breast cancer treatment response.

CONCLUSION

In conclusion, the level of calprotectin did not differ significantly between the normal population and breast cancer patients, and there was no relationship between calprotectin and chemotherapy response. Further studies should investigate the relationship between calprotectin and breast cancer treatment response, with a larger number of patients and a more homogeneous patient population.

Ethics

Ethics Committee Approval: The study was approved by the Tekirdağ Namık Kemal University of Local Ethics Committee (protocol no: 2019.206.11.03, date: 26.11.2019).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: E.B., S.Ö.G., Concept: E.B., S.Ş., Design: E.B., Data Collection or Processing: E.B., A.Ç., S.Ö.G., Analysis or Interpretation: E.B., A.Ç., Literature Search: E.B., S.Ş., Writing: E.B.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.82474
Nam Kem Med J 2023;11(1):17-21

Protective Effects of Caffeic Acid Phenethyl Ester Against Carbon Tetrachloride-induced Testicular Damage in Rats: A Histological Study

Sıçanlarda Karbon Tetraklorür ile Oluşturulan Testis Hasarına Karşı Kafeik Asit Fenetil Esterin Koruyucu Etkileri: Histolojik Çalışma

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ABSTRACT

Aim: Carbon tetrachloride (CCl_4) is a volatile organic chemical agent that can cause damage to many tissues. Caffeic acid phenethyl ester (CAPE), which is structurally similar to flavonoids, is an active component of honeybee propolis. CAPE is known for its antitoxic, antioxidant, and anti-inflammatory effects. In this study, we aimed to investigate the effects of CAPE against testicular damage caused by CCl_4.

Materials and Methods: Twenty-eight Wistar albino rats were divided into 4 groups (n=7) as, Group 1: control (5% ethanol, 1 mL/day/ip), Group 2: olive oil (0.5 mL/day over/ip), Group 3: CCl_4 (0.5 mL/kg over/ip), Group 4: CCl_4+CAPE (10 µmol/kg/day/ip). Tissue samples collected at the end of the experiment were detected in 10% formaldehyde and embedded in paraffin. Five-micron-thick sections taken from paraffin blocks were stained with hematoxylin-eosin. To evaluate testicular damage, 100 tubules from each section were randomly examined at 20x magnification under a light microscope and classified as intact, atrophic, and degenerated tubules. Sections were examined by using Leica DFC 280 light microscope and Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd. Cambridge, UK).

Results: The testicular sections of the control group and the olive oil group had a normal histological appearance. In the CCl_4 group, 55.00±4.22% of the seminiferous tubules were intact, 25.00±2.67% were atrophic and 20.00±1.88% were degenerative. In addition, multinucleated giant cells were found in the lumen of some seminiferous tubules. In the CCl_4+CAPE group, 72.14±3.91% of the tubules were intact, 16.42±2.10% were atrophic, and 11.42±2.36% were degenerative. While the number of affected tubules significantly increased in the CCl_4 group compared to the control group ($p<0.05$), the number of affected seminiferous tubules decreased significantly in the CCl_4+CAPE group compared to the CCl 4 group ($p<0.05$).

Conclusion: We think that CAPE may be useful in reducing the damaging effects of CCl_4 on the testicle.

Keywords: CAPE, carbon tetrachloride, rat, testis

ÖZ

Amaç: Karbon tetraklorür (CCl_4) birçok dokuda hasara yol açabilen, uçucu organik bir kimyasal ajandır. Yapıca flavonoidlere benzeyen kafeik asit fenetil ester (CAPE), bal arısı propolisinin aktif bir bileşenidir. CAPE'nin antitoksik, antioksidan, anti-enflamatuvar etkileri olduğu bilinmektedir. Bu çalışmada CCl_4'ün neden olduğu testis hasarına karşı CAPE'in etkilerini incelemeyi amaçladık.

Gereç ve Yöntem: Yirmi sekiz adet Wistar-albino sıçan 4 gruba ayrıldı (n=7). Grup 1: Kontrol (%5 etanol, 1 mL/gün/ip), Grup 2: Zeytinyağı (0,5 mL/gün aşırı/ip), Grup 3: CCl_4 (0,5 mL/kg gün aşırı/ip), Grup 4: CCl_4+CAPE (10 µmol/kg/gün/ip). Deney sonunda alınan doku örnekleri %10'luk formaldehitte tespit edilerek parafine gömüldü. Parafin bloklardan alınan 5 µm kalınlığındaki kesitler hematoksilen-eozin ile boyandı. Testiküler hasarı değerlendirmek için ışık mikroskobunda her kesitten x20 büyütmede rastgele 100 tübül incelenerek sağlam, atrofik ve dejenere tübüller olarak sınıflandırıldı. Kesitler, Leica DFC 280 ışık mikroskobu ve Leica Q Win Görüntü Analiz sistemi (Leica Micros Imaging Solutions Ltd. Cambridge, UK) kullanılarak incelendi.

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Received: 06.12.2022 **Accepted:** 10.01.2023

Bulgular: Kontrol ve zeytinyağı gruplarına ait testis kesitleri normal histolojik görünümdeydi. CCl_4 grubunda seminifer tubullerin %55,00±4,22'si sağlam, %25,00±2,67'si atrofik ve %20,00±1,88'i dejeneratif olarak gözlendi. Ayrıca, bazı seminer tübüllerin lümeninde multinükleer dev hücrelere rastlandı. CCl_4+CAPE grubunda ise tübüllerin %72,14±3,91'i sağlam, %16,42±2,10'u atrofik ve %11,42±2,36'sı dejeneratifti. CCl_4 grubunda etkilenen tübüllerin sayısı kontrol grubuna göre istatistiksel olarak anlamlı şekilde artarken ($p<0,05$), CCl_4+CAPE grubunda etkilenen seminifer tübüllerin sayısının, CCl_4 grubuna göre istatistiksel olarak anlamlı şekilde azaldığı tespit edildi ($p<0,05$).

Sonuç: CAPE'nin, CCl_4'ün testis üzerindeki hasar verici etkilerinin azaltılmasında faydalı olabileceğini düşünmekteyiz.

Anahtar Kelimeler: CAPE, karbon tetraklorür, sıçan, testis

INTRODUCTION

Xenobiotic carbon tetrachloride (CCl_4) is a colorless liquid with a sweet odor[1]. CCl_4, which is a highly toxic substance with potential carcinogenic effects in humans and animals, is now used extensively in the manufacture of chlorofluorocarbon refrigerants in industry and thus in air conditioning and refrigeration systems. CCl_4 has toxic effects on the kidney, heart, lung, brain, and testicular tissues, especially on the liver[2,3]. Studies have shown that CCl_4 causes spermatogenic cell damage, basal membrane separation, seminiferous tubular atrophy, expansion in the interstitial area, and a decrease in sperm count[4,5].

Caffeic acid phenethyl ester (CAPE), which is structurally similar to flavonoids, is an active component of honeybee propolis[6]. CAPE is known for its antitoxic, antioxidant, anti-inflammatory, antiviral, immunomodulatory, neuroprotective, and cytostatic effects[7,8]. Since it strongly modulates the arachidonic acid cascade compared to other propolis components, its anti-inflammatory effect is more pronounced[6]. It has been shown to block all reactive oxygen species formed by the xanthine dehydrogenase/xanthine oxidase system at a concentration of 10 µmol/L[9].

In this study, we aimed to investigate the histological changes in the testicular tissue of rats with CCl_4-induced and the curative effect of CAPE on these changes by histochemical methods.

MATERIALS AND METHODS

Experimental Animals

The 28 3-month-old male Wistar albino rats weighing 200-250 g, which were used in our study, were obtained from İnönü University Experimental Animals Production and Research Center. Approval was obtained from the Experimental Animals Ethics Committee of İnönü University Faculty of Medicine (protocol no: 2012/A-49, date: 21.03.2012). The rats were housed in rooms where the room temperature was between 24 and 27 °C, ventilation conditions were met, and the lighting was 12 hours light and 12 hours dark. The rats were fed standard pellet feed *ad libitum* throughout the study.

Experimental Groups

Randomly selected subjects were divided into 4 different groups.

1. Control group: Rats in this group were administered 5% ethanol intraperitoneally (i.p) 1 mL/day for 10 days.

2. Olive oil group: 0.5 mL/day extra olive oil was administered i.p to the rats in this group for 10 days.

3. CCl_4 group: 0.5 mL/kg CCl_4 was administered i.p to the rats in this group for 10 days.

4. CCl_4+CAPE group: 0.5 mL/kg prepared by dissolving in olive oil was administered i.p to rats in this group for 10 days by dissolving in CCl_4 followed by dissolving in 10 µmol/kg CAPE (Sigma, St. Louis, MO).

The study's 11th 5 mg/kg xylazine and 50 mg/kg ketamine i.p. were administered to the rats, and their abdomens were opened under general anesthesia with a midline incision. The testicular tissue samples were collected for histological examination.

Histological Analyses

Detection and follow-up of tissues were started for histopathological evaluation. Then, 10% formaldehyde was added to ensure good fixation of the tissues. The specimens were then divided into smaller pieces of 3-4 mm, placed in plastic tissue follow-up cassettes, and fixed in formaldehyde for 24 hours. After the fixation process was completed, the parts were rinsed in running tap water for 24 hours. They were then dehydrated in graduated alcohols, made transparent in, and embedded in paraffin. Five-micron sections were taken from paraffin blocks using a Leica RM2145 microtome. The sections were stained with hematoxylin and eosin to observe the general histological structure. To evaluate testicular damage, 100 tubules from each section were randomly examined under a light microscope at 20x magnification and classified as intact, atrophic, and degenerated tubules. Sections were examined by using Leica DFC 280 light microscope and Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd. Cambridge, UK).

Statistical Analysis

Statistical analyses were conducted via the Statistical Package for the Social Sciences (SPSS) program (SPSS for Windows version 13) and MedCalc (2007, Belgium) statistical software. All results are expressed as arithmetic mean ± standard error.

The measurable variables in all groups did not have a normal distribution according to the Shapiro-Wilk normality test (p>0.05). For this reason, the Kruskal-Wallis analysis of variance, one of the non-parametric tests, was used for the general comparison of the groups in terms of all variables, whereas the Connover test was used for the pairwise comparison of the groups. The results were considered significant at p<0.05.

RESULTS

Histopathological Evaluation

When the testicular sections of the control and olive oil groups were examined, the seminiferous tubules had a normal histological appearance. The tubules consisted of a seminiferous epithelium sitting on a distinct basal lamina. Sertoli cells and spermatogenic serial cells in the seminiferous epithelium were clearly distinguishable. While spermatogonia were located just above the basal membrane and had a round or oval shape, spermatids were observed in the lumen (Figure 1A, 1B).

In the CCl_4 group, degenerative cells with eosinophilic cytoplasm were found in some tubules, which arrested spermatocytes in different stage of division (Figure 1C). In addition, multinucleated giant cells were observed in the lumen of some seminiferous tubules (Figure 1D). In this group, 55.00±4.22% of the seminiferous tubules were intact, 25.00±2.67% were atrophic and 20.00±1.88% were degenerative. In the CCl_4+CAPE group, 72.14±3.91% of the tubules were intact, 16.42±2.10% were atrophic, and 11.42±2.36% were degenerative (Figure 1E, 1F). While the number of affected tubules significantly increased in the CCl_4 group compared to the control group (p<0.05), the number of affected seminiferous tubules significantly decreased in the CCl_4+CAPE group compared to the CCl_4 group (p<0.05) (Table 1).

DISCUSSION

Exposure to toxic substances in the environment and/or workplace is considered to be one of the main factors responsible for sperm quality decline[10]. The persistence of toxic substances in our environment and all their effects on reproductive health, especially general health, make them a public health concern.

CCl_4 is a colorless, volatile, and toxic industrial substance that is rapidly absorbed by humans and animals after being released into the air, water, and soil through toxic emissions. Studies have shown that CCl_4 causes liver[3], lung[11], kidney[12-14] and testicular damage[15] in experimental animals. Studies have reported that CCl_4 administration damages the reproductive system by causing oxidative toxicity in male rats[4,14,16].

Given the changes caused by the adverse effects of many drugs and chemical agents, the possible effects of healing agents on different tissues of the body need to be investigated. Propolis is a sticky substance with very strong antiviral, antibacterial, and antifungal effects consisting of a mixture of various oils, pollens, special resins, and waxy substances collected by honeybees from the cones and barks of trees and from buds and sprouts of plants[17]. CAPE is an active component of propolis and has been shown to be a pharmacologically safe compound with anti-inflammatory, antimitogenic, anticarcinogenic, antioxidant, and immunomodulatory effects[18-22]. This study was designed to histologically evaluate the therapeutic effects of CAPE on testicular injury induced by CCl_4.

Previous experimental studies have reported that CCl_4 exposure causes structural and functional damage in the male reproductive system[23]. Moreover, similar previous studies have shown that histopathological changes occur in testicular tissues due to CCl_4 toxicity[24]. In the CCl_4 study conducted by Türk et al.[5] with rats, it was reported that they observed degeneration in germ cells, edema, and congestion in the interstitial area. Similarly, in the study conducted by Khan and Ahmed[4] with rats, degeneration, basal membrane separation, seminiferous tubule atrophy, and expansion in the interstitial area were reported in the spermatogenic serial cells of CCl_4. In their study with rats, Horn et al.[16] reported that they observed only Sertoli cells in the seminiferous tubules as a result of loss of germ cells, vacuolization in the germinal epithelium, and interruption of meiosis with the administration of CCl_4. In our study, we classified the tubules according to the presence of

Table 1. Histopathological score values of all groups

Groups	Intact tubule	Atrophic tubule	Degenerated tubule
1. Control (%)	83.57±2.10	10.71±0.71	5.71±1.70
2. Olive oil (%)	80.00±2.43	10.71±1.70	9.28±1.30
3. CCl_4 (%)	55.00±4.22[a]	25.00±2.67[a]	20.00±1.88[c]
4. CCl_4+CAPE (%)	72.14±3.91[b]	16.42±2.10[b]	11.42±2.36[d]

[a]Statistically significant difference with control (p=0.0008).
[b]Statistically significant difference with CCl_4 (p=0.0008).
[c]Statistically significant difference with control (p=0.0025).
[d]Statistically significant difference with CCl_4 (p=0.0025).
CCl_4: Carbon tetrachloride, CAPE: Caffeic acid phenethyl ester

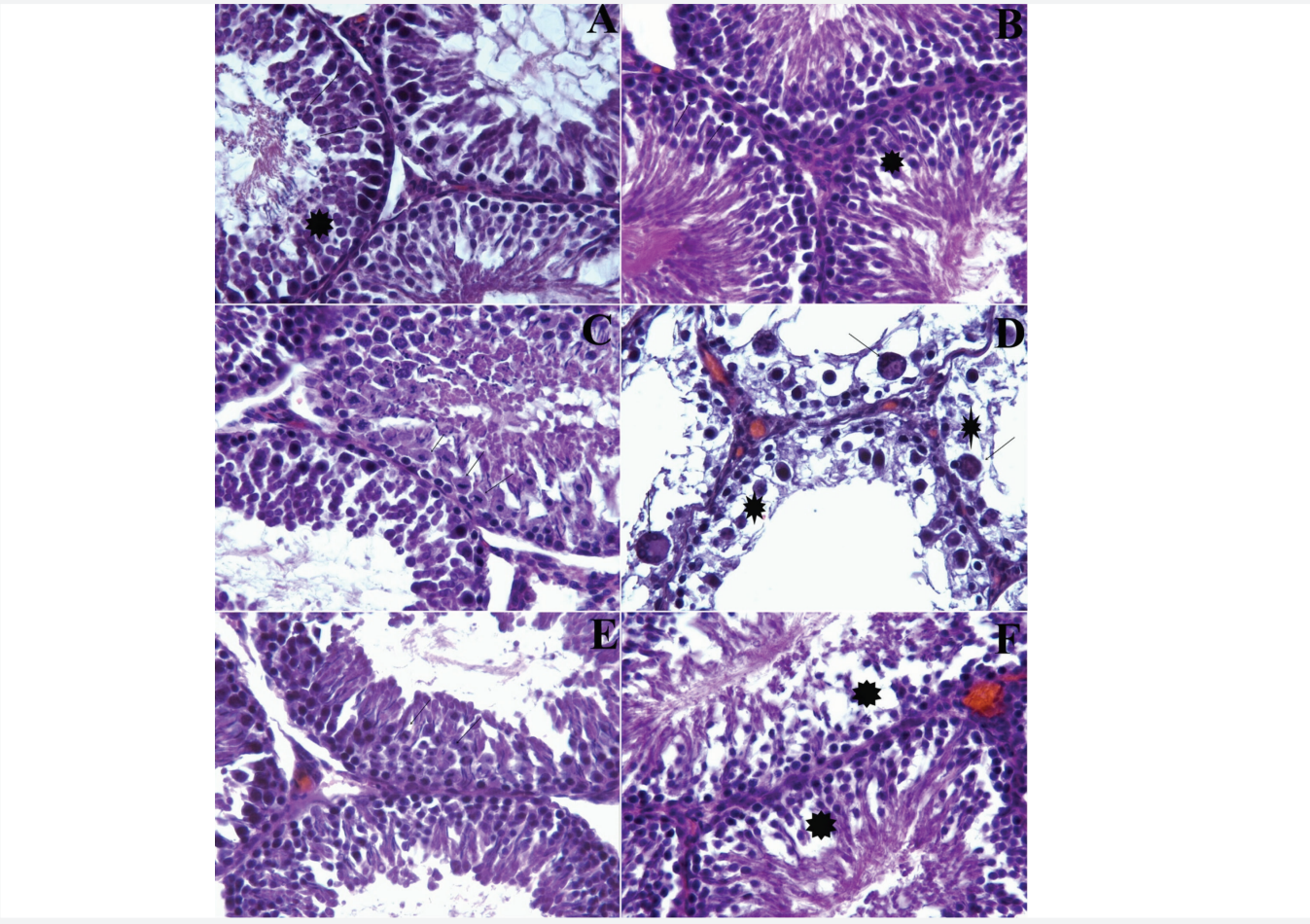

Figure 1. Control (A) and olive oil group (B), the view of Sertoli cells (arrows) and spermatogenic cells (star) in the seminiferous tubule epithelium. CCl_4 group (C), the appearance of arrested spermatocytes in different stage of division (arrows). (D) the view of germinal cell loss (asterisk) and multinuclear giant cells (arrows) CCl_4+CAPE group, (E) the view of arrested spermatocytes in different stage of division (arrows) (F) germinal cell loss (asterisk)

CCl_4: Carbon tetrachloride, CAPE: Caffeic acid phenethyl ester

degenerative cells and their atrophy and histopathologically showed the damage caused by CCl_4 to the testis. In our study, we found atrophic tubules in the testicular tissue, after CCl_4 administration, and degenerated cells with eosinophilic cytoplasm in some tubules, which paused at certain stages of meiosis and were observed in different ways. We also detected the presence of multinucleated giant cells in the lumen of some seminiferous tubules.

In our study, it was obeserved that the histological damage to the testis with the administration of CCl4 decreased with CAPE treatment. CAPE has these protective effects on the basis of antioxidant actions, but the exact mechanisms of anti-oxidant properties of CAPE are not known yet. However, it has been speculated that CAPE may affect transcription and/or translation of genes and gene products of anti-oxidant enzymes[25]. In addition, the protective effects of CAPE may be caused by its ability to block the bioactivation of CCl_4 by inhibiting CYP2E1 activity, in combination with its ability to scavenge free radicals[26]. In a study by Atik et al.[27], investigating the effects of CAPE against testicular damage caused by ischemia/reperfusion in rats, it was reported that the effect of histopathological damage decreased with CAPE application. Abdallah and El-Refaei[28] reported that the widespread inflammation, necrosis, and hemorrhage they observed in the testis when cadmium was administered to rats was alleviated by the administration of CAPE. In our study, we found that the number of affected seminiferous tubules decreased significantly in the CCl_4+CAPE group compared to the CCl_4 group.

Study Limitations

The most important limitation of our study is that it is not supported by functional recovery and biochemical parameters.

CONCLUSION

In conclusion, our study investigated the possible effect of CAPE on testicular damage by CCl_4. The results of our study show that CCl_4 causes testicular damage and that this testicular damage improves to some extent when CCl_4 and CAPE are given together. In view of these results, we think that the use of antioxidant and anti-inflammatory agents such as CAPE may have beneficial effects in the treatment of testicular damage.

Ethics

Ethics Committee Approval: Approval was obtained from the Experimental Animals Ethics Committee of İnönü University Faculty of Medicine (protocol no: 2012/A-49, date: 21.03.2012).

Informed Consent: Animal experiment.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: E.T., Design: E.T., Data Collection or Processing: B.G., N.V., A.T., H.E., Analysis or Interpretation: E.T., Literature Search: B.G., Writing: B.G.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.88709
Nam Kem Med J 2023;11(1):22-26

Evaluation of Serum TSH and Free T4 Levels in Migraine Patients

Migren Hastalarında Serum TSH ve Serbest T4 Düzeylerinin Değerlendirilmesi

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ABSTRACT

Aim: Migraine is a common neurovascular inflammatory disease that causes disability and is characterized by recurrent headache attacks. The role of thyroid regulation in migraine is poorly understood, and data are conflicting. The aim of this study is to evaluate the association of thyroid hormone levels and migraine types and headache severity.

Materials and Methods: One hundred-fifty migraine patients enrolled in this retrospective study. Demographic and clinical characteristics of the patients, migraine subtypes, frequency and severity, serum thyrothytropine (TSH), and free thyroxin levels were evaluated from records. The Migraine Disability Assessment Questionnaire (MIDAS) and Visual Analog Scale (VAS) were used to assess the severity of migraine. Data analysis was performed.

Results: The mean age was 40.40±10.84 years, and the female to male ratio was 5.1: 1. No significant relationship was found between thyroid hormone levels and headache characteristics of migraine patients and migraine severity (p>0.05). There was no significant relationship between VAS and MIDAS values and TSH levels (p=0.973).

Conclusion: Migraine and thyroid diseases are common diseases in the society. Thyroid diseases and thyroid function tests should be evaluated together when determining the characteristics of migraine and the headache severity. Our data suggest that there is no relationship between thyroid hormone levels and migraine subtypes and severity. Further studies are needed in order to confirm this association.

Keywords: Migraine, serum TSH, serum free T4

ÖZ

Amaç: Migren, tekrarlayan baş ağrısı atakları ile karakterize, dizabilitiye neden olan ve sık görülen nörovasküler enflamatuvar bir hastalıktır. Tiroid regülasyonunun migrendeki rolü tam olarak anlaşılamamıştır ve veriler çelişkilidir. Bu çalışmanın amacı tiroid hormon düzeyleri ile migren tipleri ve baş ağrısı şiddeti arasındaki ilişkiyi değerlendirmektir.

Gereç ve Yöntem: Çalışmaya 150 migren hastası dahil edildi. Hastaların demografik ve klinik özellikleri, migren alt tipleri, sıklığı ve şiddeti, serum tirotropin (TSH), serbest tiroksin düzeyleri kayıt edildi. Migrenin şiddetini değerlendirmek için Migren Dizabilite Değerlendirme Anketi (MIDAS) ve Görsel Analog Skala (VAS) kullanıldı. Verilerin analizi yapıldı.

Bulgular: Hastaların yaş ortalaması 40,40±10,84 yıl, kadın erkek oranı 5.1: 1 idi. Migren hastalarının tiroid hormon düzeyleri ile baş ağrısı özellikleri ile migren şiddeti arasında anlamlı bir ilişki bulunmadı (p>0,05). VAS ve MIDAS değerleri ile TSH düzeyleri arasında anlamlı bir ilişki saptanmadı (p=0,973).

Sonuç: Migren hastalığı ve tiroid hastalıkları toplumda yaygın görülen hastalıklardır. Migrenin özellikleri ve baş ağrısı şiddeti belirlenirken tiroid hastalıkları ve tiroid fonksiyon testleri birlikte değerlendirilmelidir. Verilerimiz, tiroid hormon düzeyleri ile migren alt tipleri ve şiddeti arasında bir ilişki olmadığını düşündürmektedir. Bu ilişkiyi doğrulamak için daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Migren, serum TSH, serum serbest T4

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Received: 08.01.2023 **Accepted:** 18.01.2023

INTRODUCTION

Migraine is a chronic, widespread neurovascular inflammatory disease characterized by recurrent moderate and severe headache attacks[1]. Photophobia, phonophobia, nausea, and vomiting are often accompanied by unilateral, throbbing, repetitive headache pain experienced for 4 to 72 hours by the patients[2]. Migraine pain not only affects the work or school performance of patients, but also causes a decrease in their quality of life, family time, and social activities. This condition, which completely or partially affects people's normal activities and lives, has been evaluated as a disability by the World Health Organization. Migraine Disability Assessment Questionnaire (MIDAS) is one of the widely used scales developed to measure disability in migraine patients[3,4]. Migraine can cause disability as well as showing significant co-morbidities with various diseases such as myocardial infarction, stroke, subclinical vascular brain lesions, patent foramen ovale, hypertension, epilepsy, asthma and psychiatric disorders[5,6]. Recently, the relationship between migraine and thyroid functions has been attempted to be established.

Subclinical hypothyroidism (SCH) is a condition characterized by normal free triiodothyronine (fT3) and free thyroxin (fT4) values and slightly increased thyrotropin (TSH) concentrations. Some studies have shown that migraine is associated with an increased risk in the development of both overt and SCH[7,8]. However, the underlying mechanisms of this relationship are still unclear. Although clinical symptoms are very few in SCH, some patients may show neuropsychiatric symptoms such as depression, anxiety, and memory disorders. There are studies that explain hormonal levels related to thyroid function, TSH, T3 and T4 blood concentrations, and migraine pathogenesis[7]. In a previous meta-analysis, serum TSH levels were found to be higher in patients with migraine compared to control groups without migraine, which was statistically significant[9]. However, when we look at the literature, the number of studies investigating the difference between serum TSH and fT4 levels and loss of working days together with pain severity are very scarce. The aim of the present study is to determine whether there is a difference between thyroid function tests (TFT) in patients diagnosed with migraine, defined migraine type and difference between TFT according to loss of working days and pain severity.

MATERIALS AND METHODS

One hundred-fifty patients admitted to Necmettin Erbakan University, Meram Faculty of Medicine, Neurology Outpatient Clinic between January 2021 and July 2022 and diagnosed with migraine (according to International Headache Society) were included in the present study. The study was approved by Necmettin Erbakan University, Meram Medical Faculty Ethics Committee (decision no: 2022/3969, date: 16.09.2022). Patients' files were analyzed retrospectively. Sixteen patients with a history of thyroid disease and using drugs related to thyroid disease, according to their records, were excluded from the present study. Patients' age, gender, biochemical serum TSH and fT4 levels, migraine type, presence of aura, frequency of attacks, duration and severity of attacks were recorded. Serum TSH and fT4 levels were studied by using standard electrochemiluminescence methods. The frequency of attacks refers to the total number of migraine attacks in the last 3 months; yet, whether the patients received treatment or not during the attacks was disregarded. Headache severity was evaluated with VAS. According to VAS 100 mL line 0-4 mL was evaluated as no pain, 5-44 mL as mild pain, 45-74 mL as moderate pain, and 75-100 mL as severe pain depending on the mark on the line[10]. MIDAS Turkish version was obtained by questioning the days on which the patients were unable to work due to the pain and the days when patients' performance decreased by at least 50%[11]. According to MIDAS, little or no disability was defined with a loss of 0-5 days, mild disability with a loss of 6-10 days, moderate disability with a loss of 11-20 days, and severe disability with a loss of 21 days or more[4]. The difference between serum TSH and fT4 values according to migraine type, the presence of aura, and the presence of a relationship with TFT in these patients, whose VAS and MIDAS values were calculated, were all taken into consideration.

Statistical Analysis

In the present study, the data obtained were analyzed using Statistical Package for Social Sciences for Windows 20.0 package program. Categorical variables were expressed as numbers (n) and the chi-square test (and/or Fisher's exact test) was used for the analysis. The Pearson correlation test was also applied to determine the correlations between serum TSH levels and migraine characteristics. Numerical variables were expressed as mean±standard deviation, and the Student's t-test was used to analyze the comparison of the means of two independent groups. A threshold level of <0.05 was considered for statistical significance in all results obtained.

RESULTS

The files of 150 migraine patients were reviewed retrospectively. Sixteen patients with a history of thyroid disease and relevant use of drugs were excluded from the present study. Out of the patients, 112 (83.6%) were female and 22 (16.4%) were male, with an age range between 18 and 65 years. The mean age was 40.40±10.84 years. The type of migraine (chronic, episodic), the presence of aura, headache severity according to VAS, disability score and demographic characteristics according to MIDAS are presented in Table 1. FT4 levels of the patients whose serum TSH level were found to be above 4.2 mU/L were reevaluated.

Eleven (8.2%) subclinical hypothyroid patients with TSH levels above 4.2 mU/L, fT4 levels within the normal range of 0.93-1.7 ng/dL, and no thyroid medication use were determined. Out of the 11 patients diagnosed with SCH, 10 were female and 1 was male.

Out of 134 migraine patients, 36 (26.9%) had migraine with aura and 98 (73.1%) had migraine without aura. The mean TSH value of patients diagnosed with migraine with aura was 2.10±1.46 mU/L, and fT4 was 1.25±0.32 ng/dL. The mean TSH value of patients with migraine but without aura was 2.24±1.58 mU/L, fT4 value was 1.16±0.17 nd/dL. However, no statistically significant differences could be determined in the TSH and fT4 values between the groups. There was no statistically significant difference between them (p=0.761, p=0.097).

Out of the migraine patients, 87 (64.9%) had episodic and 47 (35.1%) chronic migraine episodes. The mean TSH value of patients with chronic migraine was 2.33±1.61 mU/L, fT4 value was 1.19±0.29 ng/dL. The mean TSH value of patients with episodic migraine was 2.14±1.51 mU/L, fT4 value was 1.18±0.19 nd/dL. In terms of TSH and fT4 values, there was no statistically significant difference between the groups (p=0.490, p=0.887).

According to VAS, 2 patients (1.5%) had mild headache, 27 (20.1%) had moderate headache, and 105 patients (78.4%) had severe headache. Considering TSH and fT4 levels of the patients according to the severity of pain, the mean TSH value was 1.10±0.14 mU/L, fT4 value was 1.21±0.16 ng/dL in mild pain; the mean TSH value was 2.41±1.54 mU/L, fT4 value was 1.18±0.22 ng/dL in moderate pain; the meanTSH value was 2.17±1.56 mU/L, fT4 was determined as 1.18±0.23 ng/dL in severe pain, and there was no statistically significant difference among the groups (p=0.248, p=0.836).

Considering the working day loss of the patients, there were 21 patients (15.7%) with little or no disability, 43 (32.1%) with mild disability, 42 (31.3%) with moderate disability, and 28 (20.9%) with severe disability. The mean TSH value of patients with very low disability was 1.98±1.08 and fT4 value was 1.15±0.15. For those with mild disability, the mean TSH value was 2.28±1.81 and fT4 value was 1.16±0.19. For those with moderate disability, the mean TSH level was 2.06±1.27 and fT4 value was 1.21±0.17, and for those with severe disability, the mean TSH value was 2.48±1.79, and fT4 level was 1.21±0.36. There was no statistically significant difference between the groups (p=0.843, p=0.414).

TSH and fT4 levels of the patients are presented in Table 2.

Table 1. Characteristics of patients according to migraine groups

Patients	
Gender (n, %)	
Female	112 (83.6)
Male	22 (16.4)
Aura (n, %)	
Presence of aura	36 (26.9)
Absence of aura	98 (73.1)
Type (n, %)	
Episodic	87 (64.9)
Chronic	47 (35.1)
VAS (n, %)	
Mild	2 (1.5)
Moderate	27 (20.1)
Severe	105 (78.4)
MIDAS (n, %)	
0-5 days	21 (15.7)
6-10 days	43 (32.1)
11-20 days	42 (31.3)
>21 days	28 (20.9)

VAS: Visual Analog Scale, MIDAS: Migraine Disability Assessment Questionnaire

DISCUSSION

In the present study investigating TFT values in migraine patients, TSH level was above 4.2 mU/L in 134 migraine patients and fT4 level was between 0.93 and 1.7 ng/dL. We identified 11 (8.2%) patients diagnosed with SCH, who were in the normal range and did not use thyroid medication. Of the 11 patients diagnosed with SCH, 10 were female and 1 was male. In population-based studies, the prevalence of SCH varies between 4% and 15% and the diagnosis is more common in women[12]. We did not have a control group in our study, but the prevalence of SCH was similar to that in community-based

Table 2. TSH and fT4 levels of the patients

	TSH	p	fT4	p
Presence of aura	2.10±1.46	0.761	1.25±0.32	0.097
Absence of aura	2.24±1.58		1.16±0.17	
Chronic	2.33±1.61	0.490	1.19±0.29	0.887
Episodic	2.14±1.51		1.18±0.19	
VAS				
Mild	1.10±0.14	0.248	1.21±0.16	0.836
Moderate	2.41±1.54		1.18±0.22	
Severe	2.17±1.56		1.18±0.23	
MIDAS				
0-5 days	1.98±1.08	0.843	1.15±0.15	0.414
6-10 days	2.28±1.81		1.16±0.19	
11-20 days	2.06±1.27		1.21±0.17	
>21 days	2.48±1.79		1.21±0.36	

VAS: Visual Analog Scale, MIDAS: Migraine Disability Assessment Questionnaire

studies. Our female patients with SCH were more in numbers compared to male patients.

Hormones synthesized in the thyroid glands are vital for normal development and growth as well as for the development of the central nervous system. Severe deficiency of thyroid hormones results in mental retardation and ataxia during fetal and neonatal periods[13]. Basic evidence has been provided that thyroid hormones are involved in pain processing through the anterior cingulate cortex in mice with experimentally formed hypothyroidism. There are indications that thyroid hormones affect the development of pain/migraine. While hypothyroidism has been found to cause hypersensitivity to noxious thermal, but not mechanical, stimulus in mice, interestingly, pain intensity has been shown to be alleviated by T3 or T4 replacement. The mechanism underlying the effect of hormone therapy was thought to result from an improvement in the balance of glutamatergic and gamma aminobutyric acidergic transmission in the anterior cingulate cortex of hypothyroid mice[14].

In the study conducted by Bhattacharjee et al.[15], investigating the rate of SCH in patients with migraine, they determined the rate of SCH to be significantly higher in patients with migraine compared to the group without. Another study performed by Rubino et al.[16] reported a higher prevalence of migraine in patients with SCH compared to controls (46% vs. 13%, respectively). In their study, they also reported that they did not determine a statistically significant difference in serum TSH levels between SCH patients with and without migraine. The biological mechanisms underlying the relationship between SCH and migraine are unknown. Autoimmune hypothyroidism is a complex disease in which thyroid autoantigens develop on a certain genetic background after exposure to environmental factors. Thyroid autoimmunity is facilitated by single nucleotide polymorphisms in genes that regulate the immune system, such as human leucocyte antigen (HLA) genes, cytokine genes, and thyroid-specific genes. In the pathogenesis of migraine, polymorphisms play a role in some of these genes, such as tumor necrosis factor-alpha and different HLA genes[17-19].

In another study within the relevant literature, the meta-analysis results of Seidkhani-Nahal et al.[20] demonstrated statistically significantly higher TSH concentrations in patients with migraine and in controls without migraine. Based on this meta-analysis, the aim of the present study was to investigate whether there was a difference between serum TSH levels and fT4 levels in patients having migraine with or without the presence of aura, according to the severity of the headache, and type of migraine. The present study could not determine a statistically significant difference between TSH and fT4 levels of 134 migraine patients. The findings presented in the relevant literature were mostly in line with the findings of the present study. In a randomized case-control study, Bigal et al.[21] evaluated the factors associated with the transition from episodic migraine to chronic migraine. Among these factors, hypothyroidism has been found to contribute to the emergence of chronic migraine. In another study, it was shown that the rate of hypothyroidism in patients with migraine was higher than in community-based studies[7]. In addition, in the study of Rubino et al.[16], conducted with clinical subtypes of migraine, it was determined that the prevalence of migraine with and without aura was higher in patients with SCH than in the controls. In a retrospective cohort of consecutive migraine patients, which investigated the presence of migraine co-morbidities, Tietjen et al.[22] defined a subgroup with a high incidence of metabolic co-morbidities (such as hypertension, hyperlipidemia, diabetes mellitus) and hypothyroidism. Spanou et al.[23] could not determine a statistically significant relationship between headache types and thyroid hormone disorders. In terms of headache subtypes and thyroid dysfunctions, no statistically significant relationship could be determined in their study. However, a higher prevalence of thyroid dysfunction in general (20.7%) and a higher prevalence of hypothyroidism specifically (6.3%) in patients with primary headache were reported.

In a study conducted in Russia, only 5% of the migraine patients in the study were reported to have abnormal TSH levels (lower or higher than normal). Nevertheless, lower TSH values were associated with longer-term migraine attacks and had a greater impact on quality of life. Based on these findings, it was suggested that TSH levels should be confirmed in patients with severe migraine[24].

Study Limitations

The lack of a control group and the limited number of patients are the inherent limitations of the present study.

CONCLUSION

In the present study, however, no statistically significant difference could be determined between TSH and fT4 levels and the severity of headache, loss of working days, chronic and episodic migraine. The group with chronic migraine had a higher TSH value than the group with episodic migraine but there was no statistically significant difference between the groups. The pathogenesis of this association requires further research and studies with larger numbers of patients. In the present study, in 8,2% of the migraine patients, SCH was determined. As there was not a control group, comparisons with community-based studies could be made.

Ethics

Ethics Committee Approval: The study was approved by the Necmettin Erbakan University, Meram Medical Faculty Ethics Committee (decision no: 2022/3969, date: 16.09.2022).

Informed Consent: Retrospective study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: M.A., Design: M.A., Data Collection or Processing: M.A., H.Ç.B., Z.Y., Analysis or Interpretation: M.A., H.Ç.B., Z.Y., Literature Search: H.Ç.B., Z.Y., Writing: H.Ç.B., Z.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.06078
Nam Kem Med J 2023;11(1):27-34

Demographic and Clinical Features and Factors Associated with Survival in Patients with Primary Glomerulonephritis: Single Tertiary Center Experience

Primer Glomerülonefritli Hastalarda Demografik, Klinik Özellikler, Sağkalımı Etkileyen Özellikler: Tek Tersiyer Merkez Deneyimi

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ABSTRACT

Aim: Primary glomerulonephritis (GN) is a rare disease that has many different subtypes and is a significant health problem. Patients with primary GN (PGN) often do not achieve a complete cure, typically require immunosuppressive therapy, and can have serious co-morbidities due to the disease, which often progresses to end-stage renal disease (ESRD). The current study aimed to investigate the epidemiological, clinicodemographic characteristics and long-term outcomes of PGN patients.

Materials and Methods: The current study retrospectively evaluated the demographic characteristics and complaints as well as the physical examination and laboratory findings of PGN patients who were followed-up and treated in the nephrology department of our university hospital between January 2000 and June 2016.

Results: Of the 485 included patients, 265 were male (55%) and 220 were female (45%). The median age at diagnosis was 38.5 years (range; 18-77). The most frequent indication for biopsy was nephrotic syndrome (53.2%). The most common histopathological diagnoses were IgA nephritis (33.2%), focal segmental GN (31.1%), and membranous GN (19.6%), respectively. It was observed that male gender (p=0.01), systemic hypertension (p=0.01) at the time of diagnosis, proteinuria (p=0.001) in the nephrotic range, and histological diagnosis of crescentic GN (p=0.001) contributed negatively to renal survival. The mean follow-up duration after diagnosis was 59.1±48.5 months. The median overall survival was 153 (range; 1-197) months. Survival was significantly lower in patients with ESRD compared to those without ESRD (p=0.003). On clinical follow up, 48 patients died (9.9%), and 94 patients (19.3%) progressed to ESRD.

Conclusion: Clearly defining the etiology of PGN as well as determining the factors leading to ESRD may decrease morbidity and mortality.

Keywords: Primary glomerulonephritis, renal outcome, patient survival, immunosuppressive therapy, end stage renal disease

ÖZ

Amaç: Primer glomerülonefritler (GN), birçok farklı alt tipi olan ve önemli bir sağlık sorunu olan nadir bir hastalıktır. Primer GN'si (PGN) olan hastalar genellikle tam bir iyileşme elde edemezler. Tipik olarak immünosüpresif tedavi gerektirir ve sıklıkla son dönem böbrek yetmezliğine (SDBY) ilerleyen hastalığa bağlı ciddi komorbiditeler oluşabilir. Bu çalışma, PGN hastalarının epidemiyolojik, klinikodemografik özelliklerini ve uzun dönem sonuçlarını araştırmayı amaçladı.

Gereç ve Yöntem: Bu çalışmada Ocak 2000-Haziran 2016 tarihleri arasında üniversite hastanemiz nefroloji bölümünde takip ve tedavi edilen PGN'li hastaların demografik özellikleri ve şikayetleri ile fizik muayene ve laboratuvar bulguları retrospektif olarak değerlendirildi.

Bulgular: Çalışmaya dahil edilen 485 hastanın 265'i erkek (%55) ve 220'si kadındı (%45). Ortanca tanı yaşı 38,5 yıl idi (aralık; 18-77 yıl). En sık biyopsi endikasyonu nefrotik sendromdu (%53,2). En sık histopatolojik tanılar sırasıyla IgA nefriti (%33,2), fokal segmental GN (%31,1) ve

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Received: 09.01.2023 **Accepted:** 18.01.2023

membranöz GN (%19,6) idi. Erkek cinsiyet (p=0,01), tanı anında sistemik hipertansiyon (p=0,01), nefrotik düzeyde proteinüri (p=0,001) ve histolojik olarak kresentik GN (p=0,001) varlığı renal sağkalımı olumsuz etkileyen faktörlerdi. Tanı sonrası ortalama takip süresi 59,1±48,5 aydı. Ortanca genel sağkalım 153 (aralık; 1-197) aydı. SDBY olan hastalarda sağkalım, SDBY olmayanlara göre anlamlı olarak daha düşüktü (p=0,003). Klinik takipte 48 hasta (%9,9) öldü ve 94 hasta (%19,3) SDBY'ye ilerledi.

Sonuç: PGN'nin etiyolojisinin net olarak tanımlanması ve SDBY'ye yol açan faktörlerin belirlenmesi morbidite ve mortaliteyi azaltabilir.

Anahtar Kelimeler: Primer glomerülonefrit, renal sağkalım, hasta sağkalımı, immünosüpresif tedavi, son dönem böbrek hastalığı

INTRODUCTION

Glomerulonephritis (GN) refers to a group of diseases with different subtypes. GN is the third most common cause of end-stage renal disease (ESRD), following diabetes mellitus and hypertension[1-4]. However, it is the most common cause of ESRD in young adults[5]. GN can be etiologically classified as either primary or secondary. Primary GNs (PGN) are defined as diseases in which glomeruli are solely or predominantly affected by no known systemic disease or agent (e.g., vasculitis, systemic lupus erythematosus (SLE), metabolic disease, malignancy, infection, drugs). Secondary GNs are defined as diseases with glomerular damage and organ involvement due to a systemic disease or agent. The prevalence of glomerular diseases varies with race, age, geographical region, and etiological, cultural and economic differences. It is therefore important to recognize and study differences in these diseases in any geographical region[2-4].

Subtypes of PGN include IgA nephropathy (IgA-N), membranous GN (MGN), minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranoproliferative GN (MPGN), and crescentic GN (CGN)[1]. Although some clinical findings of these diseases are similar, they differ in their prevalence, pathological findings, clinical courses, and responses to treatment[1,3].

IgA-N is the most common form of PGN worldwide[1,2,4] and has a wide clinical spectrum. Often seen throughout the clinical course of IgA-N are macroscopic hematuria, asymptomatic isolated hematuria, and severe nephritic or nephrotic syndrome[1,6,7]. MGN is one of the most common causes of nephrotic syndrome in adults. Although MGN is frequently a primary (idiopathic) disease, it can also be due to secondary causes, such as malignancy, infection, drugs, and collagen tissue diseases[1,8]. Spontaneous remission may occur in the course of MGN[9]. MCD is the most common cause of nephrotic syndrome in pediatric patients. MCD and FSGS often present with nephrotic syndrome or asymptomatic proteinuria[1,10,11]. Histologically, the main problem in both MCD and FSGS lies in podocytes1. MPGN is mostly seen in children and young adults. Despite proper treatment, nearly 60% of adult PGN patients progress to ESRD within ten years[1,12]. CGN is known to cause renal failure, hematuria, and non-nephrotic proteinuria, with crescent formation being the classical morphological finding[1,13].

In the current study, we aimed to evaluate the demographic and clinical characteristics, survival rates, and factors affecting survival of patients who were diagnosed with PGN.

MATERIALS AND METHODS

The medical files of the PGN patients, who were treated and followed up in the nephrology department between January 2000 and June 2016, were retrospectively examined by the same researcher. Only patients aged 18 years and over were enrolled in this study. Patients were excluded from this study if they had secondary GN or inadequate data. MCD, MN, FSGS, MPGN, IgA-N, and CGN were accepted as the PGN.

Age, gender, age of diagnosis, and smoking status were recorded from the patient files. In addition, any drug use and systemic diseases (e.g., diabetes mellitus, systemic vasculitis, SLE, and amyloidosis) as well as the presence of swelling in the legs, macroscopic hematuria, high blood pressure, low back pain, oliguria, and uremia symptoms (e.g., nausea, vomiting, loss of appetite, fatigue) were also recorded.

Systolic and diastolic blood pressure values were recorded from patient files. According to the Eighth Joint National Committee (JNC 8) criteria, systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg, or the use of antihypertensive medication was accepted as the presence of hypertension. Hypertension was defined as being 'under control' in cases where systolic blood pressure was below 140 mmHg and diastolic blood pressure was below 90 mmHg. Oliguria was defined as daily urine output of less than 400 mL/day.

The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was used as Estimated glomerular filtration rate (eGFR) formula. Results of microscopic examination of urine sediment were recorded. The presence of three or more erythrocytes in urine sediment at high magnification was accepted as microscopic hematuria. Patients were defined as having nephrotic syndrome if they had massive proteinuria (>3.5 g/day), edema, hypoalbuminemia, and hyperlipidemia. Patients were defined as having nephritic syndrome if they had systemic hypertension, oliguria, edema and proteinuria, and hematuria. Asymptomatic urinary analysis (AUA) was defined by non-nephrotic proteinuria and/or isolated microscopic hematuria. Rapidly progressive GN was defined by rapid deterioration of kidney function within hours-days.

Patients underwent renal biopsy if they had nephritic and/or nephrotic syndrome and/or rapid deterioration of kidney function (urea/creatinine elevation and/or oliguria within hours-days). All renal biopsy samples were examined with light microscopy and immunofluorescence by the expert pathologist. Biopsy specimens were stained with hematoxylin-eosin, periodic acid-Schiff, Masson trichrome, and Jones silver methenamine stains. The presence of IgA, IgG, IgM, C3, and C1q was assessed by immunofluorescence microscopy. The biopsy indications and biopsy results were recorded. The distribution of biopsy results according to age groups was evaluated.

Conservative treatment was defined as renin-angiotensin system blockade with angiotensin converting enzyme inhibitors or angiotensin receptor blockers, blood pressure control, statin use, diuretic therapy, control of metabolic syndrome, and protein and salt restriction in the diet. Any immunosuppressive therapies (e.g., steroids, cyclophosphamide, cyclosporine, azathioprine, mycophenolate mofetil, rituximab, etc.) and observed side effects (e.g., impaired renal function, systemic infections, Cushing's syndrome, venous thromboembolism etc.) were recorded.

ESRD was defined as the need for permanent renal replacement therapy (hemodialysis, peritoneal dialysis, renal transplantation). Renal survival was defined for those who developed and did not develop ESRD. The clinical and laboratory data of the patients with ESRD were compared with those of patients who did not develop ESRD.

Statistical Analysis

The Kolmogorov-Smirnov dispersion test was used to analyze whether the data were normally distributed. Descriptive statistical methods (frequency, percentage, mean, standard deviation) were employed in evaluating the study data. The Wilcoxon sign test was used for intra-group comparisons. The Pearson's chi-square test and Fisher's exact test were carried out to investigate the factors affecting renal survival. A Kaplan-Meier analysis was used to examine the risk factors affecting patient survival and follow-up. Results were evaluated at a 95% confidence interval, and values of $p<0.05$ were considered significant. Normally distributed variables were shown as percentage or mean±standard deviation and non-normally distributed variables as median and interquartile range.

RESULTS

There were 485 patients treated due to PGNs in this study. Of the patients, 265 were male (55%) and 220 (45%) were female. Demographic and clinical characteristics are shown in Table 1. The median age at diagnosis was 38.5 years (range; 18-77). The mean follow-up period of the patients with PGNs was 59.1±48.5 months.

In terms of complaints, 299 (61.6%) patients had swelling in the legs, 42 (8.6%) had macroscopic hematuria, 91 (18.7%) had high blood pressure, 19 (3.9%) had low back pain, and 34 (7%) had oliguria, nausea, vomiting, and loss of appetite.

Laboratory findings at the time when kidney biopsy was performed are shown in Table 2. The mean systolic blood pressure was 128.9±16.1 mmHg and the mean diastolic blood pressure was 80.7±10.5 mmHg. The mean eGFR was 91.1±34.8 mL/min in cases where renal biopsy was performed.

Renal Biopsy Results

In this study, nephrotic syndrome was the most common biopsy indication [258 patients (53.2%)]. Other biopsy indications included nephritic syndrome in 95 (19.6%) patients, nephrotic + nephritic syndrome in 59 (12.2%) patients, asymptomatic urine analysis in 54 (11.5%) patients, and RPGN in 19 (3.9%) patients (Figure 1). The most common histopathological diagnosis was IgA-N (161 patients / 33.2%). Other diagnoses included FSGS (151 patients / 31.1%), MGN (95 patients / 10.9%), MPGN (53 patients / 10.9%), CGN (18 patients / 3.7%), and MCD (7 patients / 1.4%) (Figure 2).

FSGS (46.2%) and MGN (34.9%) were the most common GN subtypes followed by MPGN (10.1%), IgA-N (6.1%), and MCD (2.3%) in patients with nephrotic syndrome. IgA-N (89.5%) was the most common GN subtype in patients with pure nephritic syndrome. IgA-N (62.7%) and MPGN (25.4%) were the most common diagnoses in patients with both nephrotic range proteinurias and nephritic findings. FSGS (42.6%) were the most common GN in patients with asymptomatic urine analysis. CGN was present in 84.2% of the patients with rapid

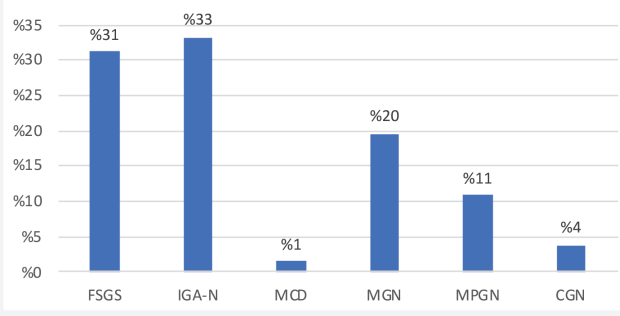

Figure 1. Diagnosis of patients according to the renal biopsy results between 2000 and 2016

CGN: Crescentic glomerulonephritis, FSGS: Focal segmental glomerulosclerosis, IgA-N: IgA nephropathy, MCD: Minimal change disease, MGN: Membranous glomerulonephritis, MPGN: Membranoproliferative glomerulonephritis

deterioration of renal function. The incidence of PGN according to year intervals (2000-2008 vs 2009-2016) is shown in Figure 3. The most common type of PGN under the age of 40 years was FSGS (41%), while MGN was (38%) in geriatric patients over the age of 65 years (Figure 4). Of the patients, 229 (49%) underwent conservative treatment and 233 (51%) received + immunosuppressive treatment in addition to conservative treatment. In terms of complications, a thromboembolic event occurred in 9 cases and 23 cases were treated with sepsis.

Renal Outcome

Ninety-four (20%) of the 459 patients with known renal outcome developed ESRD. The renal replacement therapies of these ESRD patients included hemodialysis (64 cases), peritoneal dialysis (2 cases), and renal transplantation (28 cases). When renal outcome was examined, the development of ESRD was found to be significantly higher in males than in females (p=0.011). In addition, the development of ESRD in patients with RPGN was significantly higher than in patients without RPGN (p=0.001). ESRD development was significantly higher in patients with proteinuria greater than 3.5 g/day at the time of diagnosis compared to those with proteinuria less than 3.5 g/day (p=0.001). In addition, ESRD development was significantly higher in patients with diastolic blood pressure above 90 mmHg than in those with diastolic blood pressure below 90 mmHg (p=0.011).

Table 1. Demographics and clinical characteristics of patients

Variables		n	%
Age at diagnosis; median, years (range)		38.5 (18-77)	
The mean follow-up time from diagnosis (months)		59.1±48.5	
Gender	Male	265	55
	Female	220	45
Smoker	No	359	66.6
	Current/former	126	33.4
Symptoms before diagnosis	Swelling in the legs	299	61.6
	Macroscopic hematuria	42	8.6
	High blood pressure	91	18.7
	Low back pain	19	3.9
	Oliguria and uremia symptoms	34	7
Renal biopsy indication	Nephrotic syndrome	258	53.2
	Nephritic syndrome	95	19.6
	Nephrotic and nephritic syndrome	59	12.2
	Asymptomatic urine analysis	54	11.1
	RPGN	19	3.9
Histopathology	IgA-N	161	33.2
	FSGS	151	31.1
	MGN	95	19.6
	MPGN	53	10.9
	CGN	18	3.7
	MCD	7	1.4
Renal outcome	ESRD yes	94	19.4
	ESRD no	365	75.3
	N/A	26	5.3
Renal replacement therapy in ESRD	Hemodialysis	64	13.2
	Peritoneal dialysis	2	0.4
	Kidney transplantation	28	5.7
Current status	Alive	422	87
	Exitus	48	9.9
	N/A	15	3.1

IgA-N: IgA nephropathy, CGN: Crescentic glomerulonephritis, FSGS: Focal segmental glomerulosclerosis, MGN: Membranous glomerulonephritis, MPGN: Membranoproliferative glomerulonephritis, RPGN: Rapidly progressive glomerulonephritis, MCD: Minimal change disease, N/A: Not available, ESRD: End-stage renal disease

Patient Survival

The median overall survival was 153 (range; 1-197) months (Figure 5). Within the study period, 48 of the 470 patients included died. Of them, 16 died of cardiovascular disease, 13 died of sepsis, 3 died of a thromboembolic event, and 3 died due to malignancy. The cause of death was not determined in 13 cases. When patient survival was examined, it was found that neither the presence of nephrotic range proteinuria, elevated systolic blood pressure (>140 mmHg), hematuria nor immunosuppressive treatment affected survival.

There was no significant difference in terms of survival and proteinuria (between those below and above 3.5 g/day) at diagnosis (p=0.359), systolic blood pressure (between those below and above 140 mmHg) at diagnosis (p=0.603), presence of hematuria (p=0.136). Survival was significantly lower in patients with ESRD compared to those without ESRD (p=0.003) (Figure 6).

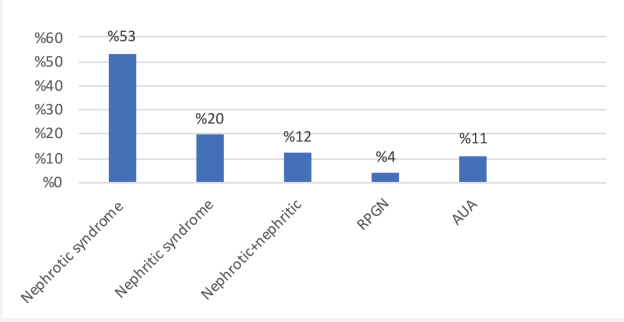

Figure 2. Indications for renal biopsy according to primary glomerulonephritis between 2000 and 2016

AUA: Asymptomatic urinary abnormalities, CGN: Crescentic glomerulonephritis, FSGS: Focal segmental glomerulosclerosis, IgA-N: IgA nephropathy, MCD: Minimal change disease, MGN: Membranous glomerulonephritis, MPGN: Membranoproliferative glomerulonephritis, RPGN: Rapidly progressive glomerulonephritis

Table 2. Laboratory findings at the time when kidney biopsy was performed

Variables	Mean±SD
Systolic blood pressure (mmHg)	128.9±16.1
Diastolic blood pressure (mmHg)	80.7±10.5
BMI (kg/m²)	27.1±9.7
Glucose (mg/dL)	96±34
BUN (mg/dL)	22±18
Creatinine (mg/dL)	1.02 (0.6-1.7)
eGFR (CKD-EPI, mL/min/1.73 m²)	91.1±34.8 mL
Triglyceride (mg/dL)	171 (115-264)
LDL-cholesterol (mg/dL)	159±78 mL
ALT (IU/L)	18 (12-26)
Calcium (mg/dL)	9.1±0.9
Serum albumin (g/dL)	3.15±0.9
Proteinuria (mg/day)	3250 (1386-7114)

BMI: Body mass index, eGFR: Estimated glomerular filtration rate, CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration equation, SD: Standard deviation, BUN: Blood urea nitrogen, ALT: Alanine aminotransferase, LDL: Low-density lipoprotein

DISCUSSION

PGN is a significant health problem due to the fact that a complete cure is hard to attain, the requirement for immunosuppressive therapy is high, serious co-morbidities occur due to the disease itself, and patients often progress to ESRD. Recent epidemiological studies have shown that cases of PGN are increasing[2,3]. The distribution and frequency of the disease is better understood by the data of the GN working groups in various countries. A common result of all studies is that the ratio of histological types in PGN varies according to

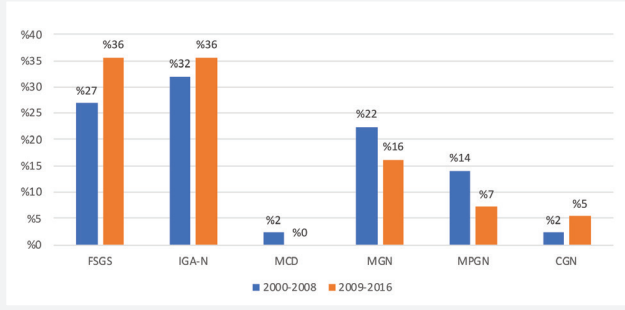

Figure 3. Diagnosis of patients according to the renal biopsy results. The incidence of primary glomerulonephritis according to year intervals (2000-2008 vs 2009-2016)

CGN: Crescentic glomerulonephritis, FSGS: Focal segmental glomerulosclerosis, IgA-N: IgA nephropathy, MCD: Minimal change disease, MGN: Membranous glomerulonephritis, MPGN: Membranoproliferative glomerulonephritis

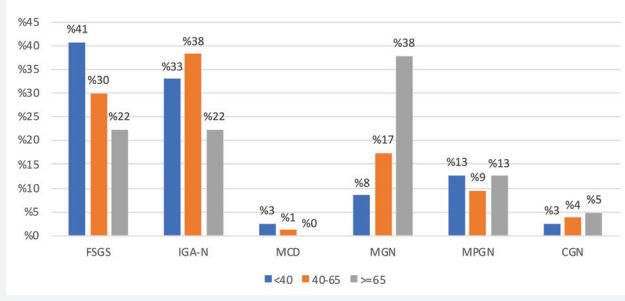

Figure 4. Distribution of primary glomerulonephritis in age groups

CGN: Crescentic glomerulonephritis, FSGS: Focal segmental glomerulosclerosis, IgA-N: IgA nephropathy, MCD: Minimal change disease, MGN: Membranous glomerulonephritis, MPGN: Membranoproliferative glomerulonephritis

race, geographic region, general characteristics of the patient group, and biopsy indication of the center[2-4].

In the current study, 485 PGN cases who were diagnosed between 2000 and 2016 were retrospectively examined. This was a single-center study with a larger number of adult patients. Another study of 293 PGN cases diagnosed between 1992 and 2000 was published from our center in 2001[14]. According to the results of the first study performed in our center, the most common PGN types were MPGN (26.6%), MGN (23.4%), and IgA-N (9.2%). However, a later study reported that

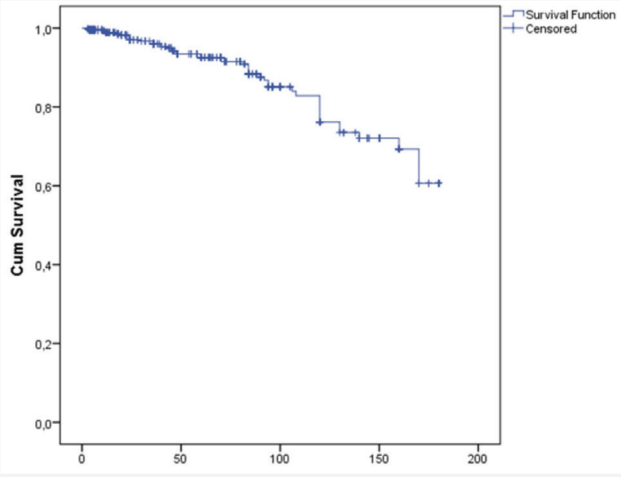

Figure 5. Overall survival curve. The median overall survival was 153 (range; 1-197) months

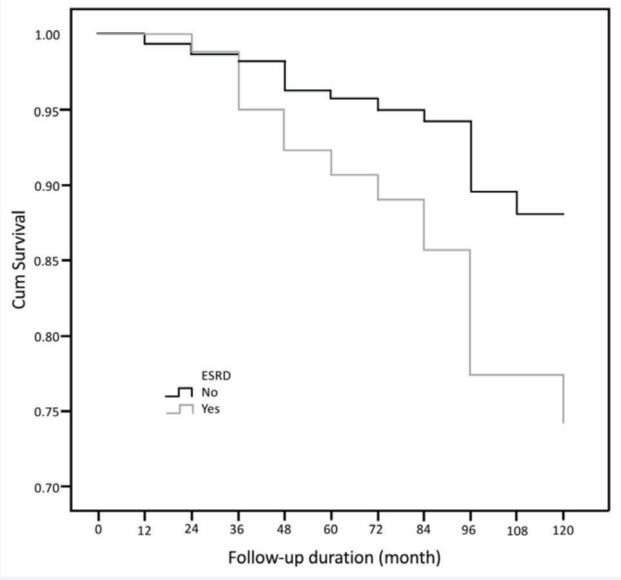

Figure 6. End-stage renal disease (ESRD) and survival curve. Survival was significantly lower in patients with ESRD compared to those without ESRD (p=0.003)

MGN was the most common, followed by FSGS and IgA-N, in Turkey. The Turkish Nephrology Association Glomerulonephritis Working Group conducted a multicenter study involving PGNs diagnosed between 1999 and 2012[15]. This study reported the most common PGNs as MGN (28.8%), FSGS (19.3%), and IgA-N (17.2%). Recently, two published studies from Turkish Society of Nephrology (TSN) GN working group show that the incidence of PGN has changed last decade in Turkey[16,17], and similar to our study, the most common PGN type was IgA-N. In the current study, IgA-N was the most common, while FSGS, MGN, MPGN were seen less frequently, respectively. As an interestingly point, the frequency of FSGS is higher in our study, unlike the studies of the TSN-GN working group. Primary FSGS occurs mostly with pure nephrotic syndrome, but in our study, a significant portion of the patients who underwent biopsy for AUA were diagnosed with FSGS. Although secondary Gn causes were excluded, considering the retrospective nature of our study, the distinction between primary and secondary FSGS may not have been fully made and it may explain this situation.

This current study had an increase in the frequency of IgA-N and FSGS, and a decrease in the frequency of MPGN may be explained by the fact that the current study had better control of secondary causes (infections) leading to glomerulopathy and/or increased biopsy frequency due to asymptomatic urine analysis.

When the distribution of histological types of PGN in the world was examined, it was found that the most common PGN type in Europe[18-20] and Asia[21,22] was IgA-N, followed by MGN, whereas in the US[23,24] and Brazil[25], it was FSGS. In a study conducted in the Czech Republic, the most common PGN was MCD. However, it should be noted that the Czech study included pediatric patients[26]. In the current study, IgA-N was the most common PGN, and FSGS was the second most common PGN, which is consistent with the European and Asian results. However, it should be noted that data after the year 2000 indicate that the frequency of FSGS is gradually increasing[2-4].

In the current study, the majority of patients underwent renal biopsy due to nephrotic syndrome, which is in parallel with international data[2-4]. Regarding studies performed in Italy[18] and Japan[22], the most common biopsy indication was asymptomatic urine analysis. The discrepancy between the current study and those in Italy and Japan may be due to the different types of urinary screening strategies. In the current study, asymptomatic urine analysis was the 4th most common biopsy indication (11.5%). This result was similar to that of the study conducted by the Turkish Nephrology Society (10.8%)[15]. Considering all patients with nephrotic syndrome in the current study, the most common diagnoses were FSGS (46.5%), MGN (34.1%), and MPGN (10.6%). Regarding GN working groups

in Italy, Spain, and Turkey, MGN was the most seen PGN in patients with nephrotic syndrome[15,18,20]. However, in Korea and Japan, MCD was the most seen GN in patients with nephrotic syndrome[22,27]. In parallel to the results of the current study, FSGS was the most common nephrotic syndrome etiology in US studies[23,24]. When the distribution of biopsy results according to age groups was evaluated in the current study, it was found that MGN was significantly higher in patients over the age of 40 years compared to younger patients. This can be explained by the fact that the peak age of MGN is in the age range of 40-60 years.

In the current study, 55% of the 485 patients diagnosed with PGN were male. The male to female ratio was about 1.2. This ratio was similar to that of the study by the Turkish Nephrology Society's Glomerulonephritis Study Group and Asia Registry. However, this ratio is slightly lower than that reported by European publications. In addition, the male to female ratio was 1.7 in the Italian GN recording system. This may be due to the fact that there is a large number of IgA-N patients in the Italian GN recording system[18].

It has been reported that having more than 1 g/day of proteinuria, hypertension (>140/90 mmHg), and severe histological lesions is significantly associated with dialysis or death in IgA-N[28]. Clinically, RPGN is characterized by a nephritic syndrome that rapidly progresses to ESRD[29]. A study conducted in the United States in 2016 reported that while ESRD was lower in women than men, there was no significant difference in mortality rates[3]. In the current study, 94 (20%) of the 459 patients with known renal output developed ESRD. Male gender, systemic hypertension at the time of diagnosis, proteinuria at the nephrotic level, and histological diagnosis of CGN were all found to negatively affect the renal outcome.

In the current study, the most common complications of nephrotic syndrome were infections (sepsis) in 13 patients and thromboembolic events in 3 patients. In a study of 1,313 cases diagnosed with nephrotic syndrome in 2012, the rate of thromboembolism was 3.3%[30]. The median overall survival time of the patients in the current study was 153±3.2 months. Within the study period, 48 of the 470 included patients died. Of these patients, 16 died of cardiovascular disease, 13 died of sepsis, 3 died of a thromboembolic event, and 3 died due to malignancy.

Study Limitations

The most important limitation of our study is that it is retrospective in nature. It may not be appropriate to infer about causality in retrospective studies, but these findings may provide a realistic picture of what is observed in daily clinical practice.

CONCLUSION

Herein, we have presented real-life data on clinical characteristic and long-term outcomes of PGN with large sample size in a single tertiary center. There are differences in the incidence and etiology of PGN in the last 2 decades. In the current study, contrary to previous studies before 2010 in Turkey, IgA-N was the most frequently observed subtype (also the most common in Asia and Europe). The difference from previous studies may be due to the increased frequency of biopsy due to asymptomatic urine analysis or better control of secondary causes (infections) that cause glomerulopathy. In conclusion, this study has revealed that male gender, systemic hypertension, proteinuria, and RPGN negatively affect kidney outcomes.

Acknowledgements

We would like to acknowledge MD, Dilara YOZGATLI for her proofreading provided for this manuscript.

Ethics

Ethics Committee Approval: This retrospectively study was approved by the İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Clinical Research Ethics Committee (no: 313708, date: 08.10.2015).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: N.P., S.T., N.S., M.R.A., Design: N.P., S.T., N.S., M.R.A., Data Collection or Processing: N.P., M.R.A., Analysis or Interpretation: N.P., S.T., N.S., M.R.A., Literature Search: N.P., M.R.A., Writing: N.P., M.R.A.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.25991
Nam Kem Med J 2023;11(1):35-41

The Immunomodulatory Effects of Ginger (Zingiber officinale) Extract on CD4 and CD8 Expression in Spleen of Diabetic Rats

Diyabetik Sıçanların Dalağında CD4 ve CD8 Ekspresyonu Üzerine Zencefil (Zingiber officinale) Ekstraktının İmmünmodülatör Etkileri

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ABSTRACT

Aim: This study aims to examine the changes of the ginger extract treatment on CD4 and CD8 expressions in the spleen tissue of rats, in which the experimental diabetes was induced with streptozotocin.

Materials and Methods: Forty Wistar albino rats were divided into 5 groups: control, sham, ginger, diabetes, diabetes+ginger. The spleen tissue sections were stained by Crossman's triple staining and the streptavidin-biotin peroxidase complex method. Diabetes was induced by 50 mg/kg streptozotocin injection intraperitoneally in the diabetes and diabetes+ginger groups. Ginger extract was administered to the ginger and diabetes+ginger groups at dose of 200 mg/kg for 30 days. Statistical measurements were analyzed in order to determine whether there was a difference between the groups in terms of weight of the spleen, the number of immunopositive CD4 and CD8 cells, and ratio of CD4/CD8.

Results: It was observed that the numerous lymph follicles were atrophied and most of the follicles did not have a germinal center in the diabetes group. Also, it was determined that ginger extract reduced degenerative changes. While it was observed that CD4 and CD8 expression was intense in the diabetes group, the intensity of CD4 and CD8 expression was decreased in the diabetes+ginger group compared to the diabetes group. In addition, it was determined that the spleen weight decreased in the diabetes group and increased in the diabetes+ginger group, which was similar to the control and sham groups.

Conclusion: In this study, it was revealed that ginger exerted a protective effect against STZ-induced diabetes in rats and had immunostimulatory effect in diabetic experimental model. Also, it can be used therapeutically in spleen problems and diabetes-related immune dysfunction.

Keywords: CD4, CD8, spleen, diabetes, ginger

ÖZ

Amaç: Bu çalışmada, zencefil ekstrakt uygulaması ile streptozotosin ile deneysel diyabet oluşturulan ratların dalak dokusunda CD4 ve CD8 salınımındaki değişiklerin incelenmesi amaçlanmıştır.

Gereç ve Yöntem: Çalışmamızda toplam kırk adet Wistar albino ırkı rat kullanıldı. Ratlar; kontrol, sham, zencefil, diyabet, diyabet+zencefil olmak üzere 5 gruba ayrıldı. Dalak doku kesitlerine Crossman'ın üçlü boyama ve streptavidin-biotin peroksidaz kompleks boyama yöntemleri uygulandı. Diyabet ve diyabet+zencefil gruplarına 50 mg/kg streptozotosinin intraperitoneal yolla enjeksiyonu ile diyabet indüklendi. Zencefil ekstraktı, zencefil ve diyabet+zencefil gruplarına 200 mg/kg dozunda 30 gün süreyle uygulandı. Gruplar arasında dalak ağırlığı, CD4 ve CD8 immünopozitif hücre sayısı ve CD4/CD8 oranı istatistiksel olarak analiz edildi.

Bulgular: Diyabet grubunda çok sayıda lenf folikülünün atrofiye uğradığı ve çoğu folikülün de germinal merkezinin olmadığı görüldü. Ayrıca, zencefil ekstraktının dejeneratif değişiklikleri azalttığı tespit edildi. Diyabet grubunda CD4 ve CD8 salınımının yoğun olduğu gözlenirken, diyabet+zencefil grubunda CD4 ve CD8 salınım yoğunluğunun diyabet grubuna göre azaldığı görüldü. Ayrıca diyabet grubunda azalan dalak ağırlığının diyabet+zencefil grubunda arttığı kontrol ve sham gruplarıyla benzer değerde olduğu tespit edildi.

Sonuç: Zencefil ekstraktının deneysel yolla diyabet oluşturulan modelde immün sistemi uyarıcı etkiye sahip olduğu görülmüştür. Elde edilen sonuçlara göre, zencefil diyabetle ilişkili dalak sorunlarında ve bağışıklık fonksiyon bozukluğunda tedavi edici olarak kullanılabilir.

Anahtar Kelimeler: CD4, CD8, dalak, diyabet, zencefil

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Received: 09.11.2022 **Accepted:** 23.01.2023

INTRODUCTION

Diabetes mellitus (DM) is a lifelong chronic disease that occurs with the body's inability to produce insulin, the absence of insulin, or the rise in blood sugar as a result of the body's inability to use insulin[1,2]. There are 2 types: type 1 DM (T1DM), which occurs mostly in childhood, and type 2 DM (T2DM), the most common type which occurs in advanced ages[2]. Immune balance is closely related to diabetes[3]. T2DM negatively affects the functioning of immune cells and increases the risk of infection. Insulin deficiency has also been observed to cause deterioration in CD4 and CD8 function[4].

Immunomodulators function as an immunostimulant in cases of immune deficiency and as an immunosuppressant in abnormally increased immunity to protect the body against harm and infection[5,6]. Studies with natural products that act as regulators by increasing or decreasing immunity are increasing[3,7,8]. Ginger (Zingiber officinale) has been used both as a spice and as a therapeutic since ancient times. The therapeutic property of ginger can be attributed to the fact that it has about 400 different components, mainly gingerols, shogaols, zingerone and paradols[5,9].

Immunity is classified as innate natural and acquired immunity. The spleen is the largest secondary lymphoid organ involved in the immunity of the organism. It hosts a large number of cells that play a role in defense, such as T and B lymphocytes, dendritic cells, macrophages and natural killer cells[10]. T lymphocytes are responsible for cellular immunity and they include two subgroups as CD4 and CD8[11].

CD4 and CD8 are very important in the pathogenesis of diabetes. Particularly in T1DM, an increase in the number of these cells or deterioration in their functions are effective. At the same time, these cells cause the destruction of islets β-cell. The increase in the number of CD4 positive cells with dysfunction causes autoimmune destruction of islet of β-cells in pancreas[12]. Therefore, CD4 T lymphocytes play an important role in the pathogenesis of T1DM[13]. T2DM, which is characterized by hyperglycemia and hyperinsulinemia, causes an increased risk for viral infection, melanoma or other different diseases. Also, it has been declared that CD8 T lymphocytes express the insulin receptor and are adversely affected by insulin and blood sugar increase. In addition, insulin is a direct influence on the function of CD8 T lymphocytes[4].

This study aimed to determine immunomodulatory effects of ethanolic extract of ginger on CD4 and CD8 expression in the spleen tissue of experimental diabetic rats.

MATERIALS AND METHODS

The study was conducted using 40 female, 206±6 g, and 4-month aged Wistar albino rats obtained from The Health and Experimental Center of Tekirdağ Namık Kemal University. All experiments were approved by the Tekirdağ Namık Kemal University Ethics Committee for Animal Experiments (meeting no: 09.11.2022/1153).

The rats were not used in any previous studies and they were housed at standard cages under temperature controlled room (22±2 °C) and were maintained on a 12-hour light/dark cycle and fed with a standard rat pellet diet and water ad libitum.

Preparation Ethanolic Extract of Ginger

Ginger fresh rhizomes were obtained from a local store and authenticated at the Department of Botany in Tekirdağ Namık Kemal University. Gingers were firstly washed and dried in a dark room. Dried ginger rhizomes were mechanically pulverized in a porcelain mortar. The resulting powder mixture was kept in 95% alcohol for 24 hours, then the mixture was filtered. This process was repeated 3 times in total. All prepared mixtures were collected together and alcohol was removed in the low speed evaporator. The prepared extract was stored in refrigerator at 4 °C. Ginger extract 200 mg/kg/bw/day was given orally to experimental rats for 30 days according to the previously mentioned methodology[14].

Induction of Diabetes

50 mg/kg of streptozotocin (STZ) (Sigma, st. Louis, MO, USA) was dissolved in 0.1 M citrate buffer (pH 4.5). Intraperitoneal injection (i.p.i.) was performed after an overnight fasting. After 3 days of the application, fasting blood glucose values were measured from the tail vein of the rats using the Accu-Chek Instant glucometer (Roche). Blood glucose values >250 mg/dL were considered an indicator for developing diabetes and rats were included in the diabetic groups[15].

Experimental Animals and Design

The experiment rats were divided into 5 groups and each group included eight animals. The total experiment protocol was maintained for 30 days. The experimental groups were as follows:

Control (n=8): No application was made (untreated group).

Sham group (n=8): Tween 80 was given to rats by oral gavage.

Ginger group (n=8): Fresh ginger extract (prepared daily) was given by oral gavage at the dose of 200 mg/kg for 30 days.

Diabetes group (n=8): This group was administered 50 mg/kg i.p.i. STZ.

Diabetes+ginger group (n=8): After diabetes was established, 200 mg/kg ginger extract was administered to this group by oral gavage for 30 days.

At the end of 30 days, the rats were euthanized under deep anesthesia and spleen tissues were removed.

Histological Procedure

Spleen tissues were fixed in 10% formalin for 48 hours. After fixation, the samples were processed for routine histological protocols and embedded in paraffin. The sections taken at 5 µm were deparaffinized in xylene, rehydrated through decreasing concentrations of ethanol and stained with Crossman's triple staining for histological examination[16].

Immunohistochemical Staining

The streptavidin biotin peroxidase complex (strepABC) method was applied to investigate CD4 and CD8 T lymphocytes immunoreactivity in the spleen. Sections of 5 µm thickness were collected on adhesive slides. The sections were processed in citrate buffer solution (pH 6.0) for 10 min in a microwave oven at 700 watts. Then, tissues were kept in 3% hydrogen peroxide (H_2O_2) for 10 min. Sections were incubated with anti-CD4 antibody (ab237722, diluted 1:150) and anti-CD8 antibody (OX-8) (ab33786, diluted 1:150). Sections were incubated with biotinylated goat anti-rabbit and rabbit anti-goat IgG for 30 min and peroxidase conjugated streptavidin (1:200) (P0397; Dako Corp., Carpinteria, CA, USA) for 10 min. After 3,3′-Diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/mL; Dako Corp.) was used as chromogen, Gill III hematoxylin was used as counterstaining. Sections were evaluated using light microscope (Olympus BX51, Tokyo, Japan). Rabbit serum without primer antibody served as the negative control. Evaluation of immunoreactivity of CD4 and CD8 were scored. Immunoreactive cells were categorized as having negative, slight, moderate, and intensive.

Assessment of Immunohistochemical Staining

The number of immunohistochemical staining of CD4 and CD8 were examined using light microscopy at X400 magnification and photographed. A total number of fifteen representative images were selected from each rat. The number of CD4 and CD8 positive and negative cells per unit area was calculated according to the following formula: (The number of positive cells/number of total cells counted in the field) x100. The number of CD4 and CD8 positive cells and ratio of CD8/CD8 were statistically analyzed[3,17,18].

Relative Spleen Weight

Fresh spleens were weighted immediately after rats were euthanized. Statistical method was used to compare change in spleen weight between groups.

Statistical Analysis

Statistical analysis was performed by using Statistical Package for the Social Sciences (SPSS) (version 20.0, IBM, SPSS Inc., Chicago, USA). Data were examined for normality distribution and variance homogeneity with the Shapiro-Wilk's test. If normally distributed, the one-way ANOVA test was applied, and the differences between the groups were analyzed with the post-hoc Tukey test. The differences were considered significant at $p<0.05$, and the means and standard errors were calculated. In the study, nonparametric tests were used as the data did not provide normal assumptions. So, the differences between the groups were analyzed with the Kruskal-Wallis, and the Mann-Whitney U test was used between the groups. Also, the differences were considered significant at $p<0.05$, and the median values (minimum-maximum) were calculated.

RESULTS

Histological Results

The control, sham and ginger groups had normal histological structure of the spleen. The tissue was surrounded by a capsule from the outside. Trabeculae separated from the capsule extended to the parenchyma of the spleen. The spleen consisted of separated lymphoid follicles (white pulp) and was surrounded by highly vascular matrix (red pulp). The white pulp was formed of periarteriolar lymphatic sheath and marginal zones. Lymphoid follicles had germinal centers and central arteriole. The red pulp consisted of network of blood cells cords (Figure 1a-c). The diabetes group was seen to have atrophied lymphoid follicles. There was no germinal center in most of the follicles (Figure 1d). The diabetes+ginger group was observed as white and red pulps, similar to those in the control, sham or ginger groups. Most of the follicles had germinal center (Figure 1e).

Immunohistochemical Results

Specific CD4 and CD8 immunoreactions were seen in all groups. However, there was a difference between the groups in terms of the severity of immunoreaction-positive cells.

CD4 and CD8 T lymphocytes were localized mostly in red pulp and few positive cells in white pulp. Slight reaction was observed in the control and sham groups in red pulp (Figure 2a, 2b, 3a, 3b). The ginger group was shown to have moderate reaction and a few slight reactions in the cells (Figure 2c, 3c). While intensive reaction was remarkable in the diabetes group (Figure 2d, 3d), moderate reaction was seen in the diabetes+ginger group (Figure 2e, 3e).

Assessment of Immunohistochemical Results

According to our findings, it was observed that the number of CD4 and CD8 immunopositive cells in the diabetes group showed significant increase compared to the control, sham and ginger groups ($p<0.0001$). After treated with ginger, it was observed that the diabetes+ginger group exhibited significant decrease as compared to the diabetes group ($p<0.0001$ for CD4 and CD8). All data of CD4 and CD8 are shown in Table 1.

In the present study, a highly significant increase was found in the CD4/CD8 ratio ($p=0.002$). There was a significant decrease in the diabetes group compared to the control ($p=0.002$) and sham groups ($p=0.001$), but there was a nonsignificant increase in the diabetes+ginger group ($p>0.05$) (Table 1).

Spleen Weight Results

When all the groups were compared in terms of spleen weight, a significant decrease was found in the diabetes group. Also, the diabetes+ginger group was compared to the diabetes group and a significant increase was detected in spleen weight ($p<0.05$). Comparison of mean values of spleen weight among the groups is shown in Figure 4.

DISCUSSION

A new one is added to the studies on immunological resources every day. The discovery of highly biostable immunomodulators without toxic side effects is great relevance. In particular, the immunomodulatory effects of plants have been a matter of curiosity that have proven biosafety in recent years[19]. In this study, we set out to investigate whether ginger has a protective effect on the spleen tissue, which has an important function on immunity in STZ induced diabetic rats.

Table 1. The number of CD4 and CD8 positive cells per unit area in all groups

	Control	Sham	Ginger	Diabetes	Diabetes+ginger
CD4	19.00	19.00	18.00	83.50[a]	21.00[bc]
CD8	15.50	16.00	16.00	80.00[a]	19.00[bc]
CD4/CD8	1.12	1.20	1.20	1.04[a]	1.12

[a]: $p<0.05$ diabetes group versus control group/diabetes group versus sham group, [b]: $p<0.05$ diabetes+ginger group versus diabetes group, [c]: $p<0.05$, diabetes+ginger group versus ginger group

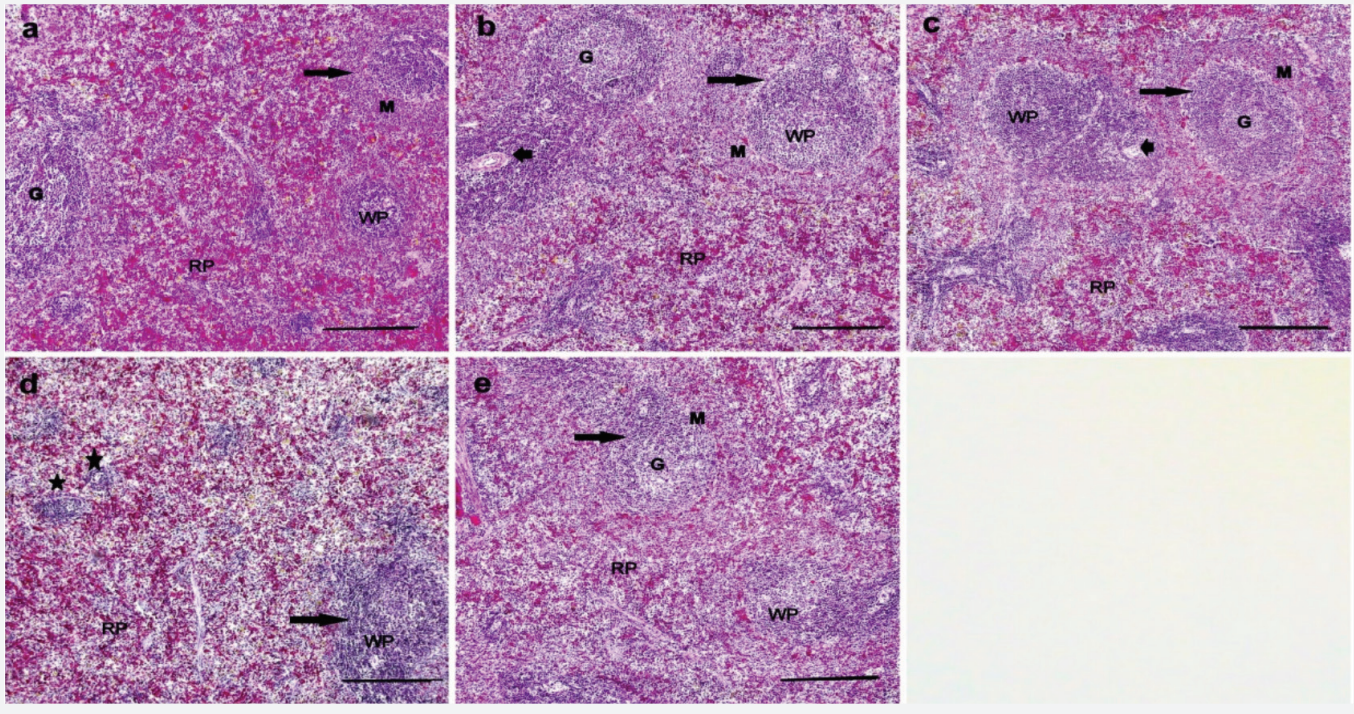

Figure 1. Histological structure of spleen tissue. The control (a), sham (b), ginger (c), diabetes (d), diabetes+ginger groups (e). Red pulp (RP), white pulp (WP), lymphoid follicles (arrow), germinal center (G), marginal zone (M), atrophied lymphoid follicles (star), periarteriolar lymphatic sheath (small arrow). Triple staining. Bar=100 µm

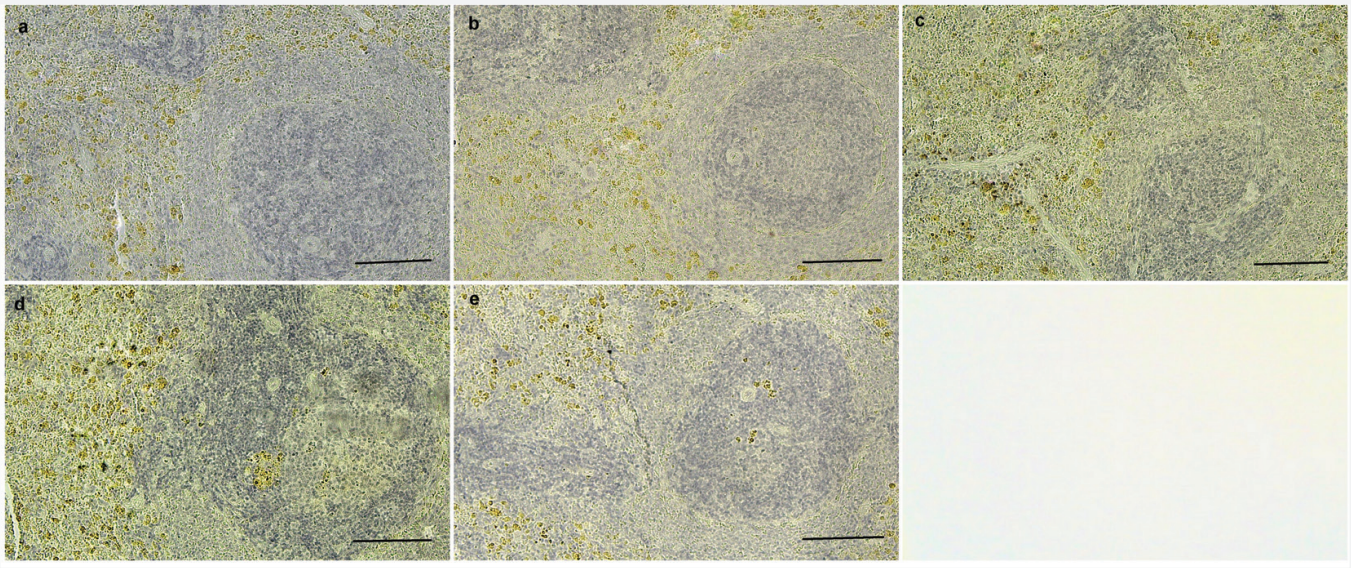

Figure 2. CD4 expression in spleen tissue. The control (a), sham (b), ginger (c), diabetes group (d), diabetes+ginger groups (e). Immunohistochemical staining. Bar=100 µm

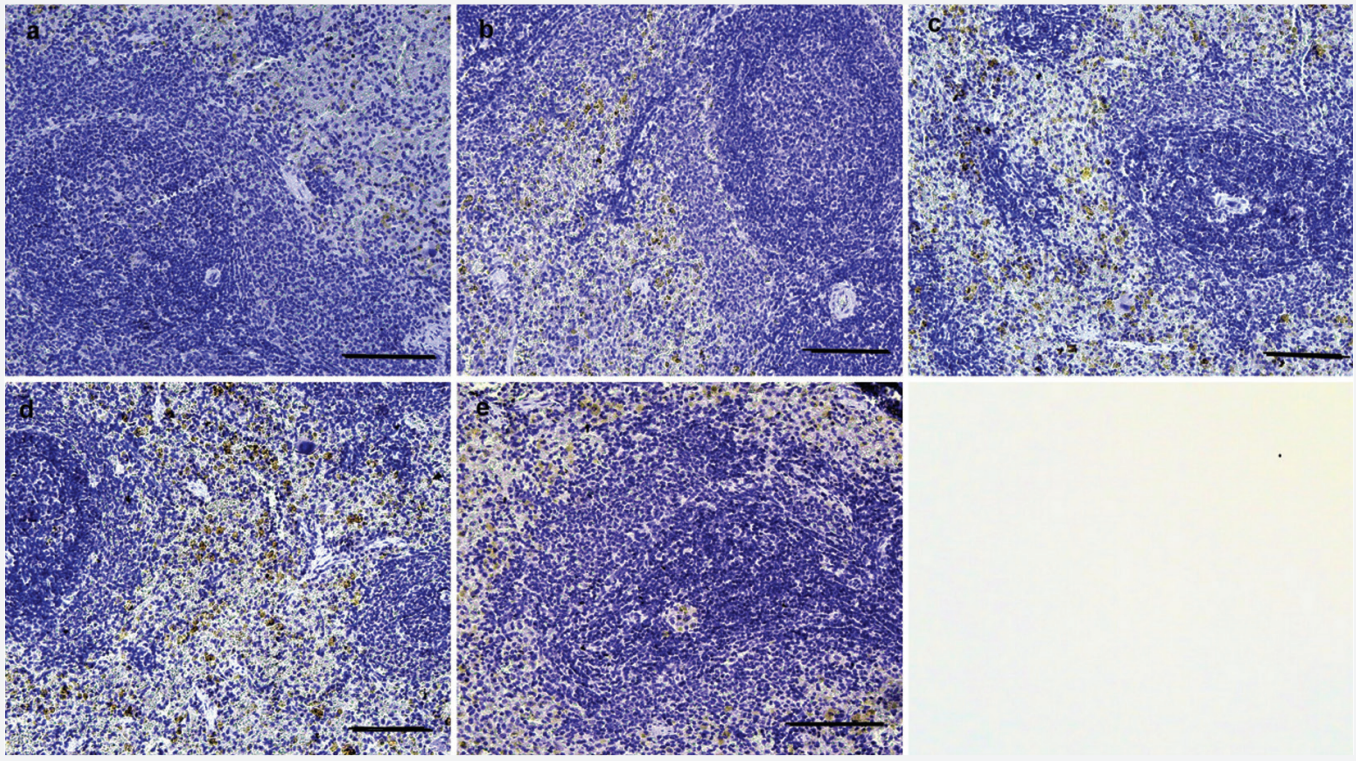

Figure 3. CD8 expression in spleen tissue. The control (a), sham (b), ginger (c), diabetes group (d), diabetes+ginger groups (e). Immunohistochemical staining. Bar=100 µm

Lymphoid organs such as the spleen are highly sensitive to various stresses, and exposure to disease causes splenic atrophy[18,20]. It was declared that germinal center of the lymphoid follicles was also dramatically reduced in diabetic rats[20,21]. Said et al.[3], (2020) declared that most follicles were atrophied with pyknotic nuclei in most of their cells. No germinal centers were seen in most of them. In animal models[3,22,23] and clinical studies[24], it was reported that ginger had a restorative effect against diabetes induced damage. In the current study, we administered ginger extract by oral gavage in diabetic rats for 30 days. We saw that ginger administration improved the histological and ultrastructural degenerative in the diabetic group. This improvement can be seen as increased number of lymph follicles in the white pulp and the appearance of the centrum germinativum.

Ginger is included in the list of safe herbs by the Food and Drug Administration. It has been reported that the use of up to 4 grams per day will not cause a pathological problem[9]. In a study conducted in patients with T2DM, it was reported that the use of ginger powder for 6-12 weeks at dosages up to 3 grams/day did not cause serious side effects[22]. It was declared that from 200 to 500 mg/kg/day for ginger extract had anti-inflammatory and antioxidative effects[9]. Also, ginger extract at the dose of 1000 mg/kg by oral administration is tolerated in pregnant rats. It was added that it exerted no adverse effects on pregnancy or the development of fetuses[25]. Before starting our experimental study, we examined many articles to design our experimental procedure, so we completed our work at the appropriate and effective dose (200 mg/kg) and time (30 days)[14]. Also, we found in our study that the dose and duration did not have serious adverse effects and complications in rats treated with ginger.

CD4 and CD8 T lymphocytes responsible for cellular immunity directly destroy harmful cells[26]. Many studies have emphasized that CD4 and CD8 T lymphocyte subtypes are effective in both T1DM and T2DM[27,28]. Miya et al.[27], (2018) investigated the effects of glucose loading on T lymphocytes in Japanese with T2DM and without diabetes. 75 g OGTT was applied to the participants. T lymphocytes were calculated at 60 and 120 minutes after fasting (12 hours, overnight). In the study, there was no difference in CD4 positive cell counts between the groups before glucose loading. However, they found that CD4 positive cell counts increased rapidly in both the T2DM and nondiabetic groups after 120 minutes of glucose loading.

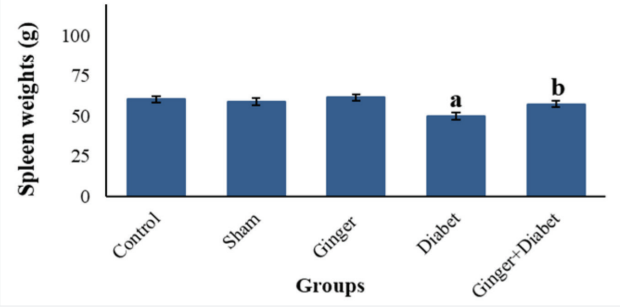

Figure 4. Comparison of the mean values of spleen weight among the groups. The control group-diabetes group p=0.007 (a), sham group-diabetes group p=0.05 (a), diabetes group-diabetes+ginger group p=0.021 (b)

The number of CD4 cells is important in the CD8 cell response. An increase in CD8 count causes an increase in CD4 count[29]. Li et al.[30], (2022) investigated CD4 expression in diabetic and normal mice. They revealed that while CD4 positive cells in the spleen, pancreas and kidney tissues were low in healthy mice, CD4 expression was significantly increased in organs of mice with DM. In the study, CD and CD8 positive cell counts of the diabetic group were found to be statistically higher than those of the control, sham and ginger groups.

Many serious side effects of synthetic drugs used in the treatment of immune system related diseases attract the attention of the consumers. Therefore, there is an increasing interest in therapeutic agents with fewer or no side effects[31]. Ginger is promising in this regard. The findings of the present study showed that ethanol extract of ginger activated T lymphocyte subtypes in term of CD4 and CD8 expression. This immunomodulatory activity of ginger may provide a future basis for the development of this plant as a source of immunoregulating substance in diabetes.

Study Limitations

Experimental diabetes was created in the study. After being treated with ginger, both the damage caused by diabetes and the anti-inflammatory effect of ginger were investigated in the spleen tissue. This study has a limitation. Immunohistochemical method was used only in this study. A more detailed evaluation can also be made using other methods such as western blot or polymerase chain reaction.

CONCLUSION

In the study, it was determined that CD4 and CD8 expression increased in rats with experimental diabetes. It was observed that the CD4 and CD8 expressions were equivalent to the control group, with the administration of ginger at 200 mg/kg for 30 days. It has been determined that ginger has an effect on eliminating the negative effects caused by diabetes and can be used as an immunomodulator.

Acknowledgments

I would like to thank Assoc. Prof. Nilay SEYİDOĞLU from Tekirdağ Namık Kemal University, Department of Physiology, for her contribution to the statistical analyses of the study.

Ethics

Ethics Committee Approval: The study was approved by the Tekirdağ Namık Kemal University of Ethics Committee for Animal Experiments (meeting no: 09.11.2022/1153).

Informed Consent: Animal experiments.

Peer-review: Externally peer-reviewed.

Financial Disclosure: This study was financially supported by a grant from Scientific Research Fund of Tekirdağ Namık Kemal University (grant number: NKUBAP.10.GA.18.147).

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.82905
Nam Kem Med J 2023;11(1):42-47

Clinical Characteristics and Risk Factors of Patients with Pediatric Amblyopia

Pediatrik Ambliyopi Hastalarımızın Klinik Özellikleri ve Risk Faktörleri

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ABSTRACT

Aim: Amblyopia is a common disease characterized by reduced visual acuity in one or both eyes during visual development in early stages of life. Satisfactory outcomes can be achieved with early diagnosis.

Materials and Methods: The study included a total of 341 pediatric patients aged three to 15 years, who presented to our clinic between February 2016 and September 2022 and were diagnosed with amblyopia. Using the follow-up files of all the patients, visual examination findings and data on birth, maternal obstetric history, family history, neurological disease history, chronic systemic disease history, and ocular surgery history were reviewed.

Results: A total of 341 patients, including 171 female and 170 male, were enrolled in the study. The mean age of the patients was 7.81±3.6 years. According to the type of amblyopia, refractive amblyopia was detected in 188 of the 341 patients (55.1%), strabismic+refractive amblyopia in 93 (approximately 27.3%), strabismic amblyopia in 55 (16.1%), and deprivation amblyopia in five (1.5%) patients. It was determined that amblyopia was most common in the age ranges of 3-6 (36.5%) and 7-10 (44.2%) years. Family history was found at a significantly higher rate in the amblyopia types presenting with strabismus compared to the refractive amblyopia type.

Conclusion: The determination of non-ocular risk factors other than the known ocular risk factors of amblyopia will allow for the early treatment of high-risk children and prevent preventable vision loss.

Keywords: Amblyopia, risk factors, refractive errors, strabismus, childhood

ÖZ

Amaç: Ambliyopi, yaşamın erken evrelerinde görme gelişimi sırasında bir veya iki gözde görme keskinliğinde azalma ile karakterize yaygın bir hastalıktır. Erken teşhis ile tedavide tatmin edici sonuçlar alınabilir. Ambliyopinin risk faktörlerinin belirlenmesi, hastalığın erken tanı konulmasını sağlayacak ve dolayısıyla oluşabilecek görme kayıplarını önleyecektir.

Gereç ve Yöntem: Şubat 2016 ile Eylül 2022 tarihleri arasında ambliyopi tanısı konulan 3-15 yaş arası toplam 341 çocuk hasta dahil edildi. Tüm hastaların takip dosyalarından tüm görme muayeneleri ve hastaların doğum, annenin obstetrik öyküsü, aile öyküsü, nörolojik hastalık öyküsü, kronik sistemik hastalık öyküsü ve oküler cerrahi öyküsü hakkındaki dosya bilgileri incelendi.

Bulgular: Bu çalışmamıza hastaların 171'i kadın ve 170'i erkek olmak üzere toplam 341 hasta dahil edildi. Hastaların ortalama yaş aralığı ise 7,81±3,6 idi. Ambliyopi türlerine göre; 341 hastadan 188 kişide refraktif ambliyopi (%55,1), 93 strabismik+refraktif ambliyopi (yaklaşık %27,3), 55 kişide strabismik ambliyopi (%16,1) ve 5 (%1,5) kişide ise deprivasyon ambliyopisi tespit edildi. Bizim çalışmada ambliyopinin en sık 3-6 yaş (%36,5) ve 7-10 yaş (%44,2) aralığında olduğu tespit edildi. Şaşılığın bulunduğu ambliyopi türlerinde aile öyküsünün, refraktif ambliyopi türüne kıyasla daha fazla olduğu görüldü.

Sonuç: Ambliyopinin bilenen oküler risk faktörleri haricindeki, non-oküler risk faktörlerinin belirlenmesi yüksek riskli çocukların erkenden tedavi edilmesini ve önlenebilir görme kayıplarının önüne geçilmesini sağlayacaktır.

Anahtar Kelimeler: Ambliyopi, risk faktörleri, kırma kusurları, şaşılık, çocukluk

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Received: 06.01.2023 **Accepted:** 24.01.2023

INTRODUCTION

Amblyopia (lazy eye) can be defined as a decrease in visual acuity that occurs often in one eye and sometimes in both eyes without any visible abnormalities in the visual pathway[1,2]. The prevalence of amblyopia has been reported to range from 1 to 5% in studies conducted in various populations and age groups across the world and 0.6 to 3.5% in those conducted in Turkey[3-6].

Amblyopia results from disturbances in visual development early in life and is classified according to the underlying cause of visual impairment. Refractive amblyopia, strabismic+refractive amblyopia, and strabismic amblyopia constitute most amblyopia cases. In addition, deprivation amblyopia is another rare cause of amblyopia associated with pathologies that can prevent vision, e.g., cataracts, corneal opacity, and ptosis[7-9].

In studies conducted to evaluate perinatal, demographic, and socio-economic risks related to amblyopia, factors such as prematurity, neonatal intensive care requirement, cesarean delivery, and familial predisposition have been defined and associated as non-ocular factors[10,11]. Amblyopia starts from risky delivery and gradually progresses until the period when visual development is completed[12]. Amblyopia has many negative effects on the patient's life, such as decreased quality of life, limited occupational choices, and increased risk of vision loss in the other eye[13]. The treatment options of amblyopia include patch, atropine eye drops, and optical punishment of the non-amblyopic eye. Although children younger than seven years constitute the age group that most benefits from treatment, there are also studies suggesting that visual acuity can be improved in amblyopic children of up to 15 years of age. However, the success rate of treatment decreases with increasing age[14-16].

Amblyopia is a disease with a high rate of treatment response, especially when detected at an early age. Therefore, the identification of risk factors that may affect the development of amblyopia can significantly contribute to the success of treatment. In this study, we aimed to determine the relationship between demographic characteristics, clinical characteristics, and risk factors (such as family history, age of onset, cesarean delivery, and prematurity) in patients diagnosed with amblyopia and followed up in our clinic.

MATERIALS AND METHODS

This study was planned retrospectively and conducted after receiving approval from the Adıyaman University Local Institutional Ethics Review Board (date: 15.11.2022, decision no: 2022/8-1). Because the study was designed retrospectively, no written informed consent form was obtained from patients.

A total of 341 pediatric patients aged three to 15 years, who were diagnosed with amblyopia between February 2016 and September 2022, were included in the study. The follow-up files of all the patients were reviewed in terms of data on birth, maternal obstetric history, family history, neurological disease history, chronic systemic disease history, and ocular surgery history. Refractive error measurement, visual acuity measurement, anterior segment examination findings, and dilated fundus examination findings, including detailed ophthalmological findings, were also recorded. In addition, the presence and degree of shifts in nine cardinal positions of gaze, primary position, and other gaze positions, as well as limitation and weakness in gaze directions were noted. The results of the cover test, prism test, Krimsky test, and head position measurements of the patients were recorded. The cases with strabismus were classified according to the direction of tropia as esotropic, exotropic, and vertical.

Both uncorrected and corrected visual acuity examinations were performed using the Lea symbols in children aged three to five years, the "E" chart in preschool and illiterate children, and the Snellen chart in school-age children.

To measure refractive error, 1% cyclopentolate was applied three times at 5-minute intervals. After 45 minutes, measurements were made using a table-mounted Topcon Autorefractor KR-800 (USA) or hand-held Pediatric Autorefractor plusoptiX A09 (Germany) if possible. In cases where these procedures could not be performed, measurements were undertaken using the Keeler Professional retinoscopy (USA).

Amblyopia was defined as the presence of at least one of the factors of unilateral amblyopia, anisometropia [hypermetropia, 1 diopter (D); ≥1.5 D astigmatism; and ≥3 D myopia), strabismus, history of strabismus surgery, and conditions that obstructed the visual axis (e.g., ptosis and congenital cataract), accompanied by a best-corrected visual acuity (BCVA) of lower than 0.63 (Snellen 20/32) or a two-line difference between eyes, without any pathologies in the eye structure or visual pathway. Bilateral amblyopia was defined as high ametropia with ≥6 D myopia, ≥4 D hyperopia, or ≥2.5 D astigmatism, or conditions obstructing the bilateral visual axis with a BCVA of lower than 0.5 (Snellen 20/32) for children aged three or four years and lower than 0.4 (Snellen 20/32) for those older than four years[17,18].

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 25.0 (SPSS Inc., Chicago, IL, USA). The normality of numerical data distribution was evaluated with the Kolmogorov-Smirnov test. Categorical data were analyzed with the chi-square test, and numerical data with the independent-samples t-test and Mann-Whitney

test. A p value of less than 0.05 was considered statistically significant.

RESULTS

This study included a total of 341 patients, of whom 171 were female and 170 were male. The mean age of the patients was 7.81±3.6 years. The gender distribution according to the types of amblyopia was as follows: 93 girls and 95 boys with refractive amblyopia (55.1%), 47 girls and 46 boys with strabismic+refractive amblyopia (27.3%), 28 girls and 27 boys with strabismic amblyopia (16.1%), and 3 girls and 2 boys with deprivation amblyopia (1.5%). In addition, unilateral amblyopia was present in 264 patients (77.4%) and bilateral amblyopia in 77 (22.69%). There was no statistically significant difference between the types of amblyopia in terms of gender (p=0.940). When evaluated according to the strabismus type, the mean age was 7.39±2.8 years in the refractive amblyopia group, 6.94±4.3 years in the strabismic+refractive amblyopia group, 6.94±3.8 years in the strabismic amblyopia group, and 4.00±1.0 in the deprivation amblyopia group. Table 1 presents the distribution of patients according to the amblyopia type and age range (3-6 years, 7-10 years, 11-14 years, and 15-18 years).

The mean BCVA was 0.62±0.44-0.61±0.43 in the patients with refractive amblyopia, 0.66±0.27-0.67±0.28 in those with strabismic+refractive amblyopia, 0.61±0.29-0.61±0.30 in those with strabismic amblyopia, and 0.64±0.33-0.61±0.34 in those with deprivation amblyopia (right, left respectively).

When the relationship between refractive error and amblyopia types was examined, the mean spherical values (1.76±3.6) - (1.92±3.6) and cylindrical values were (-0.85±1.7) - (-0.92±1.3) in refractive amblyopia; the mean spherical values (1.34±2.4) - (1.29±2.8) and cylindrical values were (-0.49±1.5) - (-0.55±1.4) in strabismic+refractive amblyopia; the mean spherical values (0.31±0.95) - (0.28±0.90) and cylindrical values were (-0.45±0.40) - (-0.50±0.42) in strabismic amblyopia; and the mean spherical values (1.40±1.3) - (1.35±1.3), and cylindrical values were (-0.25±0.3) - (-0.20±0.2) in deprivation amblyopia. (right, left respectively). The distribution of refractive errors according to the amblyopia type is shown in Table 2.

The anisometropia values were as follows: mean spherical, 1.65±2.2 and mean cylindrical, -0.95±0.92 in unilateral amblyopia and mean spherical, 0.18±0.60 and mean cylindrical, -0.15±0.24 in bilateral amblyopia. Table 3 presents the detailed anisometropia values in unilateral and bilateral amblyopia.

We detected esotropia in 68 (73%) and exotropia in 25 (27%) of the 93 patients with strabismic+refractive amblyopia. Of the 55 patients with strabismic amblyopia, 30 (54.5%) had esotropia and 25 (45.5%) had exotropia. When all the 148 patients with strabismus were evaluated together, there were 98 (66.2%) cases of esotropia and 50 (33.8%) cases of exotropia. We determined that esotropia was more likely to cause amblyopia than exotropia (p=0.015). Figure 1 shows the distribution of amblyopia cases affected by strabismus.

Of the five patients with deprivation amblyopia, four had ptosis and one had a cataract. First-degree familial amblyopia status was detected in 28 (8.2%) of the 341 patients, including 10 (5.3%) of the 188 patients with refractive amblyopia, nine (9.7%) of the 93 patients with strabismic+refractive amblyopia, and nine (16.3%) of the 55 patients with strabismic amblyopia. The rate of family history was higher in amblyopia types presenting with strabismus compared to the refractive amblyopia type (p=0.024).

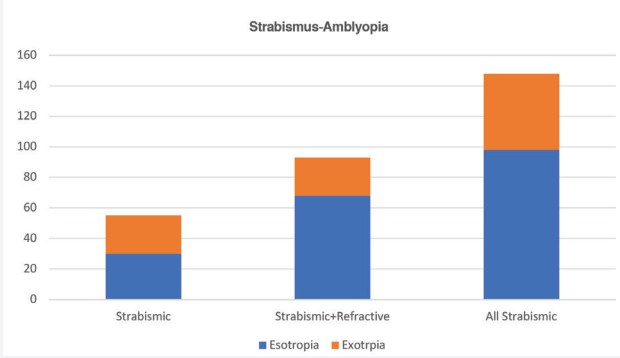

Figure 1. Distribution of strabismus in amblyopia cases associated with strabismus

Table 1. Distribution of age groups according to the amblyopia types

	Refractive	Strabismic+refractive	Strabismic	Deprivation
	Unilateral-bilateral	Unilateral-bilateral		
Age				
3-6 years	55 (16.1%) 26 (7.6%)	20 (5.9%) 12 (3.5%)	7 (2%)	5 (1.4%)
7-10 years	67 (19.7%) 20 (5.9%)	34 (9.9%) 8 (2.4%)	21 (6.3%)	-
11-14 years	20 (5.9%) 8 (2.4%)	8 (2.4%) 4 (1.2%)	18 (5.2%)	-
15-18 years	2 (0.6%) -	7 (2%) -	9 (2.6%)	
Total	134 (39.3%) 54 (15.9%)	69 (20.2%) 24 (7.1%)	55 (16.1%)	5 (1.4%)

History of preterm delivery was present in 18 patients with refractive amblyopia, 16 patients with strabismic+refractive amblyopia, and six patients with strabismic amblyopia; history of neonatal intensive care stay was detected in 18 patients with refractive amblyopia, 14 patients with strabismic+refractive amblyopia, five patients with strabismic amblyopia, and one patient with deprivation amblyopia; history of cesarean delivery was found in 45 patients with refractive amblyopia, 27 patients with strabismic+refractive amblyopia, 21 patients with strabismic amblyopia, and two patients with deprivation amblyopia; and epilepsy was detected in 10 patients with refractive amblyopia, nine patients with strabismic+refractive amblyopia, and five patients with strabismic amblyopia. The distribution of these non-ocular risk factors according to the amblyopia type is given in Table 4.

DISCUSSION

Although amblyopia is most common in the age group of three to four years, studies have found that this condition is also seen at a high rate in school-age children[19-21]. In a meta-analysis conducted by Hu et al.[22], it was reported that the prevalence of amblyopia varied between boys and girls but did not significantly differ according to geographical area, sample size, and economic status[22]. In contrast, in an amblyopia prevalence study on children, Li et al.[18] detected no significant difference between the genders. In the current study, we found that amblyopia was most common in children aged three to six years (36.5%) and seven to 10 years (44.2%). We observed no statistically significant difference in terms of gender.

Studies have shown that the most frequent ocular risk factors of amblyopia are refractive errors, strabismus, or coexistence

Table 2. Distribution of refractive errors according to the amblyopia types

	Refractive	Strabismic+refractive	Strabismic	Deprivation
Spherical	Unilateral/bilateral n=134/54	Unilateral/bilateral n=70/23	55	5
≤1.00	13 (9.7%) 3 (5.6%)	8 (11.4%) 1 (4.3%)	10 (18.2%)	1 (20%)
>1.00 to <+4.00	21 (15.6%) 7 (13%)	11 (15.9%) 1 (4.3%)	19 (34.6%)	2 (40%)
≥4.00 to <6.00	31 (23.1%) 13 (13.0%)	16 (22.8%) 6 (26.3%)	2 (3.6%)	-
≥6.00	36 (26.8%) 15 (27.8%)	16 (22.8%) 7 (30.5%)	-	-
≤-1.00	9 (6.7%) 1 (1.8%)	4 (5.7%) 1 (4.3%)	12 (21.8%)	2 (40%)
>-1.00 to <-400	10 (7.6%) 1 (1.8%)	5 (7.1%) 1 (4.3%)	10 (18.2%)	-
≥-4.00 to <-6.00	8 (6.0%) 7 (13.0%)	5 (7.1%) 2 (8.6%)	2 (3.6%)	-
≥-6.00	6 (4.5%) 7 (13.0%)	5 (7.1%) 4 (17.4%)	-	-
Astigmatism				
<1.50	44 (32.8%) 10 (18.5%)	24 (34.3%) 6 (26.3%)	36 (65.5%)	4 (80%)
≥1.50 to <2.50	56 (41.8%) 15 (27.8%)	26 (37.1%) 7 (30.4%)	14 (25.4%)	1 (20%)
≥2.50	34 (25.4%) 29 (53.7%)	20 (28.6%) 10 (43.4%)	5 (9.1%)	-

Table 3. Distribution of anisometropia according to the laterality of amblyopia

	Unilateral (n=264)		Bilateral (n=77)	
Anisometropia	**Spherical**	**Cylindrical**	**Spherical**	**Cylindrical**
<1.00	66 (25.0%)	86 (32.6%)	44 (57.2%)	51 (66.2%)
1.00 to <1.50	80 (30.3%)	100 (37.9%)	28 (36.3%)	22 (28.6%)
≥1.50	118 (44.7%)	78 (29.5%)	5 (6.5%)	4 (5.2%)

Table 4. Distribution of non-ocular risk factors according to the amblyopia types

	Refractive	Strabismic+refractive	Strabismic	Deprivation	Total
Family history	10/188 (5.3%)	9/93 (9.7%)	9/55 (16.3%)	-	8/341 (8.2%)
Premature birth	18/188 (9.6%)	16/93 (17.2%)	6/55 (10.9%)	1/5 (20%)	41/341 (12%)
Neonatal intensive care requirement	18/188 (9.6%)	14/93 (15%)	5/55 (9%)	1/5 (20%)	38/341 (11.1%)
Cesarean delivery	45/188 (24%)	27/93 (29%)	21/55 (38.1%)	2/5 (40%)	95/341 (27.8%)
Epilepsy	10/188 (5.3%)	9/93 (9.7%)	5/55 (9%)	-	24/341 (7%)

of both, as well as conditions that affect vision (such as ptosis, congenital cataract, and corneal opacity) to a lesser extent[23,24]. Studies examining the relationship between refractive error and amblyopia have shown that amblyopia is associated with refractive error in approximately 50-70% of cases. Pascual et al.[25] evaluated 3,869 children and found unilateral amblyopia in 296 (7.7%) and bilateral amblyopia in 144 (3.7%). Children with unilateral amblyopia had hyperopia of ≥2 D, astigmatism of ≥1 D, or anisometropia of 0.5 D to ≥1 D, while those with bilateral amblyopia had bilateral hyperopia of ≥3 D or astigmatism of ≥1 D. Pai et al.[26] detected both hyperopia (66.7% of patients) and astigmatism (48.1%) to be major amblyogenic risk factors for anisometropia or isoametropia. Margines et al.[27] found that 568 of 780 pediatric amblyopia cases were unilateral and 212 were bilateral, and the rates of hyperopia, myopia, and astigmatism were 75%, 15%, and 92%, respectively, in the unilateral amblyopia group. In our study, 59% of all the patients had hyperopia of ≥1 D and 64% had astigmatism of ≥1 D. Of the 264 (77.4%) patients with unilateral amblyopia, approximately 35% had hyperopia of ≥4 D, 60% had astigmatism of ≥1.5 D, 10% had myopia of ≥4 D and had anisometropia of greater than 1 D spherical in 74% and cylindrical in 68%. Of the 77 (22.69%) patients with bilateral amblyopia, approximately 59% had hyperopia of ≥4 D, 62% had astigmatism of ≥1,5 D, 25% had myopia of ≥4 D and had anisometropia of ≥1,5 D spherical in 41% and cylindrical in 43%. This is consistent with the findings reported by previous studies.

In studies on strabismus, this condition has been detected as a risk factor for amblyopia, with the rate of strabismic amblyopia ranging from 10 to 40% and that of strabismic+refractive amblyopia ranging from 20 to 25% according to various population and ages, and esotropia being determined as a factor causing a higher risk of amblyopia than exotropia[7,11,28]. Malik et al.[29] found that 67.3% of 150 patients with strabismic amblyopia had esotropia and 32.6% had exotropia. In another study, Ryu and Lambert[30] detected esotropia in 71% and exotropia in 21% of 295 patients with strabismic, strabismic+refractory, and strabismic amblyopia types. In our study, we observed that 16.1% of the amblyopia cases were strabismic and 27.3% were strabismic+refractive. Of the patients with amblyopia affected by strabismus, 66.2% had esotropia and 33.8% had exotropia, and we found that the former constituted a higher risk of amblyopia compared to the latter.

Deprivation amblyopia, which is rare (<3%) in amblyopia cases, occurs as a result of the complete or partial obstruction of the visual axis due to conditions such as congenital cataract, ptosis, vitreous hemorrhage, and corneal opacity. In our study, deprivation amblyopia was present in five patients (four with ptosis and one with cataract) (1.5%)[31–33].

Various studies suggest that a history of amblyopia, especially in first-degree relatives, increases the risk of amblyopia development[10,34]. Mocanu and Horhat[10] found that 6% of patients with amblyopia had a family history. Çakır et al.[35] detected a family history of amblyopia in 5.3% of the patients they followed up. Guimaraes et al.[36] reported that strabismus-related amblyopia was more familial than the refractive amblyopia type. In our study, the rate of those with a family history was 5.3% in the refractive amblyopia group, 9.7% in the strabismic+refractive amblyopia group, and 16.3% in the strabismic amblyopia group. We found that a positive family history was higher in the amblyopia types associated with strabismus.

Studies investigating pregnancy- and birth-related risk factors of amblyopia have shown that low birth weight, premature birth, cesarean delivery, and neonatal intensive care requirement increase the risk of amblyopia development[36-38]. In the current study, we found that 41 (12%) cases had a history of preterm delivery, 38 (11.1%) had a history of neonatal care unit admission, and 95 (27.8%) had a history of cesarean delivery.

In a study conducted with 327 patients having amblyopia, Çakır et al.[35] reported that the rate of those with a history of febrile seizures was 9.7%. In our study, epilepsy was present in 34 (7%) patients.

Study Limitations

The relatively low number of patients, retrospective design, and inclusion of the three-year-old subgroup in the sample can be considered as factors limiting this study.

CONCLUSION

Amblyopia is an eye disease that is commonly seen in childhood and responds well to treatment. In addition to known risk factors affecting refractive error, strabismus, and visual axis of amblyopia, it is very important to determine other risk factors related to family history, pregnancy, and birth. The identification of these risk factors will facilitate the early diagnosis of the disease in high-risk children, and the initiation of early treatment in these children can prevent vision loss.

Ethics

Ethics Committee Approval: The study was approved by the Adıyaman University Local Institutional Ethics Review Board (date: 15.11.2022, decision no: 2022/8-1).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.A.Y., A.B., Concept: A.A.Y., Design: A.A.Y., Data Collection or Processing: A.A.Y., Analysis or Interpretation: A.A.Y., A.B., Literature Search: A.A.Y., Writing: A.A.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.97658
Nam Kem Med J 2023;11(1):48-53

Effect of CPAP on Hemocyte Profile C-reactive Protein and Fibrinogen Levels in People with Obstructive Sleep Apnea

Obstrüktif Uyku Apnesi Olan Kişilerde CPAP'ın Hemosit Profili, C-reaktif Protein ve Fibrinojen Düzeyleri Üzerine Etkisi

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ABSTRACT

Aim: Obstructive sleep apnea syndrome (OSAS) is a common sleep disorder characterized by repeated episodes of apnea and hypopnea during sleep. The repetitive hypoxemic and hypercapnic events can lead to increased proinflammatory cytokine production, endothelial dysfunction, oxidative stress, metabolic dysregulation, and insulin resistance in OSAS patients. In previous studies, some of the hemogram values increased in patients with OSAS and a decrease in these increased values was observed with continuous positive airway pressure (CPAP) treatment. CPAP is the most effective method for treating OSAS and alleviating the patients' symptoms. The aim of this study was to assess the effect of three-month CPAP therapy on hemocyte profile in people with OSAS.

Materials and Methods: Forty patients were included in the study. Data including clinical assessment, full previous polysomnography reports, and baseline and after CPAP therapy, complete blood profile (leukocytes, neutrophils, lymphocytes, hemoglobin, hematocrit, platelets, MPV, PDW, MCV, N/L, and P/L, CRP and fibrinogen) of the participants were collected from the electronic medical record.

Results: All patients who completed the study were CPAP compliant (5.53±0.39 h/night). After three months of CPAP treatment, the mean levels of leukocytes, lymphocytes, hemoglobin, hematocrit, MPV, MCV, N/L, and P/L, CRP and fibrinogen were significantly decreased compared to baseline values.

Conclusion: Our study showed significant decrease in hemoglobin, hematocrit, leukocyte, lymphocyte, MPV, MCV, N/L, and P/L, CRP and fibrinogen after three-month CPAP therapy.

Keywords: Obstructive sleep apnea syndrome, CPAP, hemocyte profile, CRP, fibrinogen

ÖZ

Amaç: Obstrüktif uyku apne sendromu (OUAS), uyku sırasında tekrarlayan apne ve hipopne atakları ile karakterize yaygın bir uyku bozukluğudur. Tekrarlayan hipoksemik ve hiperkapnik olaylar, OUAS hastalarında artmış proenflamatuvar sitokin üretimine, endotel disfonksiyonuna, oksidatif strese, metabolik düzensizliğe ve insülin direncine yol açabilir. Daha önceki çalışmalarda OUAS'lı hastalarda bazı hemogram değerlerinde artış olmuş ve sürekli pozitif hava yolu basıncı (CPAP) tedavisi ile bu artmış değerlerde azalma gözlenmiştir. CPAP, OUAS'ı tedavi etmek ve hastaların semptomlarını hafifletmek için en etkili yöntemdir. Bu çalışmanın amacı, OUAS olan kişilerde üç aylık CPAP tedavisinin hemosit profiline etkisini değerlendirmektir.

Gereç ve Yöntem: Çalışmaya 40 hasta dahil edildi. Klinik değerlendirme, önceki tam polisomnografi raporları ve CPAP tedavisi öncesi ve sonrasında tam kan profili (lökositler, nötrofiller, lenfositler, hemoglobin, hematokrit, trombositler, MPV, PDW, MCV, N/L ve P/L, CRP ve fibrinojen dahil olmak üzere) verileri katılımcılarının elektronik tıbbi kayıtlarından toplanmıştır.

Bulgular: Çalışmayı tamamlayan tüm hastalar CPAP uyumluydu (5,53±0,39 h/gece). Üç aylık CPAP tedavisinden sonra, ortalama lökosit, lenfosit, hemoglobin, hematokrit, MPV, MCV, N/L ve P/L, CRP ve fibrinojen seviyeleri başlangıç değerlerine göre önemli ölçüde azaldı.

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Received: 13.01.2023 **Accepted:** 24.01.2023

Sonuç: Çalışmamız üç aylık CPAP tedavisinden sonra hemoglobin, hematokrit, lökosit, lenfosit, MPV, MCV, N/L ve P/L, CRP ve fibrinojen değerlerinde anlamlı azalma olduğunu göstermiştir.

Anahtar Kelimeler: Obstrüktif uyku apne sendromu, CPAP, hemosit profili, CRP, fibrinojen

INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) is characterized by repetitive episodes of partial or total collapse of the upper airway during sleep, resulting in nocturnal hypoxia, daytime sleepiness, and fatigue[1]. It is estimated that 4% of both female and male adults suffer from OSAS[2]. Intermittent hypoxia results in increased reactive oxygen species, leading to oxidative stress and systemic inflammation.

OSAS is a low-grade inflammatory disease. In previous studies, elevated inflammatory cytokines, including IL-6, IL-1, C-reactive protein (CRP), and tumor necrosis factor-alpha, have been observed in OSAS patients[3,4]. Recent studies suggest that both WBC and NLR are good indicators of inflammation[5-8]. Some studies reported that platelet was activated and aggregated in patients with OSAS, which was also relevant in inflammatio[9,10]. MPV and PDW are both useful markers of platelet activity. Recently, studies introduce PLR as a novel inflammatory marker[7,8] In recent years, many studies have focused on leukocyte subsets, red blood cell indices, platelet indices, N/L ratio, and/or P/L ratio in patients with OSAS[11-15].

Continuous positive airway pressure (CPAP) remains the optimum therapy for patients with moderate to severe OSAS. Several studies have analyzed the effect of CPAP therapy on a wide variety of biomarkers of oxidative stress and inflammation[16-20].

Therefore, we aimed to explore the effect of CPAP therapy on hemocyte profile in people with obstructive sleep apnea.

MATERIALS AND METHODS

Study Population

We performed a single-center, cross-sectional study on participants attending our sleep laboratory between May 2018 to February 2019, who had previously been diagnosed with OSAS and had three months of CPAP therapy. Demographic characteristics were collected from the electronic medical record, including age, body mass index, and past medical history.

Exclusion Criteria

Patients with diagnosed autoimmune disorders, acute respiratory tract infection in recent one month, liver or kidney disease, malignant tumor, chronic alcoholism, hyperthyroidism or hypothyroidism, inflammatory bowel disease, inflammatory connective tissue disorders, heart disease (such as coronary artery disease and heart failure), cerebrovascular accident, history of recent blood transfusion (within two weeks), or hematologic disorders such as leukemia, anemia, or myelodysplastic syndrome were excluded. Patients using drugs (including non-steroidal anti-inflammatory drugs, steroids, antibiotics, and immunosuppressive medication) and aged <18 years were also excluded.

Study Design

Data including clinical assessment, full previous PSG reports, and baseline and after CPAP therapy complete blood profile (leukocytes, neutrophils, lymphocytes, hemoglobin, hematocrit, platelets, MPV, PDW, MCV, N/L, and P/L, CRP, and fibrinogen) of the participants were collected from the electronic medical records.

CPAP Compliance

CPAP adherence (hours of use) and efficacy were evaluated by CPAP adherence tracking data downloaded from the CPAP device. We defined CPAP adherence as the average use of four or more hours per night over the three-month study.

Biochemical Parameters

Baseline and after CPAP therapy, complete blood profile in all patients was also recorded from the electronic medical record, including leukocytes, neutrophils, lymphocytes, hemoglobin, hematocrit, platelets, MPV, PDW, MCV, N/L, and P/L, CRP, and fibrinogen.

This study was approved by the Local Ethics Committee of Tekirdağ Namık Kemal University Faculty of Medicine with the number: 2019.160.09.20 (date: 24.09.2019).

Statistical Analysis

Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA) version 18 software package. All the values were calculated as the mean±standard deviation. We used paired sample t-test to compare the pre-and post-treatment data of the study group. The reported p values are 2-tailed, and a p value <0.05 was considered statistically significant.

RESULTS

A total of 40 patients were included in the study. Descriptive characteristics of OSAS patients are presented in Table 1. The

mean CPAP pressure established by automatic titration was 7.8±2.6 cmH_2O. Comparisons of before and after CPAP therapy parameters are shown in Table 2.

A paired-samples t-test was conducted to evaluate the impact of the CPAP on the hemocyte profile. There was a statistically significant decrease in hemoglobin from Time 1 [M=40.17, standard deviation (SD)=5.16] to Time 2 (M=37.5, SD=5.15), t (29)=5.39, p<0.001 (two-tailed). The mean decrease in FOST scores was 2.67 with a 95% confidence interval ranging from 1.66 to 3.68. The eta squared statistic (0.50) indicated a large effect size.

All patients who completed the study were CPAP compliant (5.53±0.39 h/night). After three months of CPAP treatment, the mean levels of leukocytes, lymphocytes, hemoglobin, hematocrit, MPV, MCV, N/L, and P/L, CRP, and fibrinogen levels were significantly decreased compared to baseline values (Figures 1 and 2). Our study showed a significant decrease in hemoglobin, leukocyte, lymphocyte, hemoglobin, hematocrit, MPV, MCV, N/L, P/L, CRP, and fibrinogen levels.

Table 1. Descriptive characteristics of participants

	Mean±SD	Range
Age; years	53.79±2.61	39-79
Male sex; n (%)	34 (85%)	
BMI; kg/m^2	31.55±1.01	24.6-40.6
AHI (h^{-1}) before CPAP	46.20±5.18	5.8-82.3
AHI (h^{-1}) after CPAP	3.28±0.96	0.2-16.8
Lowest SpO_2, %	76.95±2.13	59-92
Duration of CPAP therapy (h/day)	5.53±0.39	4.1-8
Under SpO_2 90, minute	37.16±16.01	0-275

BMI: Body mass index, AHI: Apnea-hypopnea index, SD: Standard deviation, CPAP: Continuous positive airway pressure

DISCUSSION

This study reinforces the importance of hematological evaluation as an easy complementary tool to the global approach to OSAS patients by showing that hemoglobin, hematocrit, leukocyte, lymphocyte, MPV, MCV, N/L, and P/L, CRP and fibrinogen levels significantly decreased after positive airway pressure (PAP) treatment. These findings suggest that these parameters might be used as the markers of response to treatment.

Sustained hypoxia results in increased expression of erythropoietin-inducing erythropoiesis with a consequent increase in hematological parameters[21,22]. PAP correction of respiratory events and consequent hypoxia and inflammation can translate into a decrease in hemoglobin, and hematocrit as obtained in our study.

Some studies reported that platelet was activated and aggregated in patients with OSAS, which was also relevant in inflammation. MPV and PDW are both useful markers of platelet activity. The tendency to decrease MPV could also be explained by the fact that besides PAP decreasing hypoxia and inflammation, it also improves platelet aggregability[9,10]. Our results showed a significant decrease in MPV after three months of CPAP treatment.

Neutrophils mainly mediate innate immune response by secreting mediators while lymphocytes mediate adaptive immune response by regulating inflammation[22,23]. Recent studies suggest that both NLR and PLR are good indicators of

Table 2. Comparisons of biochemical parameters after 12 weeks of CPAP treatment

n=40	Before CPAP		After CPAP		p
Variable	Mean±SD	Range	Mean±SD	Range	
Hemoglobin	14.61±1.46	10.75-16.50	14.27±1.47	9.82-17.61	0.006
Hct	43.33±4.18	32.7-51.8	42.39±3.94	29.6-49.9	0.018
MPV	8.90±0.82	7.8-10.8	8.70±0.70	7.7-10.4	0.022
Plt (10^9/L)	246.0±49.1	162-294	242.6±49.0	239.1-339	0.219
PDW	15.09±2.08	12.8-20.3	14.85±1.98	12.0-19.3	0.208
Leukocyte (10^9/L)	8.01±2.00	4.4-12.6	7.60±1.70	5.6-10.8	0.037
Neutrophil (10^9/L)	4.14±1.31	2.2-7.0	4.10±1.22	2.6-6.5	0.774
Lymphocyte (10^9/L)	2.95±0.94	1.5-5.2	2.61±0.75	1.7-4.4	0.001
N/L	1.52±0.58	0.59-3.28	1.70±0.66	0.69-3.55	0.017
P/L	91.2±31.5	40-190	98.8±32.7	50-190	0.037
Fibrinogen (mg/dL)	342.80±91.86	259-450	310.93±65.21	206-447	0.018
CRP (mg/dL)	3.50±3.33	1-14	2.16±1.71	0.2-5.6	0.007

Differences among the four groups were examined using a One-Way analysis of variance (ANOVA) or chi-square test according to the characteristics of the data distribution.

CPAP: Continuous positive airway pressure, RDW: Red cell distribution width, SD: Standard deviation, MPV: Mean platelet volume, Hct: Hematocrit, Plt: platelet, N/L: Neutrophils/lymphocytes ratio, P/L: Platelet/lymphocyte, MCV: the Average volume of red blood cells, RDW: Distribution of red blood cells, MPV: Average volume of platelets, PDW: Width of platelet distribution

inflammation[5-8]. Our study demonstrated that a significant reduction of total lymphocyte count, NLR and PLR occurred exclusively in the peripheral blood of OSAS patients who used CPAP therapy for three months. However, there was no significant decrease in neutrophil counts.

Fibrinogen is an acute-phase protein synthesized from the liver in response to infection and inflammation, and inflammatory cytokines modulate fibrinogen biosynthesis[24]. Previous studies in patients with OSAS determined that elevated fibrinogen levels were related to obesity and the

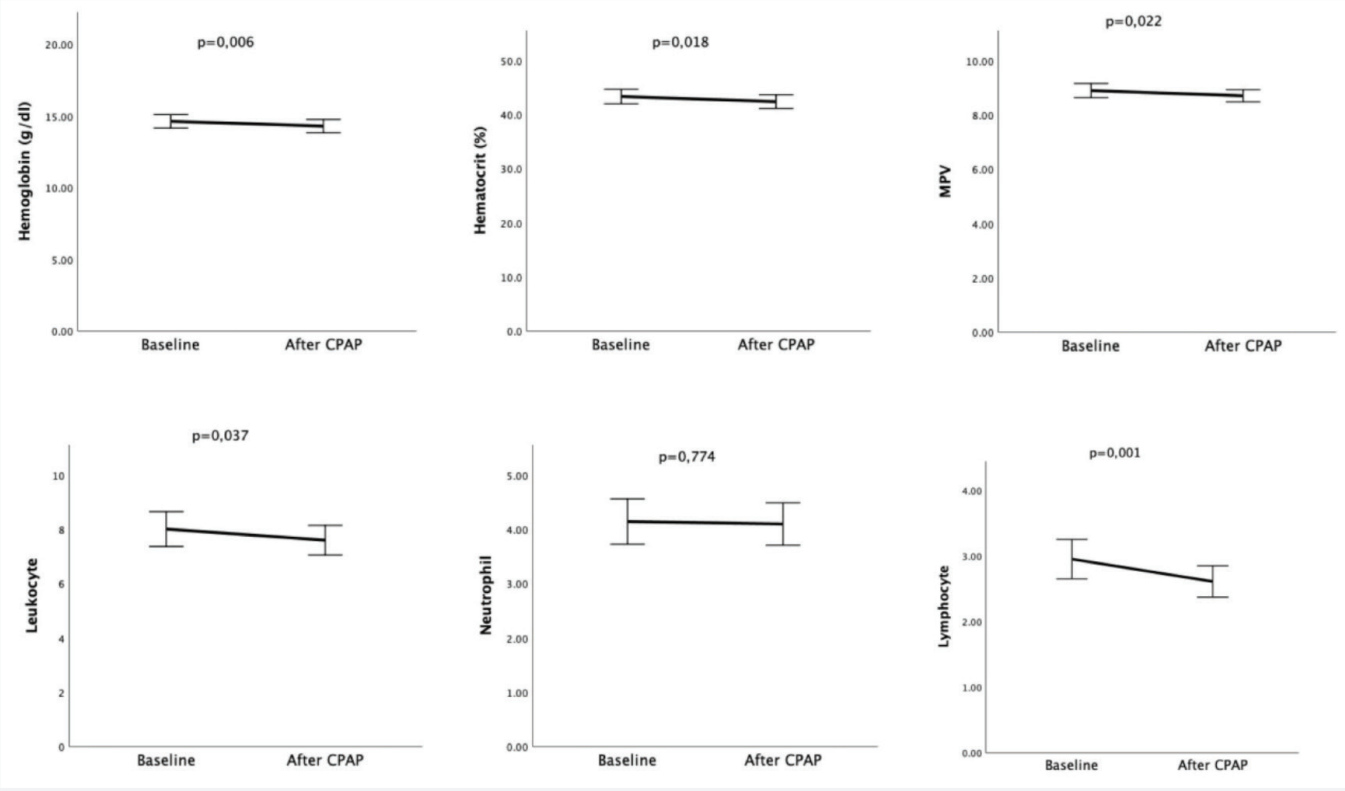

Figure 1. After three months of CPAP treatment, the mean levels of hemoglobin, hematocrit, MPV, leukocytes, neutrophils, and lymphocytes

MPV: Average volume of platelets, CPAP: Continuous positive airway pressure

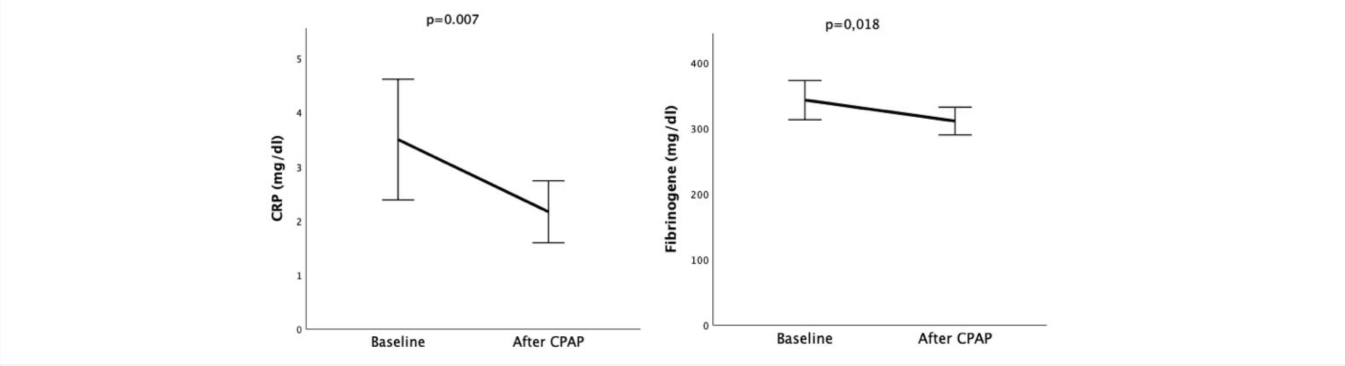

Figure 2. After three months of CPAP treatment, the mean levels of CRP and fibrinogen levels

CRP: C-reactive protein, CPAP: Continuous positive airway pressure

presence of comorbidities such as hypertension and stroke and were improved after CPAP treatment[25]. However, a recent randomized and placebo-controlled crossover trial of OSAS treatment with CPAP did not determine any significant treatment effects on elevated plasma fibrinogen levels[26]. There are inconsistencies between studies on the effect of CPAP therapy on fibrinogen levels in patients with OSAS. This may be because patients with cardiovascular diseases were not excluded from the study in some studies. Our results showed a significant decrease in fibrinogen levels after three months of CPAP treatment.

CRP is an acute-phase protein and plays an important role in innate immunity. It is a sensitive marker of inflammation and an important marker of future cardiovascular risk[27]. Previous studies have presented results, thus denoting the possible beneficial role of CPAP in reducing systemic inflammation and cardiovascular risk in OSAS patients[28-31]. This study also demonstrated that the appropriate use of CPAP therapy could significantly decrease the levels of CRP.

Study Limitations

Our study has some limitations. First, the study was retrospective. And then, our sample size was relatively small. Third, although some hematological indices were considered inflammatory markers, such as NLR and PLR, it was supposed to use classical established inflammatory markers like IL-6 as a reference for comparison during the detection process.

CONCLUSION

Hematological indices are comparatively simple, inexpensive, and practical severity markers of OSAS. Our study has established the importance of hematological evaluation as a complementary tool for diagnosis and treatment response in OSAS patients.

Ethics

Ethics Committee Approval: This study was approved by the Local Ethics Committee of Tekirdağ Namık Kemal University Faculty of Medicine with the number: 2019.160.09.20 (date: 24.09.2019).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: L.C.M., Concept: L.C.M., Design: L.C.M., Data Collection or Processing: M.Y., Analysis or Interpretation: L.C.M., Literature Search: M.Y., Writing: M.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.18894
Nam Kem Med J 2023;11(1):54-60

Internet Addiction, Psychosocial Variables and Perceived Social Support in University Students

Üniversite Öğrencilerinde İnternet Bağımlılığı, Psikososyal Değişkenler ve Algılanan Sosyal Destek

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ABSTRACT

Aim: The aim of this study was to examine socio-demographic data and perceived social support that predict internet addiction in university students.

Materials and Methods: Randomly selected 399 university students living in Istanbul, the data collection tools were delivered over the internet, were included.

Results: The frequency of internet use has been determined that 31.83% of the participants use the internet for five hours or more a day, and 72.18% use the internet seven days a week. It was determined that there was a significant difference between the internet addiction level of the participants and their age (p=0.010) and marital status (p=0.017). No significant difference was found between marital status and perceived social support level (p=0.845). It was determined that the level of perceived social support had a negative and significant effect on internet addiction at the level of -0.199 (p=0.000).

Conclusion: The results of our study indicate that as the perceived social support level in university students increases, the level of internet addiction decreases. Studies are needed to determine the mediating factors between perceived social support and internet addiction.

Keywords: Internet addiction, problematic internet use, perceived social support

ÖZ

Amaç: Bu çalışma ile üniversite öğrencilerinde internet bağımlılığını yordayan bazı sosyo-demografik veriler ile algılanan sosyal desteğin incelenmesi amaçlanmıştır.

Gereç ve Yöntem: Araştırmaya hazırlanan veri toplama araçlarının internet üzerinden ulaştırıldığı, rastgele seçilmiş, İstanbul'da yaşayan 399 üniversite öğrencisi dahil edilmiştir. Katılımcılara Sosyo-demografik Veri Formu, Young İnternet Bağımlılık Ölçeği, Çok Boyutlu Algılanan Sosyal Destek Ölçeği uygulanmıştır.

Bulgular: İnternet kullanma sıklıklarına baktığımızda katılımcıların %31,83'ünün günde beş saat ve üzerinde, %72,18'inin haftada yedi gün internet kullandıkları tespit edilmiştir. Katılımcıların internet bağımlılık düzeyi ile yaş (p=0,010) ve medeni durumları (p=0,017) arasında anlamlı bir farklılık olduğu tespit edilmiştir. Medeni durum ile algılanan sosyal destek düzeyi (p=0,845) arasında ise anlamlı bir fark saptanmamıştır. Algılanan sosyal destek düzeyinin internet bağımlılığı üzerinde -0,199 düzeyinde negatif yönlü anlamlı etkiye sahip olduğu tespit edilmiştir (p=0,000).

Sonuç: Çalışmamızın sonuçları üniversite öğrencilerinde algılanan sosyal destek düzeyi arttıkça internet bağımlılığı düzeyinin düştüğüne işaret etmektedir. Algılanan sosyal destek ve internet bağımlılığı arasındaki aracı faktörlerin belirlenmesine yönelik çalışmalara ihtiyaç bulunmaktadır.

Anahtar Kelimeler: İnternet bağımlılığı, problemli internet kullanımı, algılanan sosyal destek

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Received: 15.01.2023 **Accepted:** 24.01.2023

INTRODUCTION

The main purpose of the occurrence of the internet around the world is to increase communication. The fact that the Internet is an addictive tool was put forward in a study conducted by Goldberg[1] in 1996. Which was seen to have entered the international literature for the first time with the concept of "internet addiction", was later named with different concepts by different clinicians and researchers[2,3]. In recent years, problematic internet use has become a preferred concept in the literature[4]. In the studies performed, use for longer than 5 to 6 hours a week and for relatively long continuous periods of time are generally taken into account. Long-term use may be related to the craving for internet and/or the need to connect to the internet in case of negative emotions such as loneliness and sadness[5].

When the psychosocial factors related to the excessive use of internet are examined, loneliness appears as a research area. Chou and Hsiao[6] stated in a study they conducted that increases in internet usage rates reduce the time needed to be devoted to real social relationships and face-to-face relationships, cause social isolation, and increase loneliness. Hamburger and Ben-Artzi[7], on the other hand, emphasized that internet addiction did not increase loneliness levels and that internet addiction emerged as a result of loneliness. Social support can be briefly expressed as helping individuals by the people around them. In situations such as crisis and emotional tension, individuals need to rely on family members, friends and surroundings, who are seen as natural helpers[8,9]. Individuals whose social support needs are adequately met feel safe and have good goals. The students who cannot get the necessary social support from their environment try to fill the support gap with other methods. One of the important variables is perceived social support in the literature on internet addiction. People can seek support in the virtual world via the internet[10].

Young[11], created the first diagnostic criteria for internet addiction, argued that internet created addiction like gambling and that internet addicts had some symptoms of impulse control disorder. Pathological Internet users had "behavioral impulse control disorder" and this impulse control disorder did not involve the intake of a chemical substance. Therefore, Young[11] adapted the "pathological gambling diagnostic criterion" that best fits this definition to pathological internet use. Thus, he created and published the first serious diagnostic criteria for Internet addiction.

Many studies emphasized that the internet is a stand-alone action to improve people's social networks[12]. Individuals with high social anxiety levels, who think that communication in the virtual environment carries less risk than face-to-face communication, spend more time on the internet[13]. Sanders et al.[14], examined whether high levels of internet use were associated with social loneliness and depression in high school students, and it was found that high internet use was associated with weak social ties. However, the direction of the relationship could not be determined. In addition, the relationship between the level of internet use and depression is significant[15].

In this context, there is a need to examine the internet usage patterns of young people, who are thought to be an important risk group for problematic internet use. The aim of this study is to obtain information about the frequency and purpose of internet use among university students, to determine the relationships between internet addiction and social support. Another aim of the study is to determine the relationships between internet addiction and demographic variables such as gender, age and marital status.

MATERIALS AND METHODS

This study was designed in the general screening model based on quantitative data. The scale form prepared for the research was randomly selected and sent via e-mail to 500 university students living in Istanbul, 399 of whom participated in the research by filling out this form sent to them. Being under the age of 18 years, not being a university student and having any mental illness were determined as exclusion criteria. Ethics committee approval of the study (no: 021/2017, date: 11.01.2017) was received from Nişantaşı University, Social Sciences Institute, Social and Human Sciences Ethics Committee. The study was conducted in accordance with the Declaration of Helsinki and informed consent was obtained from the participants.

Data Collection Methods

Socio-demographic Data Form

It was created by the researcher to obtain information about the demographic characteristics of the participants, the frequency and purpose of internet use.

Young's Internet Addiction Scale

A "diagnostic questionnaire" was created by Young[11] by adapting the Pathological Gambling Criteria of the DSM-IV, then it was developed and turned into a 20-question self-report scale. In the questionnaire consisting of Likert type questions, one of the options "never", "rarely", "occasionally", "often", "very often" and "continuously" is required to be marked. Scoring is done as 0, 1, 2, 3, 4, and 5, respectively. A total score of 80 or more is defined as "internet addiction". A score between 50 and 79 is defined as "risky internet use", and those with a score of 49 and below are defined as an "average internet user" who does not have problems related to internet use. The scale was

adapted to Turkish by Bayraktar[16], and the standardized Alpha value is 0.91, the Spearman-Brown value is 0.87.

Multidimensional Scale of Perceived Social Support

The multidimensional scale of perceived social support was developed by Zimet et al.[17], as an easy-to-use, short scale, subjectively evaluating the adequacy of social support from three different sources. The scale consists of 12 items and includes 3 subgroups, each of which consists of 4 items, related to the source of the support. In the subgroups; "family" (items 3, 4, 8 and 11), "friend" (items 6, 7, 9 and 12) and "special person" (items 1, 2, 5 and 10) group. Each item was rated using a 7-point scale. Each item is scored between 1 and 7. The subscale score is obtained by adding the scores of the 4 items in each subscale, and the total scale score is obtained by adding all the subscale scores.

Statistical Analysis

In this study, the quantitative data obtained from both groups were analyzed with the Statistical Package for the Social Sciences 23 package program. Frequency, percentage and mean values were given for the demographic characteristics of the participants. Normality test was performed to see whether the measurement variables were normally distributed. The Kruskal-Wallis H and Mann-Whitney U tests were conducted to determine whether demographic characteristics differed from perceived social support sub-levels and internet addiction levels. In the last part, multivariate regression analysis was performed to determine the predictive effect of perceived social support level on internet addiction.

RESULTS

The participants were determined that 52.63% were female, 44.11% were between the ages of 21 and 24 years, and 94.74% were single.

83.71% of the participants had been using the internet for 5 years or more. 31.83% of them used the internet for 5 hours or more a day, and 72.18% of them used the internet 7 days a week.

When the participants' internet usage purposes were examined, it was detected that 41.60% used internet sometimes for homework, 37.34% mostly for research, 30.08% mostly for movies, 35.09% never for games, 40.10% always for music, and 44.61% always to establish social communication (mail, msn, etc.).

Considering the websites used by the participants, it was observed that 75.69% of them used social media (Twitter, Facebook), 57.14% of them used movie/music sites, 5.51% of them used adult sites and 8.77% of them used chat sites.

There was no significant difference between gender and family support level (p=0.169), friend support level (p=0.315), a special one's support level (p=0.528), perceived social support level and internet addiction level (p=0.790).

There was no significant difference between age and family support level (p=0.598), friend support level (p=0.355), a special one's support level (p=0.407) and perceived social support level (p=0.619). A significant difference was found between age and internet addiction level (p=0.010).

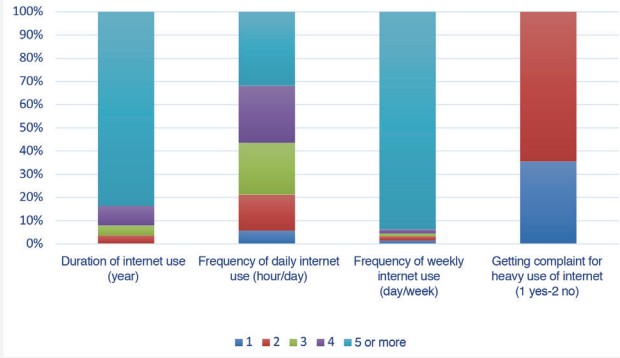

Figure 1. Internet usage frequency of participants

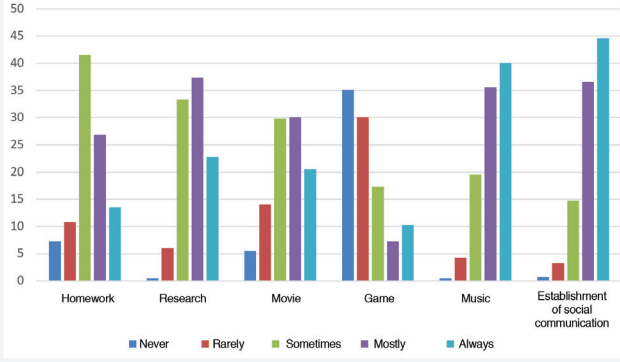

Figure 2. Distribution of participants' internet usage purposes (%)

Table 1. Distribution of demographic characteristics

		f	%
Age	18-20	146	36.59
	21-24	176	44.11
	25 years and over	77	19.30
Gender	Female	210	52.63
	Male	189	47.37
Marital status	Single	378	94.74
	Married	20	5.01
	Divorced	1	25

A significant difference was found between marital status and internet addiction level (p=0.017). Single individuals [$\bar{x}$=29.92; standard deviation (SD)=16.64] were found to have a higher level of internet addiction than married individuals ($\bar{x}$=22.52; SD=16.03).

As a result of the regression analysis performed to determine the effect of perceived social support level on internet addiction, it was revealed that the perceived social support level had a negative significant effect on internet addiction at the level of -0.199 (p=0.000). While the regression model, in which the perceived social support level is the independent variable, was found to be significant (F=16.354, p=0.000), it was revealed that this model explained 40% of the change in the level of internet addiction.

DISCUSSION

The results of our study indicate that there is a significant difference between the level of internet addiction and age and marital status in university students, and as the level of perceived social support increases, the level of internet addiction decreases. In the literature, there are many studies addressing the differences in the frequency and purpose of internet use between biological gender[18]. According to the findings of our study, gender does not affect internet addiction and perceived social support level. However, in the study conducted by Esen and Gündoğdu[19], it was determined that internet addiction scores varied according to gender, and women's internet addiction scores were lower than men. Esen and Siyez[20] remarked that the variables of gender, academic achievement, loneliness and perceived social support from the family predicted internet addiction in adolescents. While some of the studies in the literature, in parallel with the results obtained in the research, report that men are more internet addicted than women[21], some of them show that internet addiction does not show a significant difference according to gender[22]. Karasu et al.[23] examined the relationship between internet addiction of university students and social support and a statistically significant difference was found between the gender status of the students and the mean scores of internet addiction. The mean scores of internet addiction in male students were found to be statistically significant compared to female students. Different results in current studies may be due to the way of measuring internet addiction and/or variables such as cultural differences.

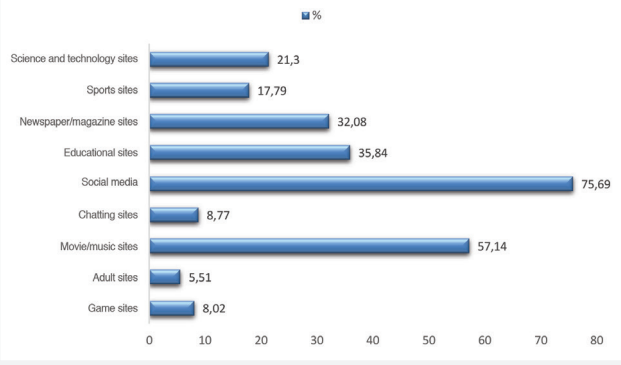

Figure 3. Rates of participants following different website content

According to the results of our study, as age increases, the level of internet addiction decreases. The studies emphasize that the majority of internet users include young adults, especially between the ages of 18 and 24 years. It should be noted that the majority of university students are in this age range[24,25]. Litwin and Landau[26] found that friendship networks decrease as age increases, family networks increase as education level decreases, and friendship networks increase as education level increases. In another study, it was found that there was a statistically significant difference between age and the perception of social support, the support of friends decreased as the age progressed, and the highest level of support from the family was perceived at the beginning of adolescence[27]. The results of the systematic review by Blasco et al.[28] indicate

Table 2. Comparison of the relationship between social support sub-levels and internet addiction by gender

Social support sublevels	Gender	N	Mean	Standard deviation	U	z	p
Family support level	Female	210	22.69	5.92	18280.500	-1.375	0.169
	Male	189	21.70	6.43			
Friend support level	Female	210	22.58	5.89	18701.500	-1.006	0.315
	Male	189	21.49	6.83			
Someone's special support level	Female	210	22.67	5.96	19133.500	-0.631	0.528
	Male	189	21.76	6.91			
Perceived social support level	Female	210	67.94	15.31	18353.500	-1.299	0.194
	Male	189	64.96	17.74			
Internet addiction level	Female	210	28.69	14.45	19539.500	-0.266	0.790
	Male	189	30.48	18.82			

Mann-Whitney U test, correlation significant at p<0.05 significance level

that internet addiction increases in new generations, and that the increase in individuality and the decrease in socialization and acculturation play a role in this result.

In our study, there was a significant difference between marital status and internet addiction level. It has been determined that single individuals have a higher level of internet addiction than married individuals. In the study of Jovic et al.[29], individuals who were married or had a partner and those who did not have a partner differed in terms of the duration and purposes of internet use. Individuals living with their partners mostly used the internet for leisure activities such as playing games as well as browsing pictures and music. While single participants spent more than 8 hours on the internet, participants who were more prone to addictive activities (playing games) were those living with their partners. In the study of Karasu et al.[23], no statistically significant difference was found between the department, class, age, family type, marital status, place of residence, mother's education, father's education, father's occupation, mother's occupation, and family income and the mean scores of internet addiction. In a study conducted by Sancar[30] on internet addiction in women, it was detected that the most frequent internet use was among engaged women.

Table 3. Comparison of the relationship between social support sub-levels and internet addiction by age groups

Social support sublevels	Age	N	Mean	Standard deviation	X^2	p
Family support level	18-20	146	22.16	6.24	1.027	0.598
	21-24	176	22.44	6.26		
	25 and over	77	21.84	5.92		
Friend support level	18-20	146	21.75	6.17	2.069	0.355
	21-24	176	22.31	6.40		
	25 and over	77	22.09	6.70		
Someone's special support level	18-20	146	21.82	6.42	1.799	0.407
	21-24	176	22.41	6.62		
	25 and over	77	22.66	6.07		
Perceived social support level	18-20	146	65.73	16.65	0.958	0.619
	21-24	176	67.15	16.71		
	25 and over	77	66.60	16.16		
Internet addiction level	18-20	146	31.66	17.48	9.278	0.010
	21-24	176	29.88	16.18		
	25 and over	77	24.73	15.39		

Kruskal-Wallis H test, correlation significant at $p<0.05$ significance level

Table 4. Comparison of the relationship between social support sub-levels and internet addiction according to marital status

Social support sublevels	Marital status	N	Mean	Standard deviation	X^2	p
Family support level	Single	378	22.24	6.14	3885.500	0.870
	Married	21	21.90	6.96		
Friend support level	Single	378	22.14	6.27	3704.000	0.602
	Married	21	20.67	7.93		
Someone's special support level	Single	378	22.25	6.41	3881.500	0.862
	Married	21	22.00	7.07		
Perceived social support level	Single	378	66.63	16.40	3868.500	0.845
	Married	21	64.57	19.52		
Internet addiction level	Single	378	29.92	16.64	2739.000	0.017
	Married	21	22.52	16.03		

Kruskal-Wallis H test, correlation significant at $p<0.05$ significance level

Table 5. Perceived social support level and internet addiction

Model	Beta	Standard error	Beta	t	p
(Stable)	42.857	3.395	-	12.624	0.000
Perceived social support level	-0.200	0.050	-0.199	-4.044	0.000

It was stated that 93% of the engaged ones used the internet every day. The second most common internet user group includes married women. It is observed that widowed/divorced women have access to the internet at a high rate of 82% every day. Less frequent use is observed to be quite low in all groups. Different results of studies showing the relationship between internet addiction levels according to marital status may be due to mediating factors such as marital satisfaction or intercultural differences.

According to the results of our study, the increase in perceived social support level in university students reduces the level of internet addiction. An increasing number of supporting groups are formed on the Internet. However, social networks play an important role in creating the perception of social support[31]. Similarly, Joinson[32] (1999) stated that the internet provided adolescents with the opportunity to establish new social relationships and adolescents who could not develop appropriate coping methods to solve the problems in family relationships preferred the internet to meet their needs for establishing close relationships. In another study, it was observed that as peer pressure levels decreased, the level of internet addiction of adolescents also decreased[21]. In addition, it was observed that as family and teacher support increased, internet addiction scores decreased. In the study conducted by Gunuc and Dogan'ın[33] on adolescents, it was observed that adolescents who spent time with their mothers had higher perceived social support and lower internet addiction. Similarly, Karaer and Akdemir[34] emphasized the importance of improving parenting, social support and emotion regulation in the prevention and treatment of internet addiction in adolescents. Also, Naseri et al.[35] stated that university students with low self-esteem were more vulnerable to internet addiction.

It is possible to say that the virtual communication environment is perceived as an environment where social relations are less risky and easier, together with the increasingly widespread use of the internet[36]. This turns the internet into one of the resources where individuals can easily find support from others. In a period when the Internet penetrates into every field of daily life and almost the real and virtual worlds compete with each other, it is seen that individuals can postpone their face-to-face relations and put the Internet in the first place among the resources they provide social support[37]. The fact that virtual social support, which is seen to be provided via the Internet, cannot be transformed into permanent relationships in real life also causes social problems, while dissatisfaction in social relations can increase the orientation to the virtual world and create a vicious circle[38]. In the study of Chou and Hsiao[6], it was found that the increase in internet use reduced the time devoted to real social relations and face-to-face relations, and caused social isolation; it was also detected that such people increased their loneliness. Hamburger and Ben-Artzi[7] found that internet addiction did not increase the level of loneliness, and that internet addiction emerged as a result of loneliness. Cui and Chi[39] revealed that the rate of internet addiction was high in students with low perceived social support and social support level, and low social support was among the risk factors for internet addiction. In addition to studies showing a negative relationship between perceived social support and internet addiction, there are also studies indicating that the internet improves social networking and increases social interaction and support[40,41]. Our study also indicates that there is a negative relationship between perceived social support and internet addiction among university students.

Study Limitations

The limitation of this study is that the sample was not screened for psychiatric symptomatology. Having a diagnosis of any mental illness was determined as an exclusion criterion. The results of the research are limited to the measurement tools used.

CONCLUSION

The results of our study indicate that as the perceived social support level of university students increases, the level of internet addiction decreases. Studies are needed to determine the mediating factors between perceived social support and internet addiction.

Ethics

Ethics Committee Approval: Ethical approval was received from the Social and Human Sciences Ethics Committee of Nişantaşı University (no: 021/2017, date: 11.01.2017).

Informed Consent: The study was conducted in compliance with the principles of Declaration Helsinki. Informed consent was obtained from all individual participants included in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: M.M., S.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.20981
Nam Kem Med J 2023;11(1):61-65

Comparison of Anastomosis Evaluation Techniques Before Ileostomy Closure in Rectal Cancer Patients

Rektal Kanser Hastalarında İleostomi Kapatılması Öncesi Anastomoz Değerlendirme Tekniklerinin Karşılaştırılması

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ABSTRACT

Aim: Postoperative ileus, stricture, abscess, and sepsis can be prevented by ensuring that there is no deterioration in the integrity of the anastomosis before closure of the protective loop ileostomy for rectal cancer. The aim of this study is to investigate which technique is more appropriate to evaluate the anastomosis before ileostomy closure.

Materials and Methods: Between 2011 and 2019, patients who underwent elective low anterior resection for rectal cancer and had a concomitant protective loop ileostomy were reviewed retrospectively. The patients included in the study were divided into 2 groups as those whose anastomosis evaluation was performed with digital rectal examination (DRE) alone and those who underwent flexible endoscopy (FE) with DRE.

Results: Ninety-nine patients were included in the study. Sixty-one of the patients were male and 38 were female. The mean age of the patients was 59.36±11.47 years. In the preoperative period, DRE+FE was applied to 67 patients and only DRE to 32 patients. Complications were detected in 10 patients after ileostomy closure (stricture and ileus in 6 patients, anastomotic leakage in 3 patients, and surgical site infection in 1 patient). Of 89 patients without complications, 66 were in the DRE+FE group and 23 were in the DRE group ($p<0.001$).

Conclusion: In order to minimize the complications related to the anastomosis, it is recommended to evaluate together with both DRE and FE, although the appropriate examination in the evaluation of anastomosis is still not clear before the protective loop ileostomy is closed.

Keywords: Rectal cancer, ileostomy reversal, endoscopy, digital rectal examination

ÖZ

Amaç: Rektum kanseri nedeniyle açılan koruyucu loop ileostominin kapatılmasından önce anastomoz bütünlüğünde bozukluk olmadığından emin olunması sayesinde postoperative ileus, striktür, abse ve sepsisten korunma sağlanabilmektedir. Bu çalışmanın amacı ileostomi kapatılmasından önce hangi tekniğin anastomozu değerlendirmede daha uygun olduğunun araştırılmasıdır.

Gereç ve Yöntem: 2011-2019 tarihleri arasında rektum kanseri nedeniyle elektif aşağı anterior rezeksiyon yapılmış ve eş zamanlı koruyucu loop ileostomi açılmış hastalar retrospektif olarak tarandı. Çalışmaya dahil edilen hastalar anastomoz değerlendirmesi sadece dijital rektal muayene (DRE) ile yapılanlar ve DRE ile birlikte fleksibl endoskopi (FE) yapılanlar olarak 2 gruba ayrıldı.

Bulgular: Çalışmaya 99 hasta dahil edildi. Hastaların 61'i erkek ve 38'i kadın idi. Hastaların yaş ortalaması 59,36±11,47 idi. Preoperatif dönemde 67 hastaya DRE+FE, 32 hastaya sadece DRE yapılmıştı. İleostomi kapatılması sonrası 10 hastada komplikasyon geliştiği tespit edildi (6 hastada striktür ve ileus, 3 hastada anastomoz kaçağı ve 1 hastada cerrahi alan enfeksiyonu). Komplikasyon izlenmeyen 89 hastanın 66'sının DRE+FE grubunda olduğu ve 23'ünün DRE grubunda olduğu görüldü ($p<0,001$).

Sonuç: Anastomoza bağlı komplikasyonları minimalize edebilmek için koruyucu loop ileostomi kapatılmadan önce anastomoz değerlendirmesinde uygun tetkik halen net olmamakla birlikte hem DRE hem de FE ile birlikte değerlendirilme yapılması önerilmektedir.

Anahtar Kelimeler: Rektum kanseri, ileostomi kapatılması, endoskopi, dijital rektal muayene

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Received: 13.01.2023 **Accepted:** 25.01.2023

INTRODUCTION

The risk of anastomotic leakage in patients with rectal cancer who underwent lower anterior resection (LAR) is between 5% and 25% and it may lead to serious complications such as abscess, fistula, and sepsis[1,2]. It is also associated with a prolonged hospital stay, an increase in postoperative mortality, and an increased risk of local recurrence[3-5]. Opening a protective loop ileostomy after LAR is a widely used and evidence-based routine[6]. In this way, with the proximal fecal diversion provided, possible complications due to postoperative anastomotic leakage are tried to be prevented. Although anastomotic leakage cannot be completely prevented with a protective loop ileostomy, it greatly reduces the incidence and impact of a clinical leak, thus eliminating the need for re-surgical or interventional intervention[7,8].

Before closing the ileostomy, it should be ensured that there is no asymptomatic anastomotic leak or stricture. The most commonly used methods to evaluate anastomosis are digital rectal examination (DRE), rigid or flexible endoscopy (FE), and contrast enema radiography. However, there is no clear consensus on which method is the most appropriate for the evaluation of colorectal anastomosis[9,10]. The necessity of contrast enema radiography in the routine evaluation of anastomotic integrity is controversial[11,12]. In a prospective study that included a group of patients evaluated with DRE after contrast enema radiography, it was reported that DRE had a sensitivity of 98.4% in detecting anastomotic pathology[13].

The aim of our study is to evaluate the differences between patients who had only DRE before closure of the protective loop ileostomy and those who had FE with DRE.

MATERIALS AND METHODS

Patients who underwent elective LAR due to rectal cancer in the general surgery clinics of University of Health Sciences Turkey, Gülhane Training and Research Hospital and Ankara University Faculty of Medicine between 2011 and 2019 and who had simultaneous protective loop ileostomy were screened retrospectively. Patient information was accessed via computer and file system. Those who had urgent surgery, who did not have the diagnosis of malignancy, whose DRE and/or FE findings and pathological diagnosis information could not be reached, those who were symptomatic at the stage of ileostomy closure, those whose anastomosis assessment (DRE/DRE+FE) was performed earlier than the 2-week period prior to ileostomy closure surgery were excluded from the study. The patients included in the study were divided into 2 groups, as those whose anastomosis evaluation was performed with DRE only and with DRE+FE.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences version 22.00. The Kolmogorov-Smirnov and Levene tests were performed for homogeneity and normality analysis of the scaled data. Since it was a two-group study, the Pearson chi-square and Fisher's exact tests were used in the evaluation of categorical data. The Student's t-test was used for scaled parametric data, and the Mann-Whitney U test for scaled non-parametric data. Binary logistic regression test was employed in one-way analysis of variance. $P<0.05$ was considered significant.

RESULTS

Ninety-nine patients were included in the study. 61.6% (n=61) of the patients were male and 38.4% (n=38) were female. The mean age of the patients was 59.36±11.47 years. In the preoperative period, DRE+FE was applied to 67 patients and only DRE was applied to 32 patients. Forty-four patients were operated after neoadjuvant therapy. Complications developed in 10 (10.1%) patients after ileostomy closure. Anastomotic leakage was observed in 3 patients, stricture and ileus in 6 patients, and surgical site infection in 1 patient. The demographic and clinicopathological characteristics of the patients are shown in Table 1 in detail.

In the examination of relationship between postoperative complications and clinicopathological data, anastomosis complication was observed in 2 patients who received neoadjuvant therapy, while no complication developed in 8 patients (p=0.017). This shows us that neoadjuvant therapy is not a risk factor for the development of anastomotic complication. In the examination of relationship between perineural invasion (PNI) and anastomosis complication,

anastomosis complication was found in 7 of 85 patients without PNI and in 3 of 4 patients with PNI (p=0.003). The number of dissected lymph nodes was similar between the group with and without complications (17.52±7.85 vs 17.80±7.92). No significant correlation was found between the stage of rectal cancer and the development of anastomotic complications (p=0.214) (Table 2).

Considering the relationship between only DRE and DRE+FE and anastomosis complications, it was seen that 66 of the 89 patients without complications were in the DRE+FE group and 23 were in the DRE group (p<0.001) (Table 3). A significant correlation was found between DRE+FE and the rate of postoperative anastomosis complications. This relationship is negative, it shows that the rate of postoperative anastomosis complications is statistically lower in patients who underwent DRE+FE (Odds ratio=0.039, 95% confidence interval: 0.005-0.323, p=0.003) (Table 4).

Table 1. Demographic characteristics of the patients	
Age, year, (mean±SD, distribution)	59.36±11.47 (31-81)
Gender, n (%)	
Male	61 (61.6%)
Female	38 (38.4%)
Neoadjuvant therapy, n (%)	
No	44 (44.3%)
Yes	55 (55.6%)
Type of surgery, n (%)	
Laparoscopic	57 (57.6%)
Open	42 (42.4%)
Preoperative DRE vs DRE+FE, n (%)	
DRE	32 (32.3%)
DRE+FE	67 (67.7%)
Complication after anastomosis, n (%)	
No	89 (89.9%)
Yes	10 (10.1%)
Complication type, n (%)	
Anastomotic leak	3 (3%)
Stricture, ileus	6 (6.1%)
Surgical site infection	1 (1%)
Management of complication, n (%)	
Percutaneous drainage	2 (2%)
Dilation	5 (5.1%)
Re-laparotomy	2 (2%)
Conventional	1 (1%)
LVI, n (%)	
No	51 (51.5%)
Yes	38 (48.5%)
PNI, n (%)	
No	92 (92.6%)
Yes	7 (7.1%)
Lymph node dissection, n (mean±SD, distribution)	17.77±7.81 (6-53)
Lymph node metastasis, n (mean±SD, distribution)	1.84±3.64 (0-24)
N Stage grade, n (%)	
N0	56 (56.6%)
N1	25 (25.3%)
N2	18 (18.2%)
T Stage grade, n (%)	
Tis	16 (16.2%)
T1	6 (6.1%)
T2	28 (28.3%)
T3	47 (47.5%)
T4	2 (2%)
Stage, n (%)	
Stage 0	15 (15.2%)
Stage 1	27 (27.3%)
Stage 2	14 (14.1%)
Stage 3	43 (43.4%)

SD: Standard deviation, LVI: Lymphovascular invasion, PNI: Perineural invasion, DRE: Digital rectal examination, FE: Flexible endoscopy

DISCUSSION

In this study, the complication rate was found to be significantly lower in the group evaluated with DRE+FE before ileostomy closure, compared to the group evaluated with DRE alone.

Ostomies opened for diversion play an important role in temporarily protecting anastomoses and minimizing peritoneal sepsis. Optimizing the timing of temporary stoma closure and evaluating anastomotic integrity prior to stoma closure are associated with minimizing major complications. The most appropriate method to evaluate the integrity of the anastomosis before the closure of the protective loop ileostomy opened with the LAR still remains unclear. Karsten et al.[14], in their retrospective study, showed that DRE and rigid sigmoidoscopy were sufficient to detect significant pathology. In a retrospective study comparing the use of FE and contrast enema in the evaluation of preoperative anastomotic integrity in rectal cancer patients, Lindner et al.[10] found endoscopic evaluation to be superior to contrast enema. In a review from the same group, when endoscopic procedure and DRE versus contrast enema evaluation were compared, it was reported that DRE and endoscopic method were the best methods for evaluating anastomotic integrity in rectal cancer patients[15].

When we examined the anastomosis complication relationships in our study, it was found that anastomotic complications were statistically significantly lower in the DRE+FE group. Complications were seen in 10 of our patients after anastomosis, and only DRE was performed in 9 of them before the ileostomy was closed. In the light of our findings, it was observed that postoperative complication rates decreased significantly thanks to FE performed together with DRE. Stricture and ileus were observed in 60% of patients who developed complications. Considering that most of the complications are only in the DRE group, it may be possible to prevent stricture and ileus that may occur in the early postoperative period and to prevent anastomotic separation by opening the fibrotic bands in the anastomosis line in the early period with FE.

When we assessed the relationship between neoadjuvant therapy and anastomosis complications in our study, it was observed that the rate of anastomosis complications was lower in patients who received neoadjuvant therapy (18.18% vs. 3.63%). Although there are studies showing that neoadjuvant therapy increases the risk of anastomotic leakage in patients who were operated with the diagnosis of rectal cancer, there are also studies claiming the opposite[16-20].

When the relationship between rectal cancer stage and complications after ileostomy closure was examined, it was observed that the complication rate was higher in stage 3 patients, but no statistically significant relationship was found between the stage and the complication rate. Different results

Table 2. Distribution of clinicopathological factors according to postoperative anastomosis complication groups

Clinicopathological factors	Number of patients (%)		p value
	Complication (-) (89 patients)	Complication (+) (10 patients)	
Age, year (mean±SD)	59.46±11.10	58.50±15.09	p=0.803†
Gender, n			
Male	56	5	p=0.426‡
Female	33	5	
Neoadjuvant therapy, n			
No	36	8	**p=0.017‡**
Yes	53	2	
Surgery type, n			
Laparoscopic	52	5	p=0.609‡
Open	37	5	
LVI, n			
No	46	5	p=0.919‡
Yes	43	5	
PNI, n			
No	85	7	**p=0.003‡**
Yes	4	3	
Lymph node dissection, n (mean±SD)	17.52±7.85	17.80±7.92	p=0.914†
Lymph node metastasis, n (median, distribution)	0 (0-17)	1.50 (0-24)	p=0.217§
N Stage grade, n			
N0	52	4	p=0.474‡
N1	22	3	
N2	15	3	
T Stage grade, n			
Tis	16	0	p=0.083‡
T1	6	0	
T2	27	1	
T3	38	9	
T4	2	0	
Stage, n			
Stage 0	15	0	p=0.214‡
Stage 1	26	1	
Stage 2	11	3	
Stage 3	37	6	

SD: Standard deviation, LVI: Lymphovascular invasion, PNI: Perineural invasion, ‡χ^2 tests; †Student's t-test; §Mann-Whitney U test

Table 3. Distribution of preoperative digital rectal examination and flexible endoscopy evaluations according to postoperative anastomosis complication groups

Anastomosis evaluation method	Number of patients, n		p value
	Complication (-)	Complication (+)	
DRE	23	9	**p<0.001†**
DRE+FE	66	1	

DRE: Digital rectal examination, FE: Flexible endoscopy, †χ^2 tests

Table 4. One-way analysis of variance of preoperative rectoscopy in the possibility of anastomotic leakage

	One-way analysis of variance			
	B	OR (95% CI)	Accuracy percentage	p value
Preoperative DRE+FE	-3.251	0.039 (0.005-0.323)	89.9%	**0.003**

DRE: Digital rectal examination, FE: Flexible endoscopy, OR: Odds ratio, CI: Confidence interval

have been reported in studies evaluating the relationship between tumor stage and anastomotic complication[21].

Study Limitations

The limitations of our study are the retrospective design and the small number of patients.

CONCLUSION

There is no consensus on the clinical examination that should be performed to ensure the safety of the anastomosis before closing the ileostomy after diverting loop ileostomy surgery with LAR for rectal cancer. In the light of our current knowledge, the recommended examination before ileostomy closure is flexible or rigid rectoscopy with DRE. We recommend conducting large-scale prospective studies to reach clearer results.

Ethics

Ethics Committee Approval: The study was approved by the University of Health Sciences Turkey, Gülhane Training and Research Hospital of Local Ethics Committee (no: E-50687469-799, date: 22.12.2022).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: E.E., Ş.E., C.A., A.B.E., Concept: E.E., A.B.E., Design: E.E., A.B.E., Data Collection or Processing: E.E., Ş.E., Analysis or Interpretation: E.E., Ş.E., Literature Search: E.E., Ş.E., C.A., A.B.E., Writing: E.E., Ş.E., C.A., A.B.E.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.02411
Nam Kem Med J 2023;11(1):66-71

A Study of Changes in Prohepcidin and Iron Levels in Patients with Liver Transplant and Chronic Viral Hepatitis

Karaciğer Naklinde ve Kronik Viral Hepatitli Hastalarda Prohepsidin ve Demir Parametrelerinin Değişiminin Araştırılması

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ABSTRACT

Aim: To study changes in hepcidin, a key protein synthesized in the liver, following chronic viral hepatitis, cirrhosis and/or liver transplant, as well as the effect of hepcidin level variation on liver function tests and iron levels.

Materials and Methods: The patient population was distributed as follows: Group 1: inactive chronic hepatitis B (n=31); Group 2: chronic hepatitis C (n=30); Group 3: decompensated cirrhosis linked to hepatitis C virus (HCV) or hepatitis B virus (HBV) (n=29); Group 4: decompensated HCV- or HBV-related cirrhosis treated by liver transplantation (n=31).

Results: The following characteristics were unequally distributed among the groups: age, Hb, AST, ALP, LDH, T. bil, albumin, total cholesterol, HDL, serum total iron binding capacity, and transferrin saturation (TS). In the two-group comparison of Groups 1 and 2, significant differences in Hb, AST, AP, albumin, and prohepcidin were observed; the latter was more elevated in HCV patients (Group 2) ($p<0.05$). Comparison between Groups 3 and 4 yielded significant differences in Hb, AST, LDH, T. bil, albumin, total cholesterol, serum iron, and TS. Prohepcidin was most elevated in Group 2. Prohepcidin level was positively correlated with ferritin and negatively with albumin and Hb in all study groups. The highest concentration of ferritin was encountered in Group 4 patients, who had undergone liver transplant, followed in decreasing order by Group 3, Group 2 and Group 1; however, no statistically significant difference could be established ($p=0.052$).

Conclusion: In our study, a significantly positive correlation between AST/ALT and prohepcidin levels in patients who had liver transplantation caused by HBV or HCV was established. This finding may be an indicator of inflammation after transplantation.

Keywords: Liver transplant, chronic viral hepatitis, prohepcidin

ÖZ

Amaç: Çalışmamızda, karaciğerde sentezlenen ve demir regülasyonunda anahtar role sahip olan hepsidinin kronik viral hepatit, siroz ve nakil sonrası hepsidin düzeyindeki değişimin karaciğer fonksiyon testleri ve demir parametreleri ile ilişkisini değerlendirmeyi amaçladık.

Gereç ve Yöntem: Hasta popülasyonu, Grup 1: inaktif kronik hepatit B (n=31); Grup 2: kronik hepatit C (n=30); Grup 3: Hepatit C virüs (HCV) veya hepatit B virüse (HBV) bağlı dekompanse siroz (n=29); Grup 4: HCV ya da HBV nedeniyle dekompanse siroz gelişip karaciğer nakli yapılan hastalar (n=31) olarak dağıtıldı.

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Received: 15.01.2023 **Accepted:** 30.01.2023

Presented in: Our study was accepted as an oral presentation at the 1st International Medicine and Therapy Congress, 2-6 October 2019.

Bulgular: Tüm guruplar ele alındığında yaş, cinsiyet, Hb, AST, ALP, GGT, LDH, T. bil; albumin, toplam kolesterol, yüksek yoğunluklu lipoprotein, SDBK ve transferrin satürasyonunda (TS) fark saptandı. Kronik inaktif HBV ile kronik HCV hastaları arasında Hb, AST, ALP, albumin ve prohepsidin düzeyleri arasında anlamlı fark saptanmış olup prohepsidin düzeyi HCV olgularında daha fazla bulundu (p<0,05). Dekompanse siroz hastaları (Grup 3) ile karaciğer Tx (Grup 4) hastaları karşılaştırıldığında Hb, AST, LDH, T. bil, albumin, toplam kolesterol, Fe ve TS arasında anlamlı fark bulundu. Prohepsidin düzeyinin özellikle Kronik HCV olgularında daha yüksek olduğu, prohepsidinin tüm gruplarda ferritin ile pozitif korelasyon içerisinde olduğu, hemoglobin ile negatif korelasyon içerisinde olduğu saptandı. Ferritinin gruplar arası dağılımına bakıldığında en fazla karaciğer nakli yapılan grupta yüksek olduğu, bunu da sırasıyla karaciğer sirozu, HCV grubu ve kronik inaktif HBV hastalarının izlediği görüldü, ancak aralarında istatistiksel olarak anlamlı bir fark saptanmadı (p=0,052).

Sonuç: Çalışmamızda HBV veya HCV'ye bağlı karaciğer nakli yapılan hastalarda AST/ALT ve prohepsidin düzeyleri arasında anlamlı pozitif korelasyon saptandı. Bu bulgu nakil sonrası enflamasyonun göstergesi olabilir.

Anahtar Kelimeler: Karaciğer nakli, kronik viral hepatit, prohepsidin

INTRODUCTION

Studies in recent years have increased our understanding of iron metabolism by identifying new molecules that play a role in iron homeostasis. Hepcidin, a small-molecule peptide hormone, was discovered to take part in the regulation of immunity and inflammation in addition to iron metabolism. This discovery led to an elucidation of the pathogenesis of different types of hereditary hemochromatosis (HH) and changed our pathophysiologic understanding of anemia in inflammation[1,2].

The observation of increased hepcidin synthesis, parallel to increased dietary iron intake, has prompted the thought that hepcidin participates in iron metabolism. Its specific role was investigated in transgenic mouse models by looking for the effects of hepcidin deficiency or excess. Results have indicated that mouse hepcidin is a negative regulator of intestinal iron absorption, placental iron transport and iron secretion by macrophages[3,4]. Hepcidin synthesis is triggered by increases in plasma levels and tissue stores of iron; hepcidin then increases iron release from macrophages and duodenal enterocytes into the plasma. While this homeostasis ensures the maintenance of plasma iron within a stable range, it prevents excessive resorption of iron and its accumulation in the tissue[5]. Hepcidin is a short-living hormone (serum half-life of several minutes) and is subjected to a complex regulation with hypoxia, anemia and iron deficiency, being the major suppressors while inflammation and iron overload are the major inducers[6,7].

Hepcidin expression increases in the case of iron deficiency and decreases with iron overload[5]. Its negative regulation of intestinal iron absorption and iron release by macrophages makes it a direct-acting mediator in the pathogenesis of anemia in chronic diseases[1]. It also has intrinsic antimicrobial activity and inflammation stimulates hepcidin expression. The liver regulates hepcidin secretion according to the presence of excessive iron stores, hypoxia, anemia, and numerous other physiologic situations. In response to such stimuli, a certain number of signals that are not yet well explained, such as transferrin receptor 2, IL-6 receptor, the *HFE* gene and hemojuvelin, intervene in receptor mechanisms that affect hepcidin secretion by the hepatocytes[8,9]. Excess iron induces free radical formation through Fenton's reaction, especially that of highly reactive hydroxyl radicals, leading to lipid, protein and DNA damage. Mitochondrial membranes are sensitive to oxidative stress; mitochondrial dysfunction leads in turn to hepatocyte injury. Hepatic stellate cells are also affected by oxidative stress. Hepatocyte injury may contribute to the transformation of stellate cells into collagen-producing cells, resulting in the development of fibrosis. Reactive oxygen radicals arising from iron excess may also create an inflammatory environment that impairs liver function[10].

The hepatotropic viruses hepatitis B (HBV) and hepatitis C (HCV) are among the main causes of chronic liver disease, progressive liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Even though the mechanisms of liver damage by chronic hepatitis is not entirely understood, excessive iron stores are known to have a role. Different authors have shown the frequent increase of serum ferritin and iron levels and transferrin saturation (TS) in cases of chronic hepatitis B (CHB) and hepatitis C (CHC)[11,12].

MATERIALS AND METHODS

Patients who were followed up at the Gastroenterology and Liver Transplantation Outpatient Clinics of the Department of Gastroenterology and Hepatology, Dokuz Eylül University Faculty of Medicine, from November 2010 to June 2011, were included in the study. The study was approved by Dokuz Eylül University Faculty of Medicine Clinical Research Ethics Committee (date: 29.09.2010, no: 2010/13-21). The patients recruited into the study had CHB or CHC, decompensated cirrhosis of the liver, or had received a liver transplant as a result of either HBV or HCV.

The study population was divided as follows: patients with inactive CHB (n=31); those with CHC (n=30); patients with decompensated cirrhosis developing in the presence of HBV or HCV (n=29); and those with decompensated HCV- or HBV-related cirrhosis treated by liver transplantation (n=31).

Diagnostic criteria were, for HCV infection: Anti-HCV antibody and HCV RNA positive (+), negative (-) Hb surface antigen (HBsAg); for chronic inactive HBV infection: HBsAg (+), anti-HBe (+), HBV DNA (-) and normal liver function test (LFT). Patients receiving antiviral treatment, those diagnosed with iron deficiency anemia, and patients receiving iron replacement therapy were deemed ineligible for the study. Also, those who had other causes of chronic hepatitis, such as Wilson's disease or HH, those with autoimmune, alcoholic or toxic hepatitis and patients with HCC or chronic renal failure were found to be ineligible for the study.

Proprietary ELISA assay kits (Prohepcidin EIA-4644 DRG Diagnostics, DRG Instruments GmbH, Marburg, Germany) were used to determine prohepcidin serum levels.

Determinations of Hb, aspartate aminotransferase (AST), alanine aminotransferase (ALT), AP, GGT, LDH, T. bil, albumin, T. chol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), Fe, serum TIBC, TS and ferritin levels were performed in the institutional biochemistry laboratory; the patients' medical charts were reviewed retrospectively with regard to hepatic disease.

Statistical Analysis

The Statistical Package for the Social Sciences 15.0 statistical package was used for data evaluation. The statistical significance threshold was set at a p value of <0.05. The Kolmogorov-Smirnov test was used for fitness of sample values to normal distribution. In the absence of normal distribution and in cases when one of the groups had less than 30 patients, it was decided to apply non-parametric tests. Descriptive statistics were expressed as the mean and standard deviation for age, Hb, albumin, T. chol, HDL, LDL, Fe, serum TIBC, ferritin and prohepcidin; median and range were reported for the other measurements. Differences among the groups for continuous variables were tested for significance with the Kruskal-Wallis test; whenever such significance was identified, two-group comparisons were performed using the Mann-Whitney U test.

RESULTS

The 83 men and 38 women in the study were aged 18-70 years. The distribution of all recorded characteristics and parameters are shown in Table 1. Significant differences among the groups were found for age, gender, Hb, AST, AP, GGT, LDH, T. bil, albumin, T. chol, HDL, TIBC, and TS.

In the two-group comparison of Groups 1 and 2, a significant difference in Hb, AST, AP, albumin and prohepcidin was observed; prohepcidin was more elevated in Group 2 (HCV patients; p=0.009). Groups 1 (chronic inactive HBV patients) and 3 (decompensated cirrhosis patients) were significantly different in AST, AP, GGT, LDH, T. bil, albumin, T. chol, HDL, TIBC, TS and ferritin levels (p<0.05).

Comparison of Group 1 to Group 4 (liver transplant patients) revealed significant differences in Hb, AP, GGT, T. bil, HDL, Fe, and ferritin (p<0.05). Ferritin was highest in liver transplant patients.

Groups 2 and 4 significantly differed in AST, AP, GGT, T. bil, albumin, T. chol, HDL, TIBC, and TS (p<0.05). Groups 1 and 4 showed differences in Hb, AP, LDH, T. bil, and albumin (p<0.05). Significant differences between Groups 3 and 4 (untransplanted vs. transplanted decompensated cirrhosis) were found in Hb, AST, LDH, T. bil, albumin, T. chol, Fe, and TS (p<0.05).

The distribution of prohepcidin levels among the four groups showed no significant differences, as shown in Table 2, although higher values were seen in Group 2 (cases of CHC). Prohepcidin level was positively correlated with ferritin and negatively with Hb across the four groups. A significant positive correlation was found between AST and ALT values and prohepsidin levels in patients who underwent liver transplantation due to HBV or HCV (p=0.046).

Ferritin levels were positively correlated with those of prohepcidin, Fe, TIBC and T but negatively correlated with albumin. The highest concentrations of ferritin were found in Group 4 patients, who had undergone liver transplant, followed in decreasing order by Group 3 (untransplanted cirrhosis), then Group 2 (HCV) and Group 1 (inactive HBV); however, no statistically significant difference could be established (p=0.052).

DISCUSSION

Numerous studies on excessive iron storage and its relationship with liver damage in chronic hepatic disease have been published; the discovery of prohepcidin has helped elucidate previously unclear aspects of iron metabolism. A study by Aoki et al.[13] on the potential role of prohepcidin in the natural history of chronic hepatitis showed a significant correlation between liver hepcidin mRNA expression and hepatic iron levels and serum ferritin, but not the intensity of liver inflammation and the stage of fibrosis, in CHC patients. These authors also pointed to a cell-mediated immune response in CHC infection, in which the secreted amounts of interferons IL-2, IL-4, IL-10, tumor necrosis factor-alpha, and γ-interferon affect hepcidin mRNA expression, while mRNA levels are not related to hepatic inflammation and correlate with hepatic iron stores and serum ferritin. These observations suggest that liver hepcidin secretion increases in response to iron stores, leading to reduced iron absorption[13]. Iron and related parameters were measured in 14,462 persons by Shan et al.[14]. They indicated that serum ferritin and Fe levels were significantly higher in

Table 1. Distribution of recorded characteristics for patients in four groups

	1. Chronic inactive HBV (n=31)	2. Chronic HCV (n=30)	3. Liver cirrhosis (n=29)	4. Liver transplant (n=31)	p
Gender					
M	61.3%	43.3%	82.8%	87.1%	
F	38.7%	56.7%	17.2%	12.9%	
Age	51.7±2.56	58.8±1.8	58.6±1.4	51.6±1.9	0.009
Hb (mg/dL)	14.6±0.2	12.5±0.3	11.9±0.3	13.3±0.3	0.001
AST	21 (15-37)	27 (13-140)	44 (13-133)	25 (13-107)	0.001
ALT	21 (12-56)	23 (9-166)	25 (3-154)	23 (8-125)	0.79
AP	70 (31-99)	93 (31-510)	107 (59-315)	134 (63-497)	0.001
GGT	22 (14-64)	23 (8-474)	47 (12-294)	50 (10-305)	0.001
LDH	171 (26-305)	205 (63-399)	202 (112-370)	176 (132-327)	0.018
T. bil	0.6 (0.2-1.5)	0.68 (0.3-2.3)	1.7 (0.8-5.9)	0.9 (0.2-5.7)	0.001
Albumin	4.5±0.05	4.2±0.6	2.7±0.1	4.3±0.07	0.001
T. chol	180±5.6	181±9.4	156±5.9	178±8.7	0.041
HDL	46.6±2.1	48.7±3.2	40.6±3.6	39.6±1.8	0.014
LDL	117±4.3	116±6.4	102±5.3	114±6.5	0.455
Fe	79±3.7	80.7±7.4	88.9±8.9	65±5.5	0.082
TIBG	328±6	348±16	279±15	315±13	0.004
TS (%)	23.7±1.1	24.5±2.3	36.2±4.3	21.7±2.2	0.022
Ferritin	59.8±10.7	128.5±34.8	131.7±25.5	138.6±26.9	0.052
Prohepcidin	91±7.2	116.9±7.4	108.4±7.9	109.9±8.8	0.087

The data are described as mean±SD or median (minimum-maximum).

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, AP: Alkaline phosphatase, GGT: γ-glutamyl transferase, LDH: Lactate dehydrogenase, T. bil: Total bilirubin, T. chol: Total cholesterol, TS: Transferrin saturation (%), HDL: High-density lipoprotein, LDL: Low-density lipoprotein, SD: Standard deviation, Fe: Iron, M: Male, F: Female, HBV: Hepatitis B virus, HCV: Hepatitis C virus

Table 2. Distribution in study groups by patient gender

Group	Male	Female
Group 1. Chronic inactive HBV (n=31)	19	12
Group 2. Chronic HCV (n=30)	13	17
Group 3. Cirrhosis (n=29)	24	5
Group 4. Liver transplant (n=31)	27	4

HCV: Hepatitis C virus, HBV: Hepatitis B virus

HCV patients compared to subjects without any liver disease and ferritin level was correlated with ALT, AST and GGT.

Lee et al.[15] compared serum prohepcidin and IL-6 levels in HCV, alcoholic liver disease and non-alcoholic steatohepatitis (NASH) patients. Both prohepcidin and IL-6 levels were significantly higher in cases of CHC compared to healthy subjects, whereas patients with alcoholic liver disease and NASH did not differ from healthy subjects in these parameters.

In another study, Olmez et al.[16] examined the correlations of plasma prohepcidin with iron and related parameters, histologic activity index (HAI) and liver fibrosis score. Prohepcidin level was reported to be higher in CHC patients compared to HB. A similar result was found for the prohepcidin/ferritin ratio, while an inverse correlation existed between prohepcidin level in CHC and the HAI and fibrosis stage; no such correlation was established for HBV subjects. No significant correlation could be established between the two groups for iron and prohepcidin levels.

Consistent with the literature, the current study also showed significantly higher prohepcidin levels in HCV than in HBV patients. No correlation between prohepcidin and AST, ALT, AP, GGT, LDH, T. bil or the lipid panel tests could be evidenced in either group. Fe, TS and ferritin levels were also higher in HCV. Despite an appearance of coordination between LFT and Fe-related parameters, no statistically significant difference was detected. As seen in Figure 1, prohepcidin was highest in Group 2 (cases of CHC). The prohepcidin level was positively correlated with ferritin and liver hepcidin was shown to increase in response to an elevation of ferritin, representing the iron stores. Also, there was an inverse relationship between prohepcidin and albumin levels, which supports their opposing functions as positive and negative acute-phase reactants, respectively.

The relationship between the ferritin elevation observed in cirrhosis patients and the developing disease complications is not yet entirely understood. The relationship between elevated

iron load and hepatocyte damage has been shown by several reports. It is also hypothesized that increased hepatic iron negatively affects the post-transplantation outcome. Stuart et al.[17] studied the post-transplantation outcomes of 282 patients who received a transplant for cirrhosis and they found iron accumulation in 37% of the patients and iron storage was found to be significantly correlated with diffuse liver disease. HFE gene mutation was not found to be widespread among patients with increased iron stores. Ferritin levels were elevated in our study, especially in CHC, cirrhosis and liver transplant patients. Published studies generally include patients with CHB instead of chronic inactive HBV as in our study, in which the ferritin levels in chronic inactive HBV were lower than in the HCV and cirrhosis patients. Compared to the latter two groups, ferritin levels were higher in cases of cirrhosis than in the HCV group, supporting a correlation between ferritin level and degree of fibrosis.

Détivaud et al.[18] found the level of fibrosis in the non-tumoral liver tissues of 36 patients operated for liver cancer (primary or secondary) or liver transplantation to be negatively correlated with hepcidin mRNA expression and urinary hepcidin levels. However, the study results were affected by patient heterogeneity and the variability in the extent of fibrosis. When patients with mild fibrosis were excluded from the analysis, only a weak correlation could be observed. There was also a positive correlation between Hb concentration and hepatic iron stores. No statistically significant differences could be identified in our study in the prohepcidin level of patients having undergone liver transplant as a result of HB or HC, those with chronic inactive HBV, those with HCV and the cases with cirrhosis (p=0.087).

Anemia following liver transplantation is attributed to many different factors, including intra-operative blood loss, medication, immune suppressive treatment, hemolysis, renal failure, aplastic anemia, Graft-versus-host disease (GvHD), and others. The incidence of such anemia varies from 4.3% to 28%. The etiology of post-transplantation anemia varies according to the time interval. On postoperative days 0-14, the most frequent causes are bleeding, sepsis, medication and hemolysis, while postoperative weeks 2-6, aplastic anemia, medication, GvHD, cytomegalovirus, and parvovirus B19 are seen. After the first 6 weeks, medications remain significant, along with iron deficiency, kidney failure and post-transplant lymphoproliferative syndrome[19]. Hemoglobin changes begin stabilizing only following the first 6 post-transplant months. Therefore, only transplant patients who had completed their first post-operative year were included in our study.

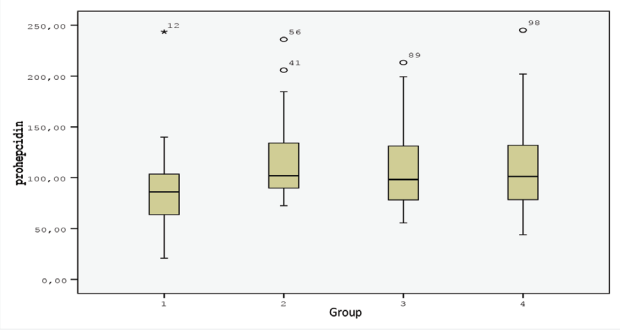

Figure 1. Distribution of prohepcidin levels among the different disease groups (Group 1=Chronic inactive HBV; Group 2=Chronic HCV; Group 3=Cirrhosis; Group 4=Liver transplant)

HCV: Hepatitis C virus, HBV: Hepatitis B virus

Studies have shown that decreased serum hepcidin levels are associated with poor survival in patients with alcoholic liver cirrhosis[20]. Since decreased hepcidin has been reported in patients with hepatic dysfunction, hepcidin levels are expected to decrease in acute liver injury[21,22]. In a study by Spivak et al.[23], hepcidin was reported to have a much shorter half-life, especially when compared to established liver function parameters such as albumin or INR. Thus, hepcidin may better reflect the dynamic changes that occur in acute liver failure. In contrast, transferrin may be a better predictor in disorders associated with a more pronounced inflammation. To summarize, ferritin levels were higher in patients with liver transplant compared to all three other groups; however, the difference was not statistically significant. On the other hand, our study revealed a positive correlation between prohepcidin levels and the patients' AST and ALT values (p=0.046).

Study Limitations

The present study has some limitations. Firstly, only the correlation with albumin and ferritin was examined, but the relationship with other acute phase reactants such as CRP, sedimentation, and fibrinogen could not be examined. In addition, we could not share the survival data as it was a prospective study.

CONCLUSION

Prohepcidin levels in our study were higher in patients with liver transplant compared to those with chronic inactive HBV and cirrhosis, while prohepcidin was even higher in patients with HCV. Our study revealed a significant correlation between prohepcidin levels in patients transplanted for HBV or HCV and patients' AST and ALT values. This correlation suggests that prohepcidin might be useful as a parameter for the post-transplant follow-up of liver reserve functionality. Additionally, this finding may be an indicator of inflammation after transplantation. Hepatic iron stores may be a prognostic factor in patients who have undergone liver transplantation.

This was a pioneering study on the role of prohepcidin, an important factor in Fe metabolism following liver transplant. Prospective, long-term studies on iron-related parameters and prohepcidin levels are needed to further elucidate the role of prohepcidin following liver transplantation.

Ethics

Ethics Committee Approval: The study was approved by Dokuz Eylül University Faculty of Medicine Clinical Research Ethics Committee (date: 29.09.2010, no: 2010/13-21).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ö.Ö, T.Ü., F.D., S.K., Concept: Ö.Ö, M.A., P.T.T., A.B., T.Ü., F.D., S.K., Design: Ö.Ö, M.A., P.T.T., A.B., T.Ü., F.D., S.K., Data Collection or Processing: Ö.Ö, P.T.T., F.Y., A.B., Analysis or Interpretation: Ö.Ö, P.T.T., F.Y., A.B., Literature Search: Ö.Ö, M.A., T.Ü., F.D., S.K., Writing: Ö.Ö, M.A., S.K.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.58076
Nam Kem Med J 2023;11(1):72-79

The Relationship Between Depression and Inflammation Markers in Patients with Metastatic Lung Cancer

Metastatik Akciğer Kanseri Hastalarında Depresyon ile Enflamatuvar Belirteçler Arasındaki İlişki

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ABSTRACT

Aim: The role of systemic inflammation in lung cancer patients is known. Diagnosis and treatment of psychiatric disorders, especially depression, can increase patients' adherence to treatment and life quality. We aimed to investigate the relationship between inflammatory markers and depression in patients with *de novo* metastatic lung cancer.

Materials and Methods: Sixty-six patients newly diagnosed with *de novo* metastatic lung cancer between January and December 2021 were included in our study. Baseline characteristics, laboratory findings, and the Beck Depression Inventory (BDI) of patients were evaluated at the pre-chemotherapy visit.

Results: Neutrophil lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR) and systemic inflammation response index (SII) were significantly higher in the group with depression. NLR, PLR, C-reactive protein to lymphocyte ratio and SII values showed a positive correlation with BDI scores, indicating depression. It was determined that the values of 3.63 for NLR, 173 for PLR and 1208 for SII could be used as cut-off values to detect depression.

Conclusion: Although the biopsychosocial approach is important in terms of disease prognosis during oncological evaluation, cancer remains the main life-threatening disease, making it difficult for clinicians to screen for depression unless the patient has an additional request. Predicting the possible risk of depression via common laboratory values measured at the time of diagnosis will significantly contribute to the treatment process of the patients.

Keywords: Depression, inflammation, lung cancer

ÖZ

Amaç: Akciğer kanseri hastalarında sistemik enflamasyonun rolü bilinmektedir. Kanser hastalarında depresyon başta olmak üzere psikiyatrik bozuklukların tanı ve tedavisi hastaların tedaviye uyumunu ve yaşam kalitesini artırabilmektedir. Bu çalışmada *de novo* metastatik akciğer kanserli hastalarda enflamatuvar belirteçler ile depresyon arasındaki ilişkinin araştırılması hedeflendi.

Gereç ve Yöntem: Ocak-Aralık 2021 tarihleri arasında yeni tanı alan *de novo* metastatik akciğer kanseri 66 hasta çalışmaya dahil edildi. Hastaların temel özellikleri, laboratuvar bulguları ve Beck Depresyon Envanteri (BDI) kemoterapi öncesi vizitte değerlendirildi.

Bulgular: Nötrofil lenfosit oranı (NLR), platelet lenfosit oranı (PLR) ve sistemik enflamasyon yanıt indeksi (SII) BDI'ye göre depresyon saptanan grupta anlamlı derecede yüksek bulundu. NLR, PLR, C-reaktif protein/lenfosit oranı ve SII değerleri, BDI puanları ile pozitif korelasyon gösterdi. NLR için 3,63, PLR için 173 ve SII için 1208 değerlerinin depresyonu saptamak için cut-off değer olarak kullanılabileceği saptandı.

Sonuç: Onkolojik değerlendirme sırasında biyopsikososyal yaklaşım hastalığın prognozu açısından önemli olmakla birlikte, kanserin yaşamı tehdit eden ana hastalık olması, hastanın ek bir isteği olmadıkça klinisyenlerin rutinde depresyon taraması yapmasını güçleştirmektedir. Tanı anında ölçülen rutin laboratuvar değerleri ile olası depresyon riskinin önceden tahmin edilmesi hastaların tedavi süreçlerine önemli katkı sağlayacaktır.

Anahtar Kelimeler: Depresyon, enflamasyon, akciğer kanseri

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Received: 16.01.2023 **Accepted:** 31.01.2023

INTRODUCTION

The second most common cancer in the world according to GLOBOCAN is lung cancer (11.4%), followed by breast cancer (11.7%). It has the first rank among cancers that cause death in the world (18%)[1]. In metastatic (Stage 4) lung cancer patients, the rate for 5-year survival is less than five percent[2]. In a meta-analysis, the frequency of major depression was found to be 15% in cancer patients, while anxiety rate was found to be 10%[3]. Although the frequency varies with the type of cancer, the highest rates of anxiety and depression are seen in lung cancer. Because metastatic patients have a poor prognosis, from the time of diagnosis, these patients tend to have anxiety and depression[4]. Diagnosis of cancer and initiation of treatment disrupt the physical, emotional, social and economic balances of the individual and family, prevent them from getting satisfaction from life and reduce their quality of life. Since the presence of depression predicts worse survival in patients with metastasis, unmanaged psychosocial difficulties may have important implications for cancer treatment[4-6].

It is known that cancer and inflammation are related to each other and the cellular immune system plays a significant role in inflammation[7]. In literature, the studies have also underlined that inflammation response may alter neurotransmission and neuroendocrine pathways that play a role in depression. In addition, recent studies have revealed that rheumatological diseases and increase in proinflammatory markers are related to a higher risk of depression[8,9].

Neutrophil lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR), C-reactive protein to lymphocyte ratio (CRP/L), neutrophil to lymphocyte, platelet ratio (NLPR), and systemic inflammation response index (SII) are biomarkers that can be obtained from the complete blood count test. As depression is a psychiatric disorder related to inflammation, the relationship between inflammation markers such as NLR, PLR and depression has also been explored by numerous studies, but the results showing the relationship between depression and NLR and PLR are controversial[10,11]. Studies have shown that depression is associated with inflammation in patients with cancer and other chronic medical diseases[12,13]. The importance of identification of depression in lung cancer patients may be essential to improve disease outcome. In spite of all efforts to screen for depression, depression is still inadequately treated[14].

The meanings that patients attribute to cancer and the way they perceive the disease affect the response to cancer, impair treatment adherence, increase the length of hospital stay and treatment costs, and may adversely affect the course of the disease. We aimed to investigate the relationship between inflammatory markers and depression in patients with de novo diagnosed metastatic lung cancer, which causes one of the highest rates of depression among all cancer types (16-29%)[4,15,16].

MATERIALS AND METHODS

Study Population and Sample

Sixty-six patients newly diagnosed with metastatic (Stage 4) lung cancer were included in our study (Figure 1). Cancer patients were recruited from the Medical Oncology Clinic of University Hospital between January and December 2021. Inclusion criteria included age of 18≥ years, willingness and ability to provide written informed consent. Patients with a history of psychiatric disorder prior to their cancer diagnosis, with a history of dementia or any other organic neurological disorders, with Eastern Cooperative Oncology Group >2 were excluded. Baseline characteristics, clinical and laboratory findings, and the Beck Depression Inventory (BDI) of patients with newly diagnosed *de novo* metastatic lung cancer were evaluated at the pre-chemotherapy visit.

The Ethics Committee of the Bezmialem Vakıf University approved this cross-sectional study with reference number 16/330 on date 22.09.2020. It was performed in accordance with the Declaration of Helsinki. All patients provided written informed consent for attendance.

The Beck Depression Inventory

It consists of 21 questions and a scoring system between 0 and 3. The severity of depression experienced by individuals is determined by the high scores obtained from the scale. Each question is scored in the range of 0-3 points and results ranging from 0 to 63 are obtained[17]. Validity and reliability studies of BDI for adaptation to the Turkish language have been conducted[18]. In the Turkish reliability and validity study, the cut-off point was 17. It was determined that scores of 17 and above on the scale might require treatment. It has been stated that it can distinguish over 90% of depressive disorders[19]. In our study, the cut-off point was 17 based on Hisli's[18] recommended cut-off scores.

Laboratory Findings and Inflammation Markers

The results of blood tests, which were routinely requested from cancer patients for treatment evaluation, were obtained from the laboratory findings. Blood tests before the chemotherapy were examined.

They were calculated as follows: NLR=Neutrophil/lymphocyte count, PLR=Platelet/lymphocyte count, NLPR=Neutrophil/(lymphocyte × platelet count), CRP/L=C-reactive protein levels/lymphocyte count, SII=Platelet × neutrophil/lymphocyte count.

Statistical Analysis

Statistical Package for Social Sciences (SPSS) 22 software (SPSS, IBM Inc. IL, USA) was used to analyze the study. The normal distribution was evaluated with the Kolmogorov-Smirnov test and Skewness-Kurtosis values. Normally distributed data are presented with mean and standard deviations in analytical evaluation; Non-normally distributed data are presented with median and minimum-maximum values. The Mann-Whitney U test was used for the comparison of the independent groups without normal distribution. The Student's t-test was employed to compare two independent groups with normal distribution. The chi-square tests were employed to compare categorical data. The Spearman correlation test was performed for correlation analysis of non-normally distributed data. Moreover, the univariate logistic regression analysis was used to determine factors predicting depression in lung cancer patients. With receiver operating characteristic (ROC) analysis, cut off values for NLR, PLR, NLPR, CRP/L and SII for depressive symptoms in cancer patients were investigated. A p value <0.05 was considered statistically significant.

RESULTS

Our study group consisted of 66 *de novo* metastatic lung cancer patients (mean age 61.97±10.74 years). Male/female (M/F) ratio was 55/11. When the education levels of the individuals were examined, it was determined that 61 (92.4%) were primary, secondary or high school graduates and 5 (7.6%) were university graduates or had PhD. Furthermore, it was determined that 8 (12.1%) patients lived in the countryside, whereas 58 (87.9%) patients lived in the city center. Forty-three of the patients (65.2%) had a previously known chronic disease, and there were 13 (19.7%) patients with polypharmacy. Clinically significant depression was endorsed by 45.5% (BDI ≥17) with a mean BDI score of 17.14±9.52. Regarding the histological type, 20 (30.3%) patients had small-cell lung carcinoma and 46 (69.7%) patients had non-small cell lung cancer. 60 (90.9%) patients were smokers, whereas 23 (34.8%) patients had a history of alcohol consumption. Descriptive characteristics of the study population are presented in Table 1.

Sixty-six patients were divided into two subgroups with the scores of BDI <17 and ≥17 as patients with depression. Patients with depression had significantly higher NLR [2.88 (1.13-14.12) vs. 4.37 (1.29-23.51), p=0.006], PLR [0.15 (0.06-0.71) vs. 0.21 (0.07-1.12), p=0.007] and SII [962 (205-3650) vs. 1510 (344-6173), p=0.002] levels compared to patients without depression (Table 1).

Spearman correlation analysis revealed that there was a significant positive relationship between BDI scores and NLR (r=0.298, p=0.015), PLR (r=0.308, p=0.012), CRP/L (r=0.254, p=0.039) and SII (r=0.353, p=0.004) (Table 2).

Since there is no shared and approved NLR cut-off value for depression in cancer patients in the literature, we consider the value of 3.5 close to the median NLR considering our study data. The univariate analysis results revealed that a statistically significant relationship was found between depression and

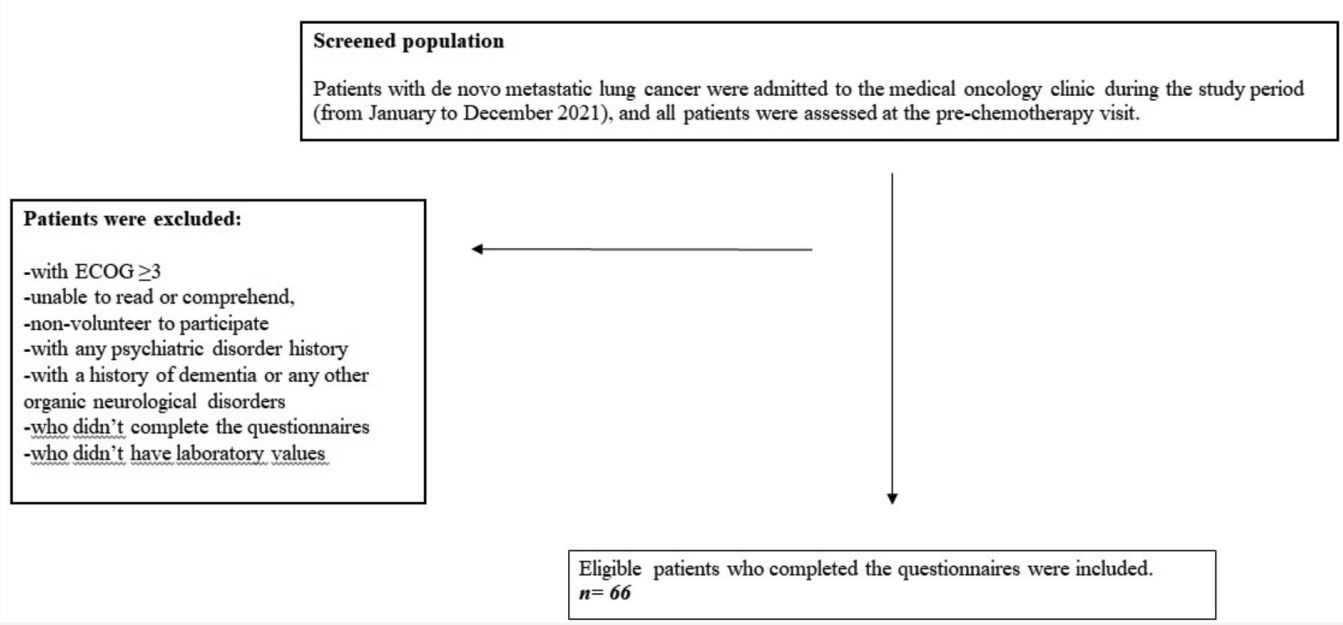

Figure 1. Flow chart of the patient selection process

ECOG: Eastern Cooperative Oncology Group

various risk factors, such as NLR >3.5 [odds ratio (OR): 3.06, 95% confidence interval: 1.12-8.37, p=0.030] and higher SII values (OR: 1.001, 95% confidence interval: 1.00-1.01, p=0.014) (Table 3).

In the ROC curve analysis performed in metastatic lung cancer patients, inflammation markers such as NLR, PLR, and SII were statistically significantly associated with depression (Figure 2). The cut-off, sensitivity, specificity and AUC values are shown in Table 4. SII had the highest AUC value for detecting depression (AUC=0.725, cut-off >1208.8, p=0.002, sensitivity 63.3%, specificity 63.9%).

Table 1. Demographic and clinical characteristics

		BDI <17	BDI ≥17	p
Age		62.7±10.92	61.1±10.7	0.56
Gender	Female Male	3 (8.3%) 33 (91.7%)	8 (26.7%) 22 (73.3%)	**0.04**
Marital status	Single Married	6 (16.7%) 30 (83.3%)	6 (20.0%) 24 (80.0%)	0.73
Education	Primary school or high school university or PhD	32 (88.9%) 4 (11.1%)	29 (96.7%) 1 (3.3%)	0.37
Smoking	Yes No	33 (91.7%) 3 (8.3%)	27 (90.0%) 3 (10.0%)	1.00
Alcohol	Yes No	15 (41.7%) 21 (58.3%)	8 (26.7%) 22 (73.3%)	0.20
Family history of lung cancer	Yes No	5 (13.9%) 31 (86.1%)	2 (6.7%) 28 (93.3%)	0.44
At least ≥1 comorbidity	Yes No	23 (63.9%) 13 (36.1%)	20 (66.7%) 10 (33.3%)	0.81
Polypharmacy	Yes No	8 (22.2%) 28 (77.8%)	5 (16.7%) 25 (83.3%)	0.57
Place of residence	Center Countryside city	33 (91.7%) 3 (8.3%)	25 (83.3%) 5 (16.7%)	0.45
Weight loss (>10%)	Yes No	14 (38.9%) 22 (61.1%)	16 (63.3%) 14 (46.7%)	0.24
Histologic classification	Small-cell Non-small cell	11 (30.6%) 25 (69.4%)	9 (30.0%) 21 (70.0%)	0.96
	All patients (n=66)	**BDI <17 (n=36)**	**BDI ≥17 (n=30)**	**p**
BDI	17.14±9.52	9.83±4.04	25.9±6.16	
Hb (g/dL)	12.90 (8.60-16.60)	13.10 (8.60-16.60)	12.80 (9.30-16.20)	0.49
TNC (×10³/µL)	6064 (2500-18100)	5550 (2500-16400)	6640 (3800-18100)	0.13
TPC (×10³/µL)	300 (95-484)	286 (124-482)	342 (95-484)	0.18
TLC (×10³/µL)	1800 (250-3800)	2200 (250-3700)	1500 (400-3800)	**0.03**
CRP (mg/L)	19.90 (0.20-150.00)	17.5 (0.20-150.0)	21.0 (0.20-149)	0.40
Albumin (mg/dL)	4.05 (3.10-5.00)	4.15 (3.10-5.00)	3.90 (3.20-5.00)	**0.09**
NLR	3.37 (1.13-23.51)	2.88 (1.13-14.12)	4.37 (1.29-23.51)	**0.006**
PLR	0.17 (0.06-1.12)	0.15 (0.06-0.71)	0.21 (0.07-1.12)	**0.007**
NLPR	0.01 (0-0,25)	0.01 (0-0.08)	0,01 (0.005-0.25)	0.06
CRP/L	0.01 (0-0.60)	0.01 (0-0.60)	0.01 (0-0.24)	0.21
SII	1177 (205-6173)	962 (205-3650)	1510 (344-6173)	**0.002**

Numbers indicate mean±standard deviation, median (minimum-maximum) or n (%).

CRP: C-reactive protein, CRP/L: C-reactive protein-to-lymphocyte ratio, Hb: Hemoglobin, NLR: Neutrophil-to-lymphocyte ratio, NLPR: Neutrophil-to-lymphocyte-platelet ratio, PLR: Platelet-to-lymphocyte ratio, TLC: Total lymphocyte count, TNC: Total neutrophil count, TPC: Total platelet count, SII: Systemic inflammation response index, BDI: Beck Depression Inventory

DISCUSSION

NLR, PLR and SII values were statistically significantly higher in the group with depression. NLR, PLR, CRP/L and SII values showed a positive correlation with BDI scores indicating depression. Considering the NLR value, it was determined that an NLR value above 3.5 was predictive of depression, and an increase in the SII value also predicted the presence of depression. Therefore, it has been determined that the values of 3.63 for NLR, 173 for PLR and 1208 for SII can be used as cut-off values to detect depression in patients with de novo metastatic lung cancer.

Our results showed no significant difference in neutrophil count between patients with and without depression, whereas the depressive patient group had significantly lower lymphocyte levels. In a study that emphasized the importance of NLR, similar to our results, lymphocyte count was significantly lower in patients with depression. On the other hand, neutrophil count was higher, controversial to our study[20]. Evaluating inflammation in relation to neutrophil or lymphocyte separately can be challenging, supporting the need to evaluate NLR, PLR, CRP/L or SII in inflammation.

Our results showed that higher depression scores were associated with increased inflammation, that can be detected by increased NLR, PLR, CRP/L and SII levels. Recently, there are some studies investigating the relationship between NLR and depression in cancer patients or other populations, which is an interesting area for clinicians nowadays. McFarland stated that there was a significant correlation between NLR and depression[21]. Besides, it has been shown that NLR and PLR levels were significantly higher in patients with major depressive disorders compared to the healthy individuals[22,23]. The PLR of patients with severe depression was found to be higher than that of patients with other types of depression (without psychotic features etc.), but there was no significant difference in NLRs among different types of depression[24]. Demir et al.[20] showed that NLR tended to be higher in patients with depression, and so they stated the fact that higher NLR values supported the approach that inflammation played critical role in the etiology of depression. A study found that after adjustment for values of hemoglobin, RDW and NLR, RDW and NLR were associated with depression independently of hemoglobin[25].

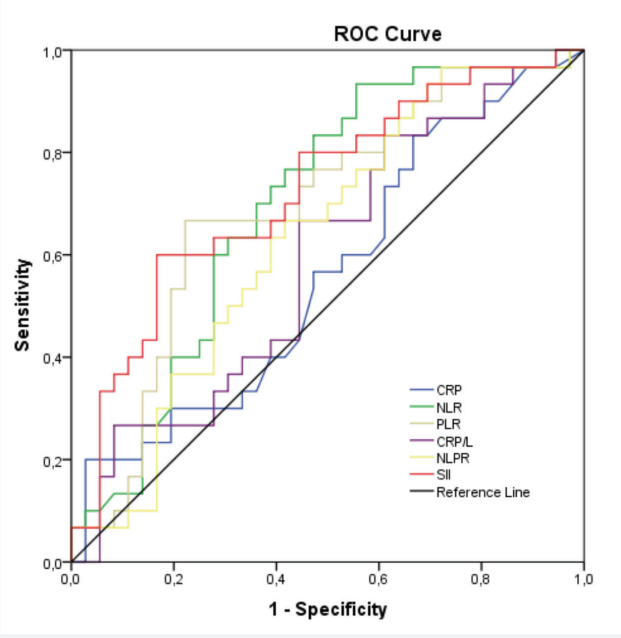

Figure 2. ROC curve analysis of inflammation markers for depression (Beck Depression Inventory ≥17)

CRP: C-reactive protein, NLR: Neutrophil to lymphocyte ratio, PLR: Platelet to lymphocyte ratio, CRP/L: C-reactive protein to lymphocyte ratio, NLPR: Neutrophil to lymphocyte-platelet ratio, SII: Systemic inflammation response index

The NLR is calculated by two types of cell counts mediating two different immune pathways. Having a phagocytic and apoptotic function neutrophil plays role at the first line of immunity[26]. As specific inflammation mediators, lymphocytes play an important role in host defense mechanisms with regulatory or protective effects. Since NLR covers both immune responses, it is expected to be more predictive and valuable than neutrophil and lymphocyte counts alone[24]. Increased neutrophil levels and decreased lymphocyte levels by production of pro-inflammatory cytokines cause elevated NLR values in different kinds of inflammatory processes. PLR is another potential and easy to measure parameter[27]. In the first-line immune response, the platelets regulate permeability of endothelium, migration of neutrophils, macrophages and other mediators. Glutamate

Table 2. Spearman correlation analysis between Beck Depression Inventory scores and laboratory findings

	r	p value
Hb (g/dL)	-0.212	0.087
Albumin (mg/dL)	-0.249*	0.044
TLC (×10³/µL)	-0.233	0.060
TNC (×10³/µL)	0.144	0.248
TPC (×10³/µL)	0.167	0.181
CRP (mg/L)	0.239	0.054
NLR	**0.298***	0.015
PLR	**0.308***	0.012
CRP/L	**0.254***	0.039
NLPR	0.193	0.121
SII	**0.353****	0.004

r: Spearman's Rho, *p<0.05, **p<0.01.

CRP/L: C-reactive protein-to-lymphocyte ratio, Hb: Hemoglobin, NLR: Neutrophil-to-lymphocyte ratio, NLPR: Neutrophil- to-lymphocyte-platelet ratio, PLR: Platelet-to-lymphocyte ratio, TLC: Total lymphocyte count, TNC: Total neutrophil count, TPC: Total platelet count, SII: Systemic inflammation response index

and serotonin pathways and other proinflammatory molecules have originated from the activated thrombocytes and so, the function of thrombocytes is modulated, resulting in alterations in pathophysiology ending up with mood disorders[28,29]. A meta-analysis mentioned above has also demonstrated that NLR and PLR levels are significantly higher in major depression and patients with mood disorders than in healthy individuals[26]. In another study it has been revealed that high NLR levels are found to be independently related to depressive symptoms in female group, but not in males[30].

The potential usage of the newly discovered CRP/L ratio as a biomarker has attracted our attention[31]. So, as an important study presenting real-life data, we examined the relationship between CRP/L ratio and depression, we found a positive

Table 3. Univariate analysis of the potential associations between patient characteristics and depression (Beck Depression Inventory ≥17) in *de novo* metastatic lung cancer patients

	Univariate regression analysis			
	OR	Lower	Upper	p value
Gender male vs female	4.00	0.95	16.7	0.06
Age	0.99	0.94	1.03	0.56
Marital status (single vs. married)	1.25	0.36	4.37	0.73
Education (primary school or high school vs. university or PhD)	3.62	0.38	34.3	0.26
Place of residency (countryside vs. city center)	0.45	0.09	2.08	0.31
Weight loss (>10%) (yes vs. no)	1.80	0.67	4.79	0.24
Smoking (ever vs. never)	0.82	0.15	4.39	0.82
Alcohol (yes vs. no)	0.51	0.18	1.45	0.21
Family history of lung cancer	0.44	0.08	2.47	0.35
At least ≥1 comorbidity (yes vs. no)	1.13	0.41	3.12	0.81
Polypharmacy (yes vs. no)	0.70	0.20	2.42	0.57
Histologic classification (small-cell vs. non-small cell)	1.02	0.35	2.94	0.96
HB (g/dL)	0.092	0.71	1.19	0.52
TNC ($\times10^3$/µL)	1.00	1.00	1.05	0.15
TPC ($\times10^3$/µL)	1.01	0.99	1.01	0.21
TLC ($\times10^3$/µL)	0.99	0.99	1.00	0.039
CRP (mg/L)	1.01	0.99	1.02	0.43
Albumin (mg/dL)	0.37	0.12	1.11	0.08
NLR (>3.5 vs. <3.5)	3.06	1.12	8.37	0.030
PLR	1.00	0.99	1.01	0.11
CRP/L	0.58	0.01	195.0	0.86
NLPR	0.66	0.00	1.12	0.19
SII	1.001	1.00	1.01	0.014

OR: Odds ratio, CRP: C-reactive protein, CRP/L: C-reactive protein to lymphocyte ratio, Hb: Hemoglobin, NLR: Neutrophil to lymphocyte ratio, NLPR: Neutrophil to lymphocyte-platelet ratio, PLR: Platelet to lymphocyte ratio, TLC: Total lymphocyte count, TNC: Total neutrophil count, TPC: Total platelet count, SII: Systemic inflammation response index

Table 4. Evaluation of depression (Beck Depression Inventory ≥17) in *de novo* metastatic lung cancer patients with ROC curve analysis

	AUC	p value	Lower bound	Upper bound	Cut-off point	Sensitivity %	Specificity %
CRP	0.56	0.403	0.42	0.700			
NLR	0.696	**0.006**	0.568	0.824	>3.63	63.3	63.9
PLR	0.693	**0.007**	0.563	0.822	>173	66.7	66.7
CRPL	0.59	0.212	0.452	0.728			
NLPR	0.632	0.066	0.498	0.767			
SII	**0.725**	**0.002**	0.601	0.849	>1208.8	63.3	63.9

AUC: Area under curve, CRP: C-reactive protein, NLR: Neutrophil to lymphocyte ratio, PLR: Platelet to lymphocyte ratio, CRP/L: C-reactive protein to lymphocyte ratio, NLPR: Neutrophil to lymphocyte-platelet ratio, SII: Systemic inflammation response index

correlation between CRP/L levels and the severity of depression in our study. However, according to univariate analysis results, CRP/L was not found to be a risk factor for depression, or a cut-off value that would shed light on the presence of depression risk could not be reached according to the ROC analysis results. Comprehensive studies are needed to clarify the relationship.

In our study, high SII values were found to be a risk factor for depression, consistent with the recent studies. Wang et al.[32] stated that after adjusting for socio-demographic and clinical features, high SII levels were found to be a risk factor for depression in patients with diabetes.

Clinical Implications

NLR, PLR, CRP/L and SII can be calculated easily and cheaply via routinely used laboratory findings. Although the biopsychosocial approach is important in terms of disease prognosis during oncological evaluation, the fact that cancer remains the main life-threatening disease makes it difficult for clinicians to screen for depression unless the patient has an additional request. Predicting the possible risk of depression via routine laboratory values measured at the time of diagnosis will greatly contribute to the treatment process of the patients. Since the diagnosis and treatment process and quality of life of patients are negatively affected by depression levels, it is important to determine the need for support in the early period to be able to screen individuals under cancer treatment for depression and to intervene in the psychosocial problems of patients at risk.

Study Limitations

This study was an important research presenting real-life data, investigating the relationship among NLR, PLR, NLPR, CRP/L and SII and depression in metastatic lung cancer patients. However, we had some limitations in our study. Patients admitted to a single center were included. This study had a small sample size. While providing valuable information for this area, reaching more patients could help us draw precise conclusions. Finally, the study's cross-sectional design could not explain causal relations well. Patients' socio-demographic, clinical characteristics, and cancer status can affect depression and inflammation processes in patients with *de novo* metastatic lung cancer; therefore, we believe these factors may not be considered independently. Longitudinal studies with larger clinical samples could provide more comprehensive findings.

CONCLUSION

Our study revealed that a high NLR, PLR, CRP/L and SII might be a predictive factor for higher depression levels in patients with metastatic cancer. Our results may underline the importance of an altered inflammation process in set of causes of depression. The current study was an important study with real-life data that evaluated the diagnostic power of inflammatory markers such as NLR, PLR, CRP/L and SII for indicating depression in cancer patients. As the management of cancer patients should also focus on providing patients with psychological support regarding its improving effect on treatment response, detecting the presence of depression or identifying patients at high risk of depression is of great importance, promisingly done easily by clinicians via inflammatory markers screening.

Acknowledgments

The authors sincerely thank Dr. Ayşe İrem YASİN and Dr. Zehra SUCUOĞLU İŞLEYEN for their help during the study period.

Ethics

Ethics Committee Approval: The Ethics Committee of the Bezmialem Vakıf University approved this cross-sectional study with reference number 16/330 on date 22.09.2020.

Informed Consent: All patients provided written informed consent for attendance.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices - Concept - Design - Data Collection or Processing - Analysis or Interpretation - Literature Search - Writing: İ.Ö.Ü., A.T.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2023.38257
Nam Kem Med J 2023;11(1):80-86

The Effect of Methylphenidate Treatment on Heart Rate Variability and Cardiac Autonomic Functions in Children with Attention Deficit Hyperactivity Disorder

Dikkat Eksikliği Hiperaktivite Bozukluğu Olan Çocuklarda Metilfenidat Tedavisinin Kalp Hızı Değişkenliği ve Kardiyak Otonomik Fonksiyonlara Etkisi

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ABSTRACT

Aim: Attention deficit hyperactivity disorder (ADHD) is the most common childhood psychiatric disorder. Psychostimulant drugs are frequently used in the treatment regimen. The possible cardiac side effects of drugs are of concern. In our study, we aimed to determine possible cardiac autonomic effects with heart rate variability (HRV) in ADHD patients receiving methylphenidate (MPH).

Materials and Methods: We used a retrospective pre-post treatment study design to measure the change in HRV parameters in ADHD patients receiving MPH therapy. A total of 49 patients (mean age, 8.3±2.5 years) diagnosed with ADHAB and 30 sex- and age-matched healthy controls (mean age, 8.2±2.7 years) were examined. Rhythm Holter recordings were made for the patients before MPH treatment and in the first month of treatment and for the control group, and HRV parameters were evaluated in the computer environment.

Results: There was no difference in age, gender, weight and height in the patient and control groups ($p>0.05$). In the analysis of time-dependent HRV parameters, SDNN, SDANN, which shows the sympathetic influence, and rMSSD, which shows the parasympathetic influence, were statistically significantly lower in the patient group than in the control group ($p<0.05$). When the patients were compared before and after the treatment, SDANN increased statistically ($p<0.05$). Besides, SDNN and rMSSD increased after the treatment, although there was no statistical significance ($p>0.05$).

Conclusion: Our study showed that there was increased sympathetic and decreased parasympathetic activity in HRV parameters in ADHD patients. Both sympathetic and parasympathetic improvement was observed with MPH treatment. Although our study shows that MPH treatment has a curative effect on cardiac autonomic functions, further studies are needed.

Keywords: Attention deficit hyperactivity disorder, methylphenidate, heart rate variability

ÖZ

Amaç: Dikkat eksikliği ve hiperaktivite bozukluğu (DEHB) en sık görülen çocukluk çağı psikiyatrik hastalığıdır. Psikostümülan ilaçlar tedavi rejiminde sık kullanılmaktadır. İlaçların olası kardiyak yan etkileri endişe vericidir. Çalışmamızda metilfenidat (MPH) tedavisi alan DEHB hastalarında kalp hızı değişkenliği (HRV) ile olası kardiyak otonomik etkileri belirlemeyi amaçladık.

Gereç ve Yöntem: MPH tedavisi alan DEHB hastalarda HRV parametrelerindeki değişikliği ölçmek için retrospektif bir tedavi öncesi-sonrası çalışma tasarımı kullandık. DEHB tanısı alan toplam 49 hasta (ortalama yaş, 8,3±2,5 yıl) ve cinsiyet ve yaşları eşleştirilmiş 30 sağlıklı kontrol (ortalama yaş, 8,2±2,7 yıl) incelendi. Hastalara MPH tedavisi öncesi ve tedavinin birinci ayında ve kontrol grubuna 24 saat ritim Holter kayıtları yapılarak bilgisayar ortamında HRV parametreleri değerlendirildi.

Bulgular: Hasta ve kontrol grubunda yaş, cinsiyet, kilo ve boy açısından fark yoktu ($p>0,05$). Zaman bağımlı HRV paramatrelerinin analizinde hasta grupta kontrol grubuna göre sempatik etkilenmeyi gösten SDNN, SDANN ve parasempatik etkilenmeyi gösteren rMSSD istatistiksel açıdan anlamlı

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Received: 12.01.2023 **Accepted:** 02.02.2023

olarak daha düşüktü (p<0,05). Hastalarda tedavi öncesi ve sonrası karşılaştırıldığında SDANN istatistiksel açıdan anlamlı olarak yüksekti (p<0,05). Yine tedavi sonrasında SDNN, rMSSD de istatistiksel anlamlılık olmamakla birlikte yüksekti (p>0,05).

Sonuç: Çalışmamızda DEHB hastalarında HRV parametrelerinde artmış sempatik ve azalmış paramsempatik aktivite olduğu görüldü. MPH tedavisi ile hem sempatik hem de parasempatik iyileşme gözlendi. Çalışmamız MPH tedavisinin kardiyak otonom fonksiyonlar üzerine iyileştirici etkisi olduğunu göstermekle beraber daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Dikkat eksikliği hiperaktivite bozukluğu, metilfenidat, kalp hızı değişkenliği

INTRODUCTION

Attention deficit and hyperactivity disorder (ADHD) is the most common neuropsychiatric disease of childhood with a rate of 9.5% in school-age children[1,2].

As ADHD causes difficulties in social and academic development in children, they also face risks such as neuropsychiatric diseases, substance abuse, and increased delinquency in adulthood[3].

The presence of hypofunction in the prefrontal cortex in the central nervous system (CNS) has been shown in the neuropathology of the disease. Different regions of the prefrontal cortex have regulatory effects on motor, sensory, behavioral and autonomic functions[4,6]. Therefore, hypofunction in the prefrontal cortex was thought to be the cause of symptoms in patients with ADHD[5,6].

The two main components of treatment are behavioral therapy and psychostimulant pharmacotherapy. Behavioral therapy is the first thing to be done, but it is often the first choice because of faster and more effective results with medical treatment[7-9]. While 50% of children with ADHD receive behavioral therapy, 75% receive psychostimulant treatment[9,10]. These drugs exert stimulating effects on the CNS by increasing the levels of norepinephrine and dopamine in the prefrontal cortex[11]. Methylphenidate (MPH) is the most used psychostimulant drug[12].

Psychostimulant drugs may cause cardiac risks such as the prolongation of cQT duration, arrhythmia and hypertension, and sudden death due to drug use has been reported[13,14]. However, this situation is controversial and some studies have not found a significant difference in terms of cardiac risks[15,16]. This may be due to the fact that the patients receiving medical treatment are children and adolescents who are cardiac healthy, and the publications are often of short duration[9]. However, in order to minimize the ultimate risks, guidelines recommend taking history, physical examination, and pre-treatment electrocardiography in evaluation[17].

Heart rate variability (HRV) in 24-hour rhythm Holter is defined as the change in heart rate from beat to beat and shows the dynamic interaction of the sinoatrial node with the autonomic nervous system (ANS)[6]. It is a simple and non-invasive method to examine the effect of ANS on the cardiovascular system (CVS)[6]. HRV is an indicator of central-peripheral neural feedback and CNS-ANS integration, and respiratory sinus arrhythmia is considered an index of cardiac vagal modulation and emotional regulation[18-20]. The standard deviation of normal sinus RR intervals (SDNN) and the standard deviation of the averages of five-minute recordings over twenty-four hours (SDANN) in the HRV analysis reflects sympathetic modulation, while the root mean square of the difference between consecutive normal RR intervals (rMSSD) and the percentage of consecutive normal sinus RR intervals that differ by more than 50 ms (pNN50) reflect parasympathetic modulation. HRV studies of ADHD show the presence of autonomic dysfunction, but the results are confusing. Although there are studies showing an increase in parasympathetic (vagal) tone, there are studies showing a decrease in vagal tone on the contrary[6,11,21].

Examination of the presence of autonomic dysfunction in children with ADHD may be useful both for a better understanding of neurobiology and for identifying patients who may be at risk for CVS[6]. As a result, treatment planning and follow-up can be done more carefully in patients at risk[6].

The aim of this study is to compare HRV with the healthy control group, assuming that children with ADHD show autonomic dysfunction, and to investigate the effect of psychostimulant MHP used in the treatment on HRV and therefore on autonomic dysfunction.

MATERIALS AND METHODS

Patients who applied to Tekirdağ Namık Kemal University Medical Hospital Child and Adolescent Psychiatry outpatient clinic for the first time between 1st of January, 2018 and 31th of December, 2020 and who were diagnosed with ADHD according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria were included in this retrospective study[22]. Forty-nine patients (38 boys and 11 girls, mean age 8.31±2.53 years, between 6-17 years of age) who were referred to the Pediatric Cardiology outpatient clinic were included. Chronic disorders of the CVS, pulmonary and/or other systems, hypertension, intolerance of MPH, not taking MPH for two days or longer, drug use affecting CVS and CNS, psychotic disorder and mental retardation were used as exclusion criteria. The study was planned on three groups. While the first group included the patients diagnosed with

ADHD before the treatment, the patients who were started on long-acting MPH therapy and evaluated in the first month of the treatment constituted the second group. The third group consisted of 30 age- and sex-matched healthy volunteers (21 boys and 9 girls, mean age 8.20±2.76 years, 6-17 years of age) who did not have any psychiatric disease, did not use drugs, and applied to the pediatric cardiology outpatient clinic due to an innocent murmur. For routine evaluation, physical examination, blood pressure, electrocardiography, and echocardiography were performed on the patient and control group. Twenty-four-hour rhythm Holter examination was performed twice, before the treatment and in the control examination in the first month of the treatment.

The patient group was assigned to one of three dose levels per day (10, 18, or 27 mg) based on long-acting MPH doses. Initial treatment of MPH was given at a dose of 0.3-0.6 mg/kg/day. In the second Holter treatment, the patients were inserted in the first month without increasing the dose. While rhythm Holter was inserted in 41 patients before the treatment, rhythm Holter control was performed in 27 patients in the first month of the treatment.

The study approval was obtained from the ethics committee of Tekirdağ Namık Kemal University non-interventional clinical studies (2021.189.06.19) and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practices (date: 12.11.2020, no: 2020/68).

Echocardiographic Studies

Echocardiographic examinations were performed using a 4V1c transducer with an ultrasound device (ACUSON SC2000, Siemens, Germany). Transthoracic echocardiography images were obtained in parasternal long-axis and short-axis images, and apical two- and four-chamber views using standard transducer positions. The following end-diastolic and end-systolic parameters were measured in parasternal long-axis view on M-mode echocardiography: interventricular septal thickness (IVSd and IVSs), LV dimensions (LVDd and LVDs), and LV posterior wall thickness (LVPWd and LVPWs) left ventricular ejection fraction (Ef) and fraction shortening (Fs).

Processing and Analysis of 24-hour Holter Recordings

While the patient and control groups continued their normal daily lives, rhythm recordings were made with a three-channel (medilogFD12 plus, Schiller, Switzerland) rhythm Holter monitor for 24 hours. All Holter recordings were reviewed by an experienced cardiologist after artifact recordings were deleted. HRV parameters were analyzed in a computer program. Physiological interpretation and measurement of HRV parameters were performed according to the standards set by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology[23].

The time domain measurement of HRV determines the heart rate at any time point or the intervals between successive normal complexes. Each QRS complex is detected on the ECG, and then all intervals (NN/normal to normal intervals) between adjacent QRS complexes resulting from sinus node depolarization and instantaneous heart rate are determined. HRV measurements were calculated using normal-to-normal ranges only. SDNN, SDANN, SDNN index, RMSSD, NN50 and pNN50 were calculated in time-based HRV parameters. SDNN (ms): the standard deviation of the time (NN interval) between consecutive normal QRS complexes. SDANN (ms): the standard deviation of the averages of five-minute recordings over twenty-four hours. SDNN index (ms): the arithmetic mean of the standard deviations of the NN intervals of five-minute recordings over twenty-four hours. RMSSD (ms): the square root of the mean of the difference of the adjacent NN intervals in a 24-hour recording. NN50: the number of intervals in which the difference between consecutive NN intervals is greater than 50 ms. pNN50 (%): the ratio of the number of NN50 to the total number of NN intervals[23].

SDNN is used for the general evaluation of HRV, SDANN is used for the long-term evaluation of HRV and it reflects the effect of the sympathetic system on HRV, while rMSSD and pNN50 for the short-term evaluation reflect the effect of the parasympathetic system on HRV[23].

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, version 22.0 (SPSS Inc. IL, USA). Continuous data were indicated as mean±standard deviation, while categorical data were presented as the number of patients. The chi-square test was used to compare categorical variables, while parametric continuous variables were compared using the Student's t-test. Data were checked for normal distribution using the Kolmogorov-Smirnov test. The correlation between two variables was calculated using the Pearson's correlation coefficient (r) analysis of variance (F). A value of $p<0.05$ was considered statistically significant.

RESULTS

Cohort Characteristics

A total of 49 patients with ADHD (mean age was 8.3±2.5 years; 38 boys and 11 girls) and 30 healthy controls (mean age was 8.2±2.7 years; 21 boys and 9 girls) were enrolled in this study. No significant differences were found between the two groups in terms of age, sex, weight, blood pressure systole and diastole, left ventricular end-diastolic dimension and ejection fraction

Table 1. Demographic and clinical characteristics of the study population

Parameters	Patients (n=49)	Controls (n=30)	p value
Sex (male/female)	38/11	21/9	0.51
Age (year)	8.3±2.5	8.2±2.7	0.85
Weight (kg)	31.1±12.1	29.2±7.3	0.48
Height (cm)	129.4±22.2	129.5±23.4	0.72
SBP (mmHg)	92.1±10.6	91.4±11.7	0.57
DBP (mmHg)	59.4±9.9	58.8±7.6	0,48
LVDd (cm)	3.9±0.4	3.7±0.3	0.34
Ef (%)	66.7±5.2	66.5±3.3	0.83

DBP: Diastolic blood pressure, Ef: Ejection fraction, LVDd: End-diastolic left ventricular (LV) dimension, SBP: Systolic blood pressure

Table 2. Heart rate variability parameters of the study population

Parameters	Patients (n=41)	Controls (n=30)	p value
Heart rate (beats/min.)	89.2±9.3	90.1±11.9	0.75
Mean NN (ms)	676.0±70.2	668.3±86.5	0.69
SDNN (ms)	124.0±34.1	165.6±113.3	**0.04**
SDANN (ms)	77.8±18.7	109.3±58.9	**0.01**
SDANN index (ms)	86.6±29.8	120.9±67.4	0.1
rMSSD (ms)	93.6±50.2	155.3±96.2	**0.04**
NN50 (count)	25051.3±13416.4	24196.6±14295.6	0.78
pNN50 (%)	22.1±13.7	24.6±13.6	0.47

NN50: Count of number of pairs of adjacent NN intervals differing by more than 50 ms, pNN50: Number of pairs of adjacent NN intervals differing by more than 50 ms divided by the total number of all NN intervals, rMSSD: Square root of the mean of the sum of the squares of differences between adjacent NN intervals, SDANN: The standard deviation of the mean of five-minute recordings over twenty-four hours, SDNN: Standard deviation of all NN intervals, SDNN index: The arithmetic mean of the standard deviations of the NN intervals of five-minute recordings over twenty-four hours during

(p>0.05). In the patient group, the MPH dose was 0.57±0.15 mg/kg/day. Table 1 shows the characteristics of the patient and the control groups.

Heart Rate Variability Findings

Analysis of the HRV data of the groups (Table 2) did not show any significant difference between the patient and control groups in terms of heart rate, Mean NN, SDANN index, NN50 and pNN50 (p>0.05). However, SDNN, SDANN and rMSSD were significantly lower in the patient group compared to the control group (p<0.05).

When HRV parameters in ADHD patients were compared between the pre- and post-treatment groups (Table 3), there was statistically significantly higher SDANN in the post-treatment group than in the pre-treatment group (p^2<0.05). There was no difference in other HRV parameters between the two groups. In addition, when we compared the post-treatment group with the control group, there was no difference between HRV parameters (p^1>0.05).

In the correlation analysis between age and MPH dose and HRV parameters (Table 4), there was no correlation between MPH dose and HRV parameters (p>0.05), there was a negative correlation between age and heart rate and a positive correlation between age and mean NN (p<0.05).

Study Power

The power results calculated according to the effect size values found using the numerical data of SDANN, SDANN and rMSSD in the available sample size using G*Power 3.1.9.2 software were 85%, 94%, 95% for SDNN, SDANN and rMSSD in the patient-control group, respectively. In addition, the power result was 81% for SDANN in the pre- and post-treatment group.

DISCUSSION

In the treatment of ADHD, the first treatment option is often the use of psychostimulant drugs such as MPH. In recent years, there has been concern about the possible CVS side effects of psychostimulant drugs. Studies on this subject are quite confusing as to whether psychostimulant drugs have a positive or negative effect on CVS. In our study, we aimed to investigate the potential benefit-harm relationship of MPH treatment in terms of CVS risks in patients with ADHD through its effect on HRV parameters, which are indicators of autonomic system dysfunction. For this purpose, 24-hour rhythm Holter recordings were analyzed in the patients before the drug and one month after they started using the drug, and in the healthy control group. When we compared the patient and healthy control groups before the treatment, SDNN and SDANN, which showed increased sympathetic effect, and rMSSD, which showed

decreased parasympathetic effect, were significantly reduced. Again, SDNN, SDANN, and rMSSD times increased in the patient group after the treatment, which showed sympathetic and parasympathetic recovery.

In a similar study, Buchhorn et al.[17] showed that rMSSD was lower in patients compared to healthy controls, supporting the decrease in parasympathetic activity, and that there was an improvement in rMSSD with MPH treatment, but this improvement was more pronounced in night HRV recordings. Similarly, Rukmani et al.[6] found rMSSD to be lower in the patient group in their study in which they compared the patient and healthy control groups. However, treatment data were not studied. There are studies reporting the opposite result. When Carvalho et al.[24] compared the patient and healthy control groups, they found rMSSD to be high in the patient group and interpreted this as an increase in parasympathetic activity. Similar results have been demonstrated in stimulant drug studies. Negrao et al.[21] found that rMSSD, which shows an increase in parasympathetic activity, increased 3 weeks after drug discontinuation in patients using MPH for ADHD. There are studies that evaluate post-treatment period, such as evaluation after treatment discontinuation. After 12 weeks of MPH treatment, Kim et al.[23] found decreased rMSSD compared to the initial values. Available data are confusing as to whether there is an increase or decrease in vagal tone with disease. The same confusion applies to how vagal healing occurs with treatment. In a recent study by Griffiths et al.[26] with a large sample (n=229 patients), although rMSSD was lower in the patient group, there was no significant difference compared to healthy controls, while low rMSSD was associated with high anxiety and social problems. Although low vagal tone has been reported in psychopathological conditions such as depression and anxiety, the relationship between this condition and ADHD appears to be weak if depression, anxiety, and mood disorders are not accompanied[27]. This may be due to the fact that ADHD is a heterogeneous group with attention deficit, hyperactivity, combined and other psychosocial disorders[27]. On the other hand, Griffiths et al.[26] showed no difference in parasympathetic activity even in ADHD subgroups in the same study. In a study evaluating short-term memory performance, children with ADHD showed excessive vagal withdrawal. If this situation is interpreted together with the study of Buchhorn et al.[17], it can be thought that there may be a daily circadian rhythm in vagal activity. This may also explain why there is a difference in vagal HRV activity at night, while there is no difference in daily measurements[28]. In a meta-analysis of eight studies (six of which were on children), Koening et al.[27] found no evidence of parasympathetic dominance or insufficiency. In our study, rMSSD values were significantly lower in the patient group compared to the healthy controls, suggesting a decrease

Table 3. Comparison of heart rate variability before and after methylphenidate treatment in ADHD (n=27)

Parameters	Controls (n=30)	Before treatment	After treatment	p^1 value	p^2 value
Heart rate (beats/min.)	90.1±11.9	88.8±9.0	89.9±10.3	0.95	0.53
Mean NN (ms)	668.3±86.5	681.2±68.1	671.4±70.1	0.88	0.40
SDNN (ms)	165.6±113.3	129.9±37.2	138.1±55.9	0.27	0.39
SDANN (ms)	109.3±58.9	80.2±19.6	107.9±74.0	0.91	**0.05**
SDANN index (ms)	120.9±67.4	91.3±32.0	98.2±58.2	0.34	0.54
rMSSD (ms)	155.3±96.2	100.7±56.7	106.4±65.8	0.19	0.68
NN50 (count)	24196.6±14295.6	26484.2±13645.6	23778.6±10235.8	0.78	0.25
pNN50 (%)	22.1±13.7	25.2±13.2	22.7±11.3	0.85	0.21

NN50: Count of number of pairs of adjacent NN intervals differing by more than 50 ms, pNN50: Number of pairs of adjacent NN intervals differing by more than 50 ms divided by the total number of all NN intervals, rMSSD: Square root of the mean of the sum of the squares of differences between adjacent NN intervals, SDANN: The standard deviation of the mean of five-minute recordings over twenty-four hours, SDNN: Standard deviation of all NN intervals, SDNN index: The arithmetic mean of the standard deviations of the NN intervals of five-minute recordings over twenty-four hours during, ADHD: Attention deficit hyperactivity disorder

p^1; Comparison of the patient group after treatment with controls.

p^2; Comparison of the patient group before and after treatment.

Table 4. Correlation between age and MPH dose with HRV variables

Parameters	Heart rate		Mean NN		SDNN		SDANN		rMSSD		NN50	
	R	p	R	p	R	p	R	p				
Age	-0.48	**0.01<**	0.49	**<0.01**	0.48	0.69	-0.06	0.95	-0.04	0.71	0.04	0.75
MPH dose	-0.95	0.59	0.08	0.63	0.09	0.60	0.32	0.06	-0.02	0.87	0.23	0.17

p value is significant if <0.05.

NN50: Count of number of pairs of adjacent NN intervals differing by more than 50 ms, pNN50: Number of pairs of adjacent NN intervals differing by more than 50 ms divided by the total number of all NN intervals, rMSSD: Square root of the mean of the sum of the squares of differences between adjacent NN intervals, SDANN: The standard deviation of the mean of five-minute recordings over twenty-four hours, SDNN: Standard deviation of all NN intervals, SDNN index: The arithmetic mean of the standard deviations of the NN intervals of five-minute recordings over twenty-four hours during, HRV: Heart rate variability, MPH: Methylphenidate

in parasympathetic activity. At the same time, although rMSSD values did not reach normal healthy control levels after treatment with MPH, they increased and were considered as parasympathetic recovery.

Studies in ADHD are mostly focused on parasympathetic involvement, and the number of studies examining sympathetic involvement is few. Similarly, results regarding whether there is an increase or decrease in sympathetic activity are mixed. Rukmani et al.[6] found low SDNN values in the patient group in their study in which they compared patients with ADHD and healthy controls, and they evaluated this situation as sympathetic dominance. A similar result was also shown in the study of Carvalho et al.[24]. The lack of screening of other sympathetic data and inclusion of a small sample were deficiencies for both studies. On the other hand, in the study of Buchhorn et al.[17] in which they compared both before and after treatment with healthy controls, there was no difference in SDNN values. On the contrary, there are studies stating that there is sympathetic insufficiency. Negrao et al.[21] found both pre- and post-treatment SDNN values higher in patients than in the healthy group and interpreted this as sympathetic insufficiency. A similar result was also found in the study of Kim et al.[25], and SDNN decreased with treatment. However, there was no comparison of healthy controls. In our study, SDNN and SDANN values were significantly lower in the patient group compared to the healthy controls, suggesting an increase in sympathetic activity. At the same time, SDNN and SDANN values, which showed sympathetic recovery, increased after treatment with MPH, and this increase was quite close to the healthy control values, especially in SDANN.

Study Limitations

The current study has some limitations. Firstly, it was conducted with a small sample size. Then, the subtypes of the patients were not evaluated, and the HRV was not reviewed again after dose increases during treatment.

CONCLUSION

As a result, the use of MPH in ADHD has a positive effect on the autonomic system with a decrease in sympathetic activity and an increase in parasympathetic activity, and it improves CVS functions. In addition, it can be said that HRV is noninvasive, reproducible and useful for possible risk assessment during treatment. Since our study was conducted with a small sample, it cannot be generalized. However, it can be a guide for larger sample studies to be done in the future.

Ethics

Ethics Committee Approval: The study approval was obtained from the ethics committee of Tekirdağ Namık Kemal University non-interventional clinical studies (2021.189.06.19) and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practices (date: 12.11.2020, no: 2020/68).

Informed Consent: Informed consent was obtained from children and their parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ö.K., S.B., Design: Ö.K., Data Collection or Processing: Ö.K., S.B., Analysis or Interpretation: Ö.K., S.B., Literature Search: Ö.K., S.B., Writing: Ö.K.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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