EISSN 1305-6441

Volume: 86 • Issue: 1 • 2023

iupress.istanbul.edu.tr/en/journal/jmed/home

Indexed in Web of Science SCOPUS

Journal of Istanbul Faculty of Medicine

İstanbul Tıp Fakültesi Dergisi

İSTANBUL UNIVERSITY PRESS

Journal of Istanbul Faculty of Medicine
İstanbul Tıp Fakültesi Dergisi

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Istanbul University, Istanbul Faculty of Medicine, Istanbul, Turkiye

CORRESPONDENCE ADDRESS

Istanbul University, Istanbul Faculty of Medicine Dean's Office,
Publication Commission, 34093 Capa, Fatih, Istanbul, Turkiye
Phone: +90 (212) 414 21 61
E-mail: itfdergisi@istanbul.edu.tr
https://dergipark.org.tr/tr/pub/iuitfd
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PUBLISHER

Istanbul University Press
Istanbul University Central Campus,
34452 Beyazit, Fatih, Istanbul, Turkiye
Phone: +90 212 440 00 00

The publication languages of the journal is English.

This is a scholarly, international, peer-reviewed and open-access journal published quarterly in January, April, July and October.

Publication Type: Periodical

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Address: Istanbul University, Istanbul Faculty of Medicine Deanery, Turgut Özal Cad. 34093, Çapa, Fatih, Istanbul, Turkiye
Phone: +90 212 414 21 61
E-mail: itfdergisi@istanbul.edu.tr

Publisher: Istanbul University Press
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Technical Note	1500	No abstract	15	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	5	1	1

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All acronyms and abbreviations used in the manuscript should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text.

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Book section: Suh KN, Keystone JS. Malaria and babesiosis. Gorbach SL, Barlett JG, Blacklow NR,

editors. Infectious Diseases. Philadelphia: Lippincott Williams; 2004.p.2290-308.

Books with a single author: Sweetman SC. Martindale the Complete Drug Reference. 34th ed. London: Pharmaceutical Press; 2005.

Editor(s) as author: Huizing EH, de Groot JAM, editors. Functional reconstructive nasal surgery. Stuttgart-New York: Thieme; 2003.

Conference proceedings: Bengisson S. Sothemin BG. Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; 1992. pp.1561-5.

Scientific or technical report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, et al. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Early Treatment Diabetic Retinopathy Study KidneyInt: 2004. Report No: 26.

Thesis: Yılmaz B. Ankara Üniversitesindeki Öğrencilerin Beslenme Durumları, Fiziksel Aktivitelerive Beden Kitle İndeksleri Kan Lipidleri Arasındaki Ilişkiler. H.Ü. SağlıkBilimleriEnstitüsü, DoktoraTezi. 2007.

Manuscripts accepted for publication, not published yet: Slots J. The microflora of black stain on human primary teeth. Scand J Dent Res. 1974.

Epub ahead of print articles: Cai L, Yeh BM, Westphalen AC, Roberts JP, Wang ZJ. Adult living donor liver imaging. DiagnIntervRadiol. 2016 Feb 24. doi: 10.5152/dir.2016.15323. [Epub ahead of print].

Manuscripts published in electronic format: Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) 1995 Jan-Mar (cited 1996 June 5): 1(1): (24 screens). Available from: URL: http:/ www.cdc.gov/ncidodlElD/cid.htm.

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Address: Istanbul University, Istanbul Faculty of Medicine Deanery, Turgut Özal Cad. 34093, Çapa, Fatih, Istanbul, Turkiye
Phone: +90 212 414 21 61
E-mail: itfdergisi@istanbul.edu.tr

Publisher: Istanbul University Press
Address: Istanbul University Central Campus, 34452 Beyazıt, Fatih/Istanbul, Turkiye
Phone: +90 212 440 00 00

Journal of Istanbul Faculty of Medicine
İstanbul Tıp Fakültesi Dergisi
Volume: 86 • Issue: 1 • 2023

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RESEARCH ARTICLE

Journal of Istanbul Faculty of Medicine
İstanbul Tıp Fakültesi Dergisi

EDITORIAL

Dear Colleagues,

It gives us great pleasure to wish you a happy new year 2023 from the Journal of Istanbul Faculty of Medicine (J Ist Faculty Med). As we left behind a beautiful and successful year, we are starting a new year with a new issue.

Journal of Istanbul Faculty of Medicine, is a peer-reviewed international journal indexed by ESCI, DOAJ, EBSCO, SCOPUS, and ULAKBİM TR-DİZİN. With great enthusiasm and energy, we are looking forward to being indexed by PUBMED and MEDLINE.

We have a great and collaborative editorial board and section editors who are all highly experienced in their areas of expertise. Our editorial team strives to publish manuscripts at the highest scientific level in all fields of medicine. The journal publishes original experimental and clinical research articles, reports of rare cases, letters to the editors, and invited papers written by authors with a prominent place in the international literature in their field.

In the last few years, we had significant changes in the journal. As of the latest issue of 2021, the publication language of our journal has been changed to English, and beginning from the first issue of 2022; we will continue to be published with our new logo and cover design. In 2023, we plan to start the graphical/visual abstract submission, which is intended to be optional initially.

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We express our gratitude to all the authors who chose our journal to publish their scientific research, our reviewers who did not refuse our referee invitation, our editors, our readers, and IU Press for their interest and efforts in our journal.

We hope we will continue publishing remarkable articles contributing to the literature in the coming years.

Sincerely,

Prof. Dr. Ayşe Kubat Üzüm
Prof. Dr. Birsen Karaman
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RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1211888
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

ASSOCIATING *eNOS* GENE VARIANTS WITH COVID-19 SUSCEPTIBILITY IN THE TURKISH POPULATION

TÜRK POPÜLASYONUNDA COVID-19 DUYARLILIĞI İLE *e*NOS VARYANTLARININ İLİŞKİSİ

Naci ŞENKAL[1], Yasemin OYACI[2], Timurhan CEBECİ[1], Hilal KONYAOĞLU[1], Murat KÖSE[1], Mustafa ÖNEL[3], Alpay MEDETALİBEYOĞLU[1], Gözde YEŞİL SAYIN[4], Mustafa PEHLİVAN[5], Sacide PEHLİVAN[2], Ümmihan İŞOĞLU-ALKAÇ[6], Tufan TÜKEK[1]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine Istanbul, Turkiye
[2]Istanbul University, Istanbul Faculty of Medicine, Department of Medical Biology Istanbul, Turkiye
[3]Istanbul University, Istanbul Faculty of Medicine, Department of Microbiology, Istanbul, Turkiye
[4]Istanbul University, Istanbul Faculty of Medicine, Department of Medical Genetics Istanbul, Turkiye
[5]Gaziantep Univesity, Faculty of Medicine, Department of Haematology, Gaziantep, Turkiye
[6]Istanbul University, Istanbul Faculty of Medicine, Department of Physiology, Istanbul, Turkiye

ORCID IDs of the authors: N.S. 0000-0001-7072-8724; Y.O. 0000-0002-1338-0087; T.C. 0000-0002-0966-2436; H.K. 0000-0003-3036-9143; M.K. 0000-0002-7487-9287; M.Ö. 0000-0002-3987-6611; A.M. 0000-0002-5829-9186; G.Y.S. 0000-0003-1964-6306; M.P. 0000-0002-6692-085X; S.P. 0000-0003-1272-5845; Ü.İ.A. 0000-0003-1992-0109; T.T. 0000-0002-4237-1163

Cite this article as: Senkal N, Oyaci Y, Cebeci T, Konyaogu H, Kose M, Onel M, et al. Associating *eNOS* gene variants with COVID-19 susceptibility in the Turkish population. J Ist Faculty Med 2023;86(1):1-6. doi: 10.26650/IUITFD.1211888

ABSTRACT

Objective: COVID-19 is a serious respiratory and vascular disease that impairs the protective function of the endothelial barrier. Endothelial nitric oxide synthase (eNOS), the most important isoform for nitric oxide (NO) production, is mostly expressed in endothelial cells. Therefore, this study aims to evaluate whether *eNOS* G894T and variable tandem repeat number (VNTR) functional variants show predisposition to developing COVID-19.

Materials and Methods: The study includes a total of 384 subjects (284 COVID-19 patients and 100 healthy controls). Two *eNOS* gene variants (G894T and VNTR) were genotyped using the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods, with the results being evaluated using statistical methods.

Results: A significant association has been identified between *eNOS* G894T and COVID-19. For the eNOS G894T variant, the T/T genotype (p=0.035) and T allele carriers (p=0.030) appear to have an increased risk of developing COVID-19. The eNOS G894T G/G genotype (p=0.030) was more common in the control group compared to the patient group. No significant difference was found between groups regarding the *eNOS* VNTR genotype and allele frequencies (p>0.05). The genotypes of

ÖZET

Amaç: Ciddi bir solunum ve damar hastalığı olan COVID-19 hastalığında endotel bariyerinin koruyucu işlevi bozulmaktadır. Nitrik oksid (NO) üretimi için en önemli izoform olan endotelyal NO sentaz (eNOS), çoğunlukla endotel hücrelerinde eksprese edilir. Bu nedenle, bu çalışma, eNOS G894T ve değişken ardışık tekrar sayısı (VNTR) fonksiyonel varyantlarının COVID-19 hastalığının gelişimine yatkınlık oluşturup oluşturmadığını değerlendirmeyi amaçladı.

Gereç ve Yöntem: Bu çalışmaya toplam 384 birey (284 COVID-19 hastası ve 100 sağlıklı kontrol) dahil edildi. İki *eNOS* gen varyantı (G894T ve VNTR), polimeraz zincir reaksiyonu (PCR) ve/veya kısıtlama fragman uzunluğu polimorfizmi (RFLP) yöntemleri ile genotiplendi. Sonuçlar istatistiksel yöntemlerle değerlendirildi.

Bulgular: eNOS G894T ile COVID-19 hastalığı arasında anlamlı bir ilişki tespit edildi. eNOS G894T varyantı için, T/T genotipi ve T aleli taşıyıcılarının COVID-19 hastalığı için artan riske sahip olduğu görüldü (sırasıyla, p=0,035 ve p=0,030), eNOS G894T G/G genotipi, kontrol grubunda hasta grubuna göre daha yaygındı (p=0.030). eNOS VNTR genotipi ve allel frekansları açısından gruplar arasında anlamlı farklılık yoktu (p>0.05). Bu varyantlar için

Corresponding author/İletişim kurulacak yazar: Timurhan CEBECİ – timurhancebeci19@gmail.com

Submitted/Başvuru: 01.12.2022 • **Revision Requested/Revizyon Talebi:** 01.01.2023 •
Last Revision Received/Son Revizyon: 06.01.2023 • **Accepted/Kabul:** 07.01.2023 • **Published Online/Online Yayın:** 26.01.2023

the patient and control groups for these variants were in Hardy-Weinburg equilibrium (HWE).

Conclusion: These results provide evidence supporting the hypothesis that the eNOS G894T variant is associated with an increased risk of developing COVID-19 in the Turkish population. These findings may lead to the emergence of new treatment options. Further research is required to understand the molecular mechanisms involved in the pathogenesis of the disease.

Keywords: COVID-19, nitric oxide synthase, variant, RFLP-PCR

hasta ve kontrol gruplarının genotipleri HWE'deydi.

Sonuç: Bu sonuçlar, eNOS G894T varyantının Türk popülasyonunda artan COVID-19 riski ile ilişkili olduğu hipotezini destekleyen kanıtlar sağlamıştır. Bulgularımız yeni tedavi seçeneklerinin ortaya çıkmasına yol açabilir. Hastalığın patogenezinde yer alan moleküler mekanizmaları anlamak için daha fazla araştırmaya ihtiyaç vardır.

Anahtar Kelimeler: COVID-19, nitrik oksit sentaz, varyant, RFLP-PCR

INTRODUCTION

COVID-19 is caused by the coronavirus 2 (SARS-CoV-2) and is a respiratory and vascular disease with severe symptoms (1). About 50% of COVID-19 patients are asymptomatic or mildly symptomatic. However, 3-10% of patients require hospitalization, up to 20% of which may result in death (2). A COVID-19 infection involves a cytokine storm, in which large amounts of proinflammatory cytokines are released and an aggressive inflammatory response is observed. In this stage, proinflammatory cytokines increase in the circulatory system (3). The clinical picture shows cell death and diffuse endotheliitis directly related to viral infection to occur in patients who eventually die as a result of rapid worsening and multi-organ failure (4). This indicates COVID-19 to impair the protective function of the endothelial barrier, which allows a systemic viral invasion. Furthermore, altered vascular barrier integrity has been linked to pulmonary endothelial cell injury and the initiation and spread of acute respiratory distress syndrome (ARDS), which is the leading cause of death in COVID patients (4, 5). High levels of D-dimers, which are produced from the coagulation and fibrin degradation caused by endothelial cell death, indicate a poor prognosis in COVID-19 patients. Conversely, a COVID infection may end without thrombotic complications in patients with mild symptoms. This suggests the integrity of endothelial cells to be important in preventing the occurrence of COVID-19 (6).

Nitric oxide (NO) is produced from L-arginine through nitric oxide synthase (NOS) and has an important function in vascular tone and blood pressure control, and NOS has three different types: endothelial (*e*NOS), inducible (*i*NOS), and neuronal (*n*NOS) (7), *e*NOS is mostly expressed in endothelial cells. Systemic hypertension, platelet aggregation, and a number of vascular diseases including thrombosis and atherogenesis have been observed in *e*NOS-/- mice (8). The vasodilatory and platelet aggregation-inhibiting properties of eNOS-derived NO appear to act as an antiviral defense mechanism. By inactivating viral replication proteins, NO either directly or indirectly inhibits viral replication and protein production in host cells (9), SARS-CoV being one of these (10). The *eNOS* gene is localized on the human chromosome 7q36, which consists of 27 exons and encodes a protein of 1,203 amino acids. Many variants have been identified in the *eNOS* gene, with the most investigated variants being G894T in exon 7 (rs1799983) and variable tandem repeat number (VNTR) in intron 4 (rs61722009). These variants can alter the expression and activity of the *e*NOS enzyme (11). Therefore, this study aims to evaluate whether *e*NOS G894T and VNTR functional variants show predisposition to COVID-19 development.

MATERIALS AND METHODS

Study population

The study sample includes 234 unrelated PCR-confirmed patients with COVID-19 and 100 healthy controls. The patients with COVID-19 and the controls were recruited from the internal medicine department of a university research hospital in Türkiye. The diagnosis was confirmed by a positive result obtained from the real-time reverse transcriptase polymerase chain reaction (RT-PCR) of oropharyngeal and nasopharyngeal swab samples taken from patients with a suspected infection. The control group was composed of individuals who, alongside their families, have not been exposed to the virus and/or disease and have had a negative RT-qPCR test for over 7 months. All subjects in this study are older than 18 years and are part of the Turkish population from Türkiye's Marmara region. The university clinical research ethics committee approved the protocol of the study (Date: 08.05.2020, no:09), and the study was carried out in accordance with the Declaration of Helsinki.

Genotyping

DNA isolation was performed from the blood taken from the groups in accordance with the manufacturer instructions using a commercial kit. *e*NOS G894T and the VNTR variants were genotyped using the polymerase chain reaction (PCR) and/or restriction fragment length polymorphism (RFLP) methods, as previously described (12). The *e*NOS G894T variant was identified through PCR using the following primers: (F) 5′ CATGAGGCTCAGCCCCAGAAC-3′ and (R) 5′-AGTCAATCCCTTTGGTGCTCAC-3′. The PCR products were digested overnight by the MboI enzyme (Invitrogen, Carlsbad, CA, USA) at 37°C. Next, the fragments were separated on a 2% agarose gel electrophoresis and visualized using ultraviolet light. The 206 bp products had

a consistent restriction site, resulting in a 119 bp and an 87 bp fragment. The *eNOS* VNTR variant was evaluated through PCR amplification using the primers (F) 5'-AGG-CCCTATGGTAGTGCCTTT-3' and (R) 5' TCTCTTAGTGCT-GTGGTCAC-3'. The amplified products were separated by electrophoresis on a 2% agarose gel and visualized using ethidium bromide staining. The wild-type allele had five tandem repeats of 27 and 420 bp, while the mutant allele had four tandem repeats of 27 and 393 bp.

Statistical analysis

Statistical analysis was performed using the program SPSS (version 22.0) for Windows (SPSS Inc., Chicago, IL, USA). The differences between groups were analyzed using logistic regression analysis, with the odds ratio (OR) at a 95% confidence interval (CI) also being calculated. The *e*NOS genotype distribution of groups was compared using the chi-square test, with Fisher's exact test being used as needed. The Hardy-Weinberg equilibrium (HWE) was then calculated, with a *p*-value less than 0.05 being considered statistically significant.

RESULTS

The 334 subjects (234 COVID-19 patients and 100 healthy controls) were evaluated for the *eNOS* G894T and VNTR variants. The distribution of the genotypes and alleles of the *eNOS* G894T and VNTR variants is presented in table 1. The *eNOS* G894T genotype and allele comparison between groups showed a significant difference. For *e*NOS G894T, a higher frequency was observed for the G/T (*p*=0.035; OR=9.469, 95% CI[1.170, 76.603]) and T/T genotypes (*p*=0.013; OR=8.313, 95% CI[1.106, 62.494]) in the patients compared to the controls. The *e*NOS G894T variant G/G genotype was more common in the control group compared to the patient group (*p*=0.030; OR=9.883, 95% CI[1.243, 78.578]).

The prevalence of the genotypes 4b/4b, 4a/4b, and 4a/4a profiles for the *e*NOS VNTR variant were 69%, 28.2%, and 2.8% in patients, and 68%, 28%, and 4% in the control group, respectively. No significant difference was observed between groups regarding the *e*NOS VNTR genotype and allele distribution (p>0.05). The genotypes of both groups regarding the *e*NOS G894T and VNTR were in HWE.

DISCUSSION

COVID-19 has a heterogeneous clinical phenotype, in which severe symptoms such as endothelial inflammation, thromboembolic complications, acute respiratory distress syndrome (ARDS), and multiple organ failure

Table 1. Genotype and allele distribution of *eNOS* gene variants in patients and controls

eNOS G894T	Patients n=284 (%)	Controls n=100 (%)	OR Exp (B)	95% CI	p*
Genotypes		n=100 (%)			
G/G	146 (51.4)	63 (63)	9.883*	1.243-78.578*	**0.030***
G/T	116 (40.8)	36 (36)	9.469*	1.170-76.603*	**0.035***
T/T	22 (7.8)	1 (1)	8.313[&]	1.106-62.494[&]	**0.013[&]**
Alleles					
G	408 (71.8)	164 (81.0)	1.672[&]	1.123-2.489[&]	**0.011[&]**
T	160 (28.2)	38 (19.0)			
HWEp	0.875	0.089			
eNOS VNTR	**Patients n=284 (%)**	**Controls n=100 (%)**	**OR Exp (B)**	**95% CI**	**p***
Genotypes					
4b/4b	196 (69.0)	68 (68.0)	0.696[&]	0.205-23.62[&]	0.519[&]
4b/4a	80 (28.2)	28 (28.0)	1.179*	0.663-2.095*	0.576*
4a/4a	8 (2.8)	4 (4.0)	1.076*	0.293-3.953*	0.912*
Alleles					
4b	472 (83.1)	164 (82.0)	0.927[&]	0.607-1.413[&]	0.744[&]
4a	96 (16.9)	36 (18.0)			
HWEp	0.962	0.606			

HWEp: *Hardy*-Weinberg Equilibrium *:OR (95%CI) adjusted for age and gender, [&]Fisher's Exact Test. Statistically significant results are in bold.

may occur, as well as asymptomatic or mild findings (13). The endothelial cell layer between the blood and tissues forms an anatomical barrier against viruses in the body. The endothelium is found in many organs in the human body and provides vascular homeostasis through the interaction of the vessel walls and cells in the lumen. The endothelium also balances the production of NO, which is the most important compound in vasodilators, and thus regulates vascular tone (14). Permeable viruses such as viremic SARS-CoV-2 (80–100μnm in size) have the ability to invade the local tissue underneath the endothelium, and endothelial dysfunction is usually seen in infections caused by highly pathogenic coronaviruses (15). This feature of SARS-CoV-2 infections damages pulmonary and other vascular endothelia (7). In endothelial dysfunction, the bioavailability of NO generally decreases, while endothelin-1 (ET1), angiotensin II (Ang II), and other similar vasoconstrictor substances increase (16).

NO plays a role in regulating immune responses against pathogens and also regulates the cellular function, growth, and death of various immune cells (17). Although NO has a protective role in viral infections, it may contribute to the molecular mechanism of COVID-19. Pathophysiological conditions that allow NO release to lead to the formation of reactive oxygen species (ROS) (18). Excess ROS produced by endothelial and epithelial cells and leukocytes is important in ARDS progression and lung injury. The majority of vascular NO production is mediated by *e*NOS. The *eNOS* gene is highly polymorphic and has several variants, such as single nucleotide polymorphisms (SNPs), insertions/deletions, VNTRs, and microsatellites (19). The *e*NOS G894T variant resulting from a substitution of thymine for guanine at position 894 in exon 7 of the *eNOS* gene causes a glutamine-aspartate exchange at position 298 of the protein (20). This functional variant leads to posttranslational modifications. The presence of the allele-specific primers (ASP) in this variant reduces *e*NOS levels by reducing the binding of *e*NOS to caveolin-1 (21). This effect results in reduced *e*NOS activity and NO formation (21). *In vitro* studies have reported that carriers of the variant allele reduce platelet NO production compared to those carrying the wild allele. As another variant in the current study, the 4b/4a VNTR variant in intron 4 transcriptionally regulates *e*NOS by altering small interfering RNA (siRNA) formation (22). The most common alleles in this variant are those with five (4b variant) or four (4a variant) copies of the 27-bp DNA fragment (23). *In vitro* studies have indicated the 4b variant to show higher amounts of siRNA in endothelial cells, resulting in lower *e*NOS mRNA levels compared to the 4a variant (24). Various studies are found to have examined the associations these variants have with different diseases, such as essential hypertension, prostate cancer, colorectal cancer, and recurrent spontaneous abortion (25-28). Zhao et al. reported *e*NOS G894T to be related to *Mycoplasma pneumoniae* in Chinese children (29). A meta-analysis study found *e*NOS G894T genotype frequencies and the T allele to differ between high-altitude pulmonary edema patients and controls in Asians (30). A study on enterovirus 71 (EV71) found the T allele of *e*NOS G894T to be related to EV71 infection (31). That same study found low NO levels for people infected with EV71 who have the T allele. The *e*NOS G894T and VNTR genotype and allele distribution have been reported to not differ between sepsis patients and controls in Turkish patients (32). Koskela et al. reported the *e*NOS G894T TT genotype to be associated with the severity of Puumala hantavirus (PUUV) infection, with infected patients possessing the TT homozygous genotype to show more severe clinical presentations and longer hospital stays compared to other genotypes (33). A study comparing severe COVID-19 and mild COVID-19 patients found the distribution of the *e*NOS G894T genotype frequencies to not differ between the two groups (34). A previous study also found the genotype and allele distribution of *e*NOS G894T and VNTR to not different between PCR-negative COVID-19 patients and controls (35).

This study has investigated the association of *eNOS* G894T and intron 4 VNTR variants with the risk of COVID-19 and has found a significant difference to exist between patients and controls regarding *e*NOS G894T, with the *e*NOS G894T TT genotype and T allele being more common in patients compared to controls. The patients carrying the T allele were also found to be predisposed to developing COVID-19. The study has shown the G894T variant in exon 7 to cause a mature NOS protein that is sensitive to intracellular division, which may reduce functional *e*NOS activity in those possessing the T allele (36). The *e*NOS VNTR variant genotype and allele distribution revealed no difference between the patient and control groups.

This study has certain limitations. First, it was carried out only with patients living in a particular area of Türkiye. Different results may be obtained by studying people living in different regions of Türkiye. Second, the study only evaluated two variants in the *eNOS* gene. Other functional variants in the *eNOS* gene may also contribute to disease occurrence. In addition, gene-environment interactions may also need to be evaluated.

In conclusion, having sufficient knowledge about the etiology and pathogenic processes associated with COVID-19 is just as important as knowing the factors that play a role in the disease. This information can contribute to estimating the risk of COVID-19 disease and taking more effective measures. Genetic variants play a role in the susceptibility to many diseases such as cancer, autoimmune diseases, and infections. Studies have shown various genetic variants and environmental factors to influence the course of COVID-19. The findings from this

study support the hypothesis that *e*NOS G894T is linked to the occurrence of COVID-19. Individuals who carry the *e*NOS G894T, T/T genotype, and T allele were found to be more likely to develop COVID-19, and this study's findings may lead to the emergence of new treatment options. Further research is required to understand the molecular mechanisms involved in the pathogenesis of the disease.

Ethics Committee Approval: This study was approved by Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 21/05/2020, No: 61754).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- Ü.İ.A., N.S., H.K., T.C., A.M., M.K., Y.O.; Data Acquisition- G.B., G.Y.S., H.K.; Data Analysis/Interpretation- M.P., G.Y.S., H.K.; Drafting Manuscript- N.S., Y.O., A.M.; Critical Revision of Manuscript- S.P., N.S., M.Ö.; Final Approval and Accountability- N.S., T.T.; Material or Technical Support- T.T., M.P., M.O., Ü.İ.A.; Supervision- T.T., S.P.

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

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

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RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1175498
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

REGIONAL INVOLVEMENT IN LEFT VENTRICULAR STRAIN IN PATIENTS RECOVERED FROM COVID-19 PNEUMONIA

COVID-19 PNÖMONİSİ GEÇİRMİŞ HASTALARDA SOL VENTRİKÜL STRAİN DEĞERİNİN BÖLGESEL TUTULUMU

Ekrem Bilal KARAAYVAZ[1], Berat ENGİN[2], Pelin KARACA ÖZER[1], Zeynep Gizem DEMİRTAKAN[1], Elif AYDUK GÖVDELİ[1], Türker DEMİRTAKAN[4], Derya BAYKIZ[1], Alpay MEDETALİBEYOĞLU[3]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Cardiology, Istanbul, Turkiye
[2]Manavgat State Hospital, Department of Cardiology, Antalya, Turkiye
[3]Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine, Istanbul, Turkiye
[4]Kanuni Sultan Suleyman Educational Research Hospital, Istanbul, Turkiye

ORCID IDs of the authors: E.B.K. 0000-0002-0105-6167, B.E. 0000-0001-7805-375X; P.K.Ö. 0000-0002-1085-5462; Z.G.D. 0000-0003-2401-3837; E.A.G. 0000-0002-6595-4812; T.D. 0000-0002-4075-5518; D.B. 0000-0003-0666-6631; A.M. 0000-0002-5829-9186

Cite this article as: Karaayvaz EB, Engin B, Karaca Ozer P, Demirtakan ZG, Ayduk Govdeli E, Demirtakan T, et al. Regional involvement in left ventricular strain in patients recovered from COVID-19 pneumonia. J Ist Faculty Med 2023;86(1):7-13.
doi: 10.26650/IUITFD.1175498

ABSTRACT

Objective: COVID-19 patients with cardiovascular involvement have been shown to have a worse prognosis compared to those without cardiovascular compromise. This study aimed to investigate whether left ventricular (LV) global and regional strain is impaired in patients with COVID-19 with or without pneumonia after discharge.

Materials and Methods: Seventy-eight consecutive COVID-19 patients diagnosed by PCR test were enrolled in this cross-sectional study during their first follow-up visit to an outpatient clinic. All patients underwent two-dimensional echocardiography and speckle tracking echocardiography (STE) at the first follow-up visit. The patients were divided into two groups with or without pneumonia, and they were compared with the healthy control group.

Results: A total of 123 subjects were included in the study (78 with COVID-19 and 45 in the control group). Admission and follow-up hs-troponin-T concentrations were similar in both the control group and patients with varying severity of COVID-19. LV ejection fraction (EF) was similar in all groups. However, LV global longitudinal strain (GLS) was significantly lower in subjects with pneumonia compared to the control group and subjects without pneumonia. Regional strain analysis showed that subjects with pneumonia had significantly lower strain values at mid-anterior, mid-anteroseptal, apical-inferior, apical-lateral, and apex regions than subjects without pneumonia or the control group.

Conclusion: LV GLS and the regional strain were significantly impaired in COVID-19 patients with pneumonia compared to

ÖZET

Amaç: Kardiyovasküler tutulumu olan COVID-19 hastalarının, kardiyovasküler tutulumu olmayanlara göre daha kötü prognoza sahip olduğu gösterilmiştir. Bu çalışma, pnömonisi olan ve olmayan COVID-19 hastalarında taburculuk sonrası sol ventrikül (LV) global ve bölgesel strain değerinin bozulup bozulmadığını araştırmaktadır.

Gereç ve Yöntem: Bu kesitsel çalışmaya, PCR testi ile tanı konulan ve polikliniğimize başvuran 78 hasta dahil edildi. Tüm hastalara ilk muayenelerinde iki boyutlu ekokardiyografi ve speckle tracking ekokardiyografi (STE) yapıldı. Hastalar pnömonisi olan ve olmayan olarak iki gruba ayrıldı ve sağlıklı kontrol grubu ile karşılaştırıldı.

Bulgular: Çalışmaya toplam 123 kişi alındı (78 COVID-19 hastası, 45 sağlıklı kontrol). Hem kontrol grubunda hem de farklı ciddiyetteki COVID-19 hastalarında ilk ve takip hs-troponin-T değerleri benzerdi. Sol ventrikül ejeksiyon fraksiyonu (EF) tüm gruplarda benzerdi. Bununla birlikte, sağlıklı kontroller ve pnömonisi olmayan hastalara kıyasla pnömonisi olan hastalarda LV global longitudinal straini (GLS) anlamlı ölçüde daha düşüktü. Bölgesel strain analizi, pnömonisi olmayan hastalara ve kontrol grubuna kıyasla pnömonisi olan hastaların mid-anterior, mid-anteroseptal, apikal-inferior, apikal-lateral and apeks bölgelerinde önemli ölçüde daha düşük strain değerlerine sahip olduğunu göstermiştir.

Sonuç: Pnömonisi olmayan hastalara ve sağlıklı kontrollere kıyasla pnömonisi olan COVID-19 hastalarında sol ventrikül global ve bölgesel strain önemli ölçüde bozulmuştur. Bu bulgu, pnömonisi olan COVID-19 hastalarının, gizli sol ventrikül tutulumunun tes-

Corresponding author/İletişim kurulacak yazar: Zeynep Gizem DEMİRTAKAN – zeyneptopcak@gmail.com

Submitted/Başvuru: 14.09.2022 • **Revision Requested/Revizyon Talebi:** 26.09.2022 •
Last Revision Received/Son Revizyon: 12.10.2022 • **Accepted/Kabul:** 21.11.2022 • **Published Online/Online Yayın:** 27.01.2023

those without pneumonia or in to control group. This finding indicates that COVID-19 subjects with pneumonia should undergo strain measurement to detect concealed LV involvement.

Keywords: COVID-19, pneumonia, left ventricle, regional strain

piti için strain ekokardiyografi ile değerlendirilmesi gerektiğini göstermektedir.

Anahtar Kelimeler: COVID-19, pnömoni, sol ventrikül, bölgesel strain

INTRODUCTION

COVID-19 is a multisystem disease that predominantly affects respiratory system (1). During the course of the disease, several systems including cardiovascular system are involved in many aspects (2, 3). First patients in Wuhan diagnosed with COVID-19 had myocardial involvement with an increase in high-sensitivity troponin I (hs-cTnI) levels (4, 5). Most of those patients with myocardial involvement were further admitted to the intensive-care unit (ICU), which indicates a more serious course of COVID-19 occurs when myocardial involvement is added to the pulmonary involvement (4).

Patients with cardiovascular risk factors are shown to be susceptible to more serious disease with wide-range of cardiac and cardiovascular involvements such as myocarditis, myocardial infarctions, and pulmonary embolism (6-9). Moreover, COVID-19 patients with elevated cardiac markers tend to have unfavorable prognosis (10). Although cardiac involvement by elevated cardiac biomarkers is observed in a substantial subset of patients with poor prognosis, transthoracic echocardiography (TTE) is normal or near-normal in many of these patients.

Strain imaging, which measures regional myocardial deformation of the ventricular myocardium, has been shown to detect myocardial involvement in hypertension, diabetes mellitus, and various systemic diseases prior to apparent changes in TTE (11-13). Limited data encourages the utilization of strain imaging for detection of myocardial involvement in COVID-19 patients. Recently, it was demonstrated that left ventricular global longitudinal strain (LV-GLS) was decreased in patients hospitalized for severe COVID-19 in comparison to non-severe COVID-19 patients despite similar LV ejection fraction (EF) (14). However, extensive evidence is still lacking concerning the role of strain imaging to detect myocardial involvement in patients with COVID-19 especially after recovery.

This study purposed to investigate the subclinical cardiovascular involvement by regional speckle tracking after recovery in COVID-19 patients without apparent myocardial involvement on TTE.

MATERIALS AND METHODS

Study design and participants

Seventy-eight consecutive COVID-19 patients who were diagnosed by PCR test admitted to our institute and discharged between March 2020 and June 2020 were enrolled in this cross-sectional study during their first follow-up visit to the outpatient clinic. Forty-five healthy individuals were enlisted as the control group. Patients with a history of prior cardiovascular disease (ischemic heart disease, heart failure, dilated or hypertrophic cardiomyopathy, and severe valvular disease) and those with pre-existing systemic disease that has cardiovascular involvement were taken out of the study.

Demographic features of the study group and the agents administered for the treatment of COVID-19 were recorded for each patient. Venous blood samples were drawn upon admission and at first follow-up visit in the outpatient clinic for complete blood count and measurement of high-sensitive troponin-T (hs-TnT), NT-proBNP, C-reactive protein (CRP), and serum ferritin concentration. All patients underwent TTE at the first follow-up visit. Patients with apparent cardiac involvement and regional wall abnormality on TTE were excluded. Written informed consent was taken from all participants. The study was conducted in accordance with the Helsinki declaration and approved by the local ethics committee (Date: 25/09/2020, No: 2020/1185).

Participants were categorized into three groups as follows: the control group, patients without pneumonia, and patients with pneumonia.

Echocardiography and strain imaging

2D TTE was performed on all patients with a iE33xMATRIX ultrasound system (Philips Medical Systems, Andover, Massachusetts) with a X5-1 (1-5 MHz) transducer according to the guidelines recommendations. All views were recorded as digital images and then reanalyzed. Global longitudinal strain (GLS) of the LV were measured by 2D STE in all patients. The CMQ option of the Philips IE33, QLAB 10.8.5 software was used for deformation analysis. Three consecutive cardiac cycles from the apical 4-, 3-(long axis), and 2-chamber views were obtained at 42–56 frames per second and then stored. First, for each view, the operator placed three points (two points at the base of the LV and one point at the apex) at the end of diastole. The endocardial and epicardial borders were then automatically traced by the software. Adjustments were made by the operator if required. Each wall of the LV was segmented into three (base, mid, and apical) equal parts automatically, and 17 segmental strain curves were obtained to give the so-called bull's-eye plots (figure 1).

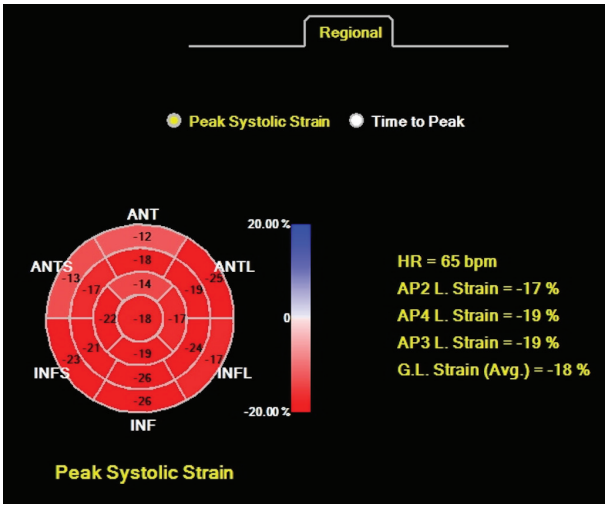

Figure 1: Bull's eye image of LV regional longitudinal strain with speckle-tracking imaging

Statistical analysis

All analyses were performed on SPSS v21 (SPSS Inc., Chicago, IL, USA). Shapiro-wilk test was used for testing data distribution. Data are presented as mean ± standard deviation or median (min-max) for continuous variables according to the normality of distribution and as frequency (percentage) for categorical variables. Mann Whitney U and Kruskall Wallis tests were used for group comparisons. Categorical variables were compared using the Pearson chi-square test. Pearson and Spearman's correlation analysis were performed to identify the relation between the myocardial strain and markers of myocardial involvement and severity of COVID-19. P value of <0.05 was accepted to be statistically significant.

RESULTS

One hundred twenty-three subjects were enrolled in the study (78 in COVID-19, 45 in the control group). Table 1 shows demographic characteristics and laboratory measurements of COVID-19 patients and the control groups. COVID-19 patients and control group were similar with respect to age, diabetes mellitus, hypertension, chronic obstructive pulmonary disease, smoking and heart rate. Admission and follow-up hs-TnT concentrations were similar in both the control group and in patients with varying severity of COVID-19.

Table 2 shows standard TTE measurements. Subjects with COVID-19 had higher left atrium (LA) diameter compared to control group. Like LA diameter, E/A ratio was lower in subjects with COVID-19 compared to control group. Nevertheless, LV EF was similar in all groups. LV-GLS was lower in subjects with pneumonia compared to control group and subjects without pneumonia. Regional strain analysis, as shown in table 3, showed that subjects with pneumonia had significantly lower strain values at mid-anterior, mid anteroseptal, apicoinferior, apicolateral and apical regions compared to control group and to subjects without pneumonia.

ROC curve analysis demonstrated that hs-TnT value above 7.065 pg/ml on admission was predictive for a decrease in LV-GLS with 54.1% sensitivity, 76.3% specificity (p=0.008, AUC 0.679, 95% CI 0.557-0.801) (figure 2).

DISCUSSION

This observational cross-sectional study sought to evaluate changes in LV global and regional strain in COVID-19 patients with apparently normal or near normal LV EF. Our findings reveal that, although not statistically significant, COVID subjects with pneumonia have higher hs-TnT concentration both on admission and follow-up compared to control group and to subjects without pneumonia. The patients and control group had similar LV EF. However, LV-GLS and regional strain was significantly impaired in COVID-19 patients with pneumonia compared to the other groups. Given the worse prognosis in COVID-19 patients with cardiovascular involvement, this finding indicates that COVID-19 subjects with pneumonia should undergo strain measurement for detection of concealed LV involvement.

COVID-19 is a multi-systemic disease predominantly affecting respiratory system. Among the systems affected by COVID-19, cardiovascular involvement has been shown to predict mortality. Conventionally, cardiac biomarkers including Troponin-I and –T and cardiovascular imaging have been used to evaluate cardiovascular involvement in COVID-19. Elevation in cardiac biomarkers have been reported to increase mortality in these patients (10). However, in a substantial proportion of patients, 2D-TTE is normal despite a clear elevation in cardiac biomarkers. The study of Clark et al. which enrolled 22 collegiate athletes with prior mildly symptomatic or asymptomatic COVID-19 infection has reported late gadolinium enhancement by CMR in 9% of the subjects (18). Given the predictive role of cardiovascular involvement on mortality in COVID-19, advanced imaging techniques including STE may be helpful to identify patients with cardiovascular involvement, but apparently normal on TTE.

STE is one of the advanced echocardiographic techniques which may provide further data concerning the LV function in patients with COVID-19. Accumulated evidence indicates that LV GLS can be used to estimate global LV myocardial tissue damage (17, 19). Regional and global LV function can be determined using strain analysis. Several studies have shown that strain analysis could be utilized to detect LV ischemia even in asymptomatic patients (20). However, its utility in COVID-19 patients with normal LVEF has not been investigated extensively.

Table 1: Demographic features and laboratory measurements of the study groups

	Control group (n=45)	Without pulmonary involvement (n=35)	With pulmonary involvement (n=43)	p-value
Age (year)	46.5±13.3	48.3±13.7	50.7±13.7	0.340
Gender, n (%) Male Female	14 (31.1%) 31 (68.9%)	17 (48.6%) 18 (51.4%)	26 (60.5%) 17 (39.5%)	0.021
HT, n (%)	11 (24.4%)	13 (37.1%)	11 (25.6%)	0.401
DM, n (%)	6 (13.3%)	5 (14.3%)	8 (19%)	0.740
COPD, n (%)	3 (6.7%)	4 (11.4%)	3 (7%)	0.699
Smoker, n (%)	16 (35.6%)	9 (25.7%)	9 (20.9%)	0.295
HR (bpm)	76.2±13.5	78.9±12.8	82.1±13.7	0.136
Laboratory findings at hospital admission				
Hgb (gr/dl)	13.3 (11-16)	13.1 (8-17)	12.8 (8-17)	0.043
Leukocyte (10^3/µl)	7 (4.4-13.5)	5.9 (0.8-9.8)	4.6 (3.6-16)	0.060
Lymphocytes (10^3/µl)	2.2 (1.1-6.9)	1.5 (0.2-2.7)	1 (0.1-5)	
Hs-troponin-T (pg/ml)	3.5 (3-30)	4 (2-59)	6.1 (2-37)	0.713
Pro-BNP (pg/ml)	34.5 (4-209)	80.8 (7-711)	78 (12-793)	0.245
CRP (mg/L)	2 (0-24)	23.5 (1.6-235)	29.3 (0-127)	
D-dimer (µg/L)	475 (210-1330)	450 (270-7340)	900 (320-18550)	0.182
Ferritin (ng/ml)	32 (3-212)	942 (167-1718)	1396 (338-1654)	0.411
Laboratory findings after discharge				
Hgb (gr/dl)	13 (11-17)	12.8 (8.5 -15.9)	13.2 (8.8-16.4)	0.135
Leukocyte (10^3/µl)	6.9 (4.4-13.5)	5.9 (2.4-10.8)	6.8 (4.1-19.6)	0.052
Lymphocytes (10^3/µl)	2.3 (1.1-6.9)	1.8 (0.5-3.8)	2.3 (1.1-4.5)	0.007
Hs-troponin-T (pg/ml)	3.5 (3-30)	3.8 (1-59)	5.9 (2-37)	0.198
Pro-BNP (pg/ml)	34 (4-803)	56.2 (5-1674)	64.2 (5-621)	0.413
CRP (mg/L)	2 (0-24)	1.4 (0-17)	3.3 (0-39)	0.065
D-dimer (µg/L)	435 (210-1330)	315 (180-920)	365 (170-2600)	0.175
Ferritin (ng/ml)	32 (3-212)	34.2 (7-359.5)	160 (51-1010)	0.018
Treatment				
Hydroxychloroquine, n (%)	-	35 (100%)	43 (100%)	
Azithromycin, n (%)	-	12 (34.3%)	21 (48.8%)	
Favipiravir, n (%)	-	5 (14.3%)	10 (23.3%)	
Steroid, n (%)	-	0	5 (11.6%)	
Immune modulator, n (%)	-	7 (20%)	10 (23.3%)	
Antibiotics, n (%)	-	17 (48.6)	17 (39.5%)	
Hospital stay (days)	-	3.2 (0-18)	6.1 (0-33)	

DM: Diabetes Mellitus, COPD: Chronic obstructive pulmonary disease, HR: Heart Rate, CRP: C-reactive protein

Baycan et al. have studied STE in 100 patents admitted with COVID-19 (14). The authors divided COVID-19 patients into subgroups according to the severity of pulmonary involvement They reported that LV GLS and right ventricular (RV) longitudinal strain were significantly impaired in COVID-19 patients with severe pulmonary involvement compared to patients with non-severe pulmonary involvement. The authors also reported that LV GLS and RV longitudinal strain was independently predictive for mortality (14).

Our findings show that LV GLS and regional strain is significantly impaired in patients with pneumonia compared to control group and those without pneumonia. Although there were no statistically significant differences in hs-TnT concentration among the groups; patients with

Table 2: 2D Transthoracic echocardiography and global longitudinal strain analysis of the groups

	Control group (n=45)	Without pulmonary involvement (n=35)	With pneumonia (n=43)	p-value
LVEDV (ml)	127.1±25.5	139.1±23.6	139.9±24.9	0.030[b]
LVESV (ml)	49.0±14	53.9±13.9	55.7±10.2	0.145
EF (%)	65.1±4.3	65.3±5.1	64.5±4.6	0.721
LV mass index (ml/m^2)	99.2±24.4	102.4±27.6	100.3±24	0.872
LVEDD (mm)	44±4.1	45.6±3.8	45.7±4	0.103
LA (mm)	32.9±4.3	35.9±4.3	36.6±4.4	0.001[a,b]
RV (mm)	25.9±2.6	26.8±2.1	27.3±2.2	0.028[b]
RA (mm)	31.1±2.9	31.2±3.4	31.9±3	0.428
E/A ratio	1.2±0.4	1±0.3	0.9±0.3	0.000
E/e' ratio	8.5±2.6	9±3.8	8.9±2.6	0.618
LVGLS (%)	-18.1±2.8	-18.1±3.8	-15.5±3.2	0.000[b,c]
LVS-2C (%)	-18±3.8	-18.5±5.6	-14.5±6.8	0.002[b,c]
LVS-3C (%)	-17.4±3.7	-17.7±4.4	-14.6±4.1	0.001[b,c]
LVS-4C (%)	-19.1±3.6	-18.9±4.7	-16.2±3.8	0.001[b,c]
LAVI	18.8±6.3	20±7.6	18±5.5	0.473
TAPSE (mm)	22.6±3.4	21.8±3.5	20.9±3.8	0.81
sPAP (mmHg)	18±4.7	26±5.5	26.6±4.7	0.000[a,b]

[a]: p<0.05 between the control group and the subjects without pneumonia; [b]: p<0.05 between the control group and the subjects with pneumonia; [c]: p<0.05 between the subjects without pneumonia and the subjects with pneumonia

Table 3: Regional strain analysis of the study groups

	Control group (n=45)	Without pulmonary involvement (n=35)	With pneumonia (n=43)	p-value
Basal anterior (%)	-19.6±9.4	-20.9±7.5	-18.6±9,5	0.543
Basal anteroseptal (%)	-18.1±6.7	-16.5±8.5	-14.1±8.8	0.066
Basal inferoseptal (%)	-16.7±6.5	-14.6±7.2	-14.8±6.9	0.294
Basal inferior (%)	-20.2±9.3	-15.4±9.1	-17±6.4	0.035[a]
Basal inferolateral (%)	-19.8±8.3	-20±9.2	-18±7.8	0.494
Basal anterolateral (%)	-20.9±7.5	-20.3±9.4	-19.5±6.3	0.688
Mid anterior (%)	-18.1±9.6	-17.7±9.9	-12.6±9	0.015[b]
Mid anteroseptal (%)	-21.4±7.8	-17.9±12.5	-15.2±10.2	0.020[b]
Mid inferoseptal (%)	-23.5±6.5	-23.3±8.9	-20±8.4	0.079
Mid inferior (%)	-20.3±8.8	-21.5±9.3	-19.6±9.8	0.679
Mid inferolateral (%)	-19.3±8.2	-19.3±11.3	-16.1±10.9	0.263
Mid anterolateral (%)	-21.8±9	-20±8.6	-18±9.3	0.150
Apical anterior (%)	-16.2±7.5	-16.6±7.4	-14±5.6	0.179
Apical septal (%)	-22.3±5.9	-22.2±8.8	-19.5±8.7	0.168
Apical inferior (%)	-22.1±8.8	-25±6.2	-19.4±7.1	0.007[c]
Apical lateral (%)	-17.5±8	-19.6±6.4	-14±5.4	0.001[b,c]
Apex (%)	-18.7±5	-20.1±4.8	-16.1±3.3	0.000[b,c]

[a]: p<0.05 between the control group and the subjects without pneumonia; [b]: p<0.05 between the control group and the subjects with pneumonia; [c]: p<0.05 between the subjects without pneumonia and the subjects with pneumonia

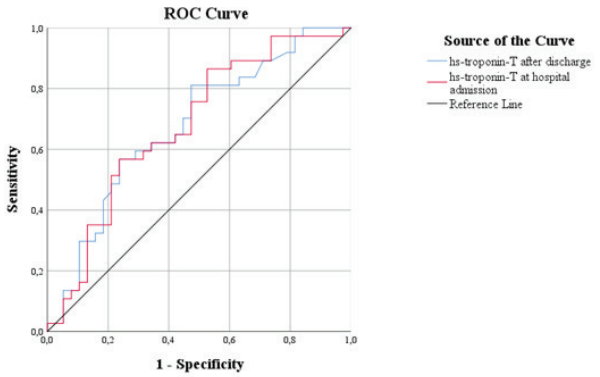

Figure 2: ROC curve demonstrating the sensitivity and specificity of high-sensitive TroponinT for predicting a decrease in LV global longitudinal strain

pulmonary involvement tended to have slightly increased hs-TnT concentrations compared to those without pulmonary involvement. With this in mind, we suggest that COVID-19 patients with pulmonary involvement should not only receive 2D-TTE, but also undergo strain echocardiography to detect myocardial damage which is indicated by LV GLS and regional strain.

In our study, COVID-19 patients and the control group had similar LVEF. LV GLS and regional strain is significantly impaired in COVID-19 patients with pneumonia compared to those without pneumonia or to healthy control group. Strain analysis may be helpful in detection of cardiovascular involvement of COVID-19 patients with normal LVEF.

CONCLUSION

Cardiovascular involvement has been shown to predict mortality in COVID-19. LV-GLS and regional strain was significantly impaired in COVID-19 patients with pneumonia compared to those without pneumonia or to healthy control group. COVID-19 subjects with pneumonia should undergo strain measurement for detection of concealed LV involvement.

Ethics Committee Approval: This study was approved by Istanbul University Clincial Research Ethics Committee (Date: 25.09.2020, No: 2020/1185).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- E.B.K., B.E., D.B.; Data Acquisition- B.E., P.K.Ö., E.A.G., A.M.; Data Analysis/Interpretation- B.E., Z.G.D., T.D.; Drafting Manuscript- E.B.K., P.K.Ö., E.A.G.; Critical Revision of Manuscript- B.E., Z.G.D., T.D., D.B., A.M.; Final Approval and Accountability- E. B.K., B.E., P.K.Ö., Z.G.D., E.A.G., T.D., D.B., A.M.; Material or Technical Support- P.K.Ö.; Supervision- E.B.K.

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

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

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Society of Echocardiography, American Society of Nuclear Cardiology, Heart Rhythm Society, Society for Cardiovascular Angiography and Interventions, Society of Cardiovascular Computed Tomography, Society for Cardiovascular Magnetic Resonance, and the Society of Thoracic Surgeons. J Am Coll Cardiol 2019;73(4):488-516. [CrossRef]

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DOI: 10.26650/IUITFD.1168211
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

TURKISH DIALYSIS HEALTHCARE PROVIDERS' PSYCHOLOGICAL RESPONSE TO COVID-19

TÜRK DİYALİZ SAĞLIK ÇALIŞANLARINDA COVID-19'A BAĞLI GÖRÜLEN RUHSAL TEPKİLER

Irmak POLAT[1], Mehmet Şükrü SEVER[2], Erol DEMİR[2], Halil YAZICI[2], Serkan Kubilay KOÇ[3], Rabia PAPİLA[3], Mine ÖZKAN[1]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Psychiatry, Istanbul, Turkiye
[2]Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine, Division of Nephrology, Istanbul, Turkiye
[3]Private clinic, Istanbul, Turkiye

ORCID IDs of the authors: I.P. 0000-0003-3631-6018; M.Ş.S. 0000-0002-6074-2239; E.D. 0000-0003-0128-5645; H.Y. 0000-0003-2526-3483; S.K.K. 0001-8634-1583; R.P. 0000-0002-6909-3159; M.Ö. 0000-0002-2981-9541

Cite this article as: Polat I, Sever MS, Demir E, Yazici H, Koc SK, Papila R, et al. Turkish dialysis healthcare providers' psychological response to COVID-19. J Ist Faculty Med 2023;86(1):14-27. doi: 10.26650/IUITFD.1168211

ABSTRACT

Objective: COVID-19 has been a stressful experience for healthcare providers (HCPs) and created additional distress for dialysis HCPs due to patients' higher risk of infection, symptom severity, and death. This study aims to investigate Turkish dialysis HCPs' levels of psychological difficulties during COVID-19's initial outbreak.

Materials and Methods: The study has recruited physicians, nurses, and healthcare workers in dialysis centers. The participants completed an online survey that includes the screening questionnaire, Depression Anxiety Stress Scale-21 (DASS-21), and Multidimensional Scale of Perceived Social Support (MSPSS). The study conducts the chi-square test, Fisher's exact test, Mann-Whitney U test, Kruskal Wallis H test, Spearman correlation, and linear regression analyses.

Results: The study involves 953 respondents, with nurses making up the majority (n=465, 48.8%), followed by healthcare workers (n=402; 42.2%) and physicians (n=86; 9%). HCPs' most significant concerns were getting infected with COVID-19 and transmitting the disease to their loved ones. Single participants, those without children, those who had trouble finding equipment, and those worried about being able to find equipment in the future, being in contact with COVID-19 (+) people, those whose tobacco and alcohol use increased, and those who declared sleep, appetite, and/or somatic problems had higher DASS-21 scores. When compared respectively to healthcare workers and physicians, nurses were found to be more worried about getting COVID-19 (94.6% compared to 90.6% and 84.7%; $p < 0.001$), experience equipment shortages (52.9% compared to 29.4% and 26.3%; $p<0.001$), have sleep (62.2% compared to 43.5% and

ÖZET

Amaç: COVID-19, sağlık çalışanları için psikolojik sorunların başlamasına veya kötüleşmesine yol açmıştır. Hastaların enfeksiyon, semptom şiddeti ve ölüm riskinin daha yüksek olması nedeniyle COVID-19, diyaliz çalışanları üzerinde ek bir stres oluşturmuştur. Bu çalışmada, salgının erken döneminde Türk diyaliz çalışanlarındaki psikolojik zorlanma ve ilişkili etmenleri araştırmayı amaçladık.

Gereç ve Yöntem: Çalışmaya Türkiye'deki özel ve kamu diyaliz merkezlerinden doktor, hemşire ve yardımcı sağlık personelleri katılmıştır. Katılımcılar, COVID-19'la ilişkili sorular, Depresyon Anksiyete Stres Ölçeği-21 (DASÖ-21) ve Çok Boyutlu Algılanan Sosyal Destek Ölçeği'ni (ÇBASDÖ) içeren çevrimiçi bir anket doldurmuşlardır. Ki-Kare, Fisher's exact, Mann-Whitney-U, Kruskal Wallis, Spearman korelasyon ve lojistik regresyon analizleri uygulanmıştır.

Bulgular: Çoğunluğu hemşireler (n=465; %48,8) olmakla birlikte, yardımcı sağlık personeli (n=402; %42,2) ve doktoların (n=86; %9) yanıtlarından eksiksiz olan toplam 953 yanıt analize alınmıştır. Enfekte olmak ve COVID-19'u çevresindekilere bulaştırmak en büyük endişe kaynakları olarak saptanmıştır. DASÖ-21 puanları bekar, çocuğu olmayan, koruyucu ekipman bulmakta güçlük çeken veya ileride bulma kaygısı yaşayan, COVID-19(+) kişilerle temas halinde olan; sigara ve alkol kullanımını artıran; yeni başlayan uyku, iştah ve somatik sorunlar bildiren katılımcılarda daha yüksek bulunmuştur. Enfeksiyonu kapmak (%94,6) vs. (%90,6) vs. (%84,7); $p<0,001$] ve ekipman sorunuyla ile ilgili endişeler [(%52,9) vs. (%29,4) vs. (%26,3); $p<0,001$], uyku [(%62,2) vs.(%43,5) vs. (%34); $p<0,001$, sırasıyla] ve somatik sorunlar [(%58,4) vs. (%50) vs. (%28,2); $p<0,001$] ve DASÖ-21 puanları [(5-21) vs. (3-15) vs.

Corresponding author/İletişim kurulacak yazar: Irmak POLAT – irmakpolat@gmail.com

Submitted/Başvuru: 31.08.2022 • **Revision Requested/Revizyon Talebi:** 06.09.2022 •
Last Revision Received/Son Revizyon: 14.12.2022 • **Accepted/Kabul:** 17.12.2022 • **Published Online/Online Yayın:** 31.01.2023

34%; p<0.001) and somatic problems (58.4% compared to 50% and 28.2%; p<0.001), and higher DASS-21 scores (Range=5-21 compared to 3-15 and 0-12; p<0.001).

Conclusion: Worries and lifestyle changes associated with the outbreak are seen to have been be related to psychological difficulties. An adequate level of knowledge, self-protection, and social support are essential issues for HCPs. While this study recommends that HCPs express and share their worries, institutions should also focus on the psychological status of their staff and provide immediate interventions.

Keywords: COVID-19, dialysis, healthcare providers, psychological response

(0-12); p<0,001] hemşirelerde doktor ve sağlık çalışanlarına göre daha yüksektir.

Sonuç: Salgına yönelik endişeler ve yaşam şekli değişiklikleri psikolojik zorluklarla ilişkili saptanmış olup, yeterli bilgi düzeyi, bulaştan korunma ve sosyal desteğin sağlık çalışanları için önemli konular olduğu görülmektedir. Sağlık çalışanlarının endişelerini yakınları, meslektaşları ve amirlerine/yöneticileriyle paylaşması önerilirken; kurumların da personelin psikolojik durumuna dikkat göstermesi ve gereğinde hızlı girişimler sağlaması önemlidir.

Anahtar Kelimeler: COVID-19, diyaliz, sağlık çalışanları, ruhsal tepki

INTRODUCTION

In December 2019, the world became alarmed by many cases of life-threatening atypical pneumonia caused by a novel retrovirus known as SARS-CoV-2 and called the coronavirus disease 2019 (COVID-19) (1). As the disease and resultant deaths continued to spread uncontrollably, WHO declared COVID-19 to be a pandemic on March 11, 2020 (2).

COVID-19 has been quite a stressful experience and has presented several compelling situations to healthcare providers. The rapid increase of those confirmed/suspected of having COVID-19, uncertainty about its diagnostic and therapeutic aspects as well as outcomes, shortages of personal protective equipment (PPE) and supplies, decreased numbers of actively working colleagues due to either quarantine or sick leaves, as well as an infodemic shaped by contradictory commentaries and speculations in the news, press, and social media formed the leading reasons for distress (3-6). As a result, the COVID-19 outbreak caused either an onset or worsening of existing psychological disorders in healthcare providers (HCPs). Reports from the epicenter and other countries revealed primary care workers involved in the diagnosis, treatment, and care of COVID-19 patients to have varying rates of increased mental health problems (7, 8).

Dialysis HCPs had already been regarded as a group vulnerable to stress and burnout even outside of crises and disasters (9-11). Dialysis nurses in particular spend a long time with patients who often unrealistically view dialysis centers as a threat toward their safety and survival (12). Dialysis HCPs' attempts to provide emotional support as well as the physical demands of their workload (e.g., complex technical content of the procedures, infection risks) also increase their psychological burden (9).

Alongside these difficulties well-known to dialysis treatment, COVID-19 caused additional distress to dialysis HCPs due to their patients being at greater risk of infection, symptom severity, and death due to their comorbidities, immunosuppression, and older age (13). In addition, contraction and transmission of the disease among HCP was another critical issue to closely monitor due to Wuhan reporting varying infection rates between 6.4%-12.1% in the medical staff at dialysis centers in just the brief period after cases started emerging (5, 14).

HCPs' psychological problems are associated with the risk of adversely affecting team spirit and creating disorganization, unfavorable treatment outcomes, medical errors, and patient dissatisfaction. These may also lead to burnout and thus distinguishing staff's level of psychological distress and taking adequate precautions are necessary (15, 16).

This study aims to investigate Turkish dialysis HCPs' levels of depression, anxiety, and stress during the early outbreak period.

MATERIALS AND METHODS

Participants

A convenience sample of healthcare providers, including physicians, nurses, and healthcare workers such as technicians, secretaries, drivers, and cleaning and security staff from private and public outpatient dialysis centers in Turkiye, were contacted to participate in the study. No specific exclusion criteria were set, and all volunteers among the desired sample over 18 years of age and eligible to read and mark the answers could participate.

Procedure

This cross-sectional study was conducted using the online survey method. The researchers transferred the screening questions and inventories to a form on Google Documents, and access links were sent to healthcare providers by contacting the directors of private and public dialysis centers, personal connections, and social network groups, as well as social media and messaging applications such as WhatsApp, Telegram, Instagram, and Facebook. The researchers combined the invento-

ries as a single form, and all participants filled out this final form. Incomplete or abandoned forms were not evaluated. Written informed consent was presented on the first page, and the participants were asked to check a box denoting their acceptance and then to continue with the questions. Attention was paid to anonymity. Only one data entry submission was permitted per person, and the researchers have kept all information confidential. The Ethics Committee of Istanbul University Faculty of Medicine approved the study protocol (Date: 08.05.2020, No: 09). The study has been conducted in accordance with the Declaration of Helsinki.

Instruments

Screening questionnaire

Designed by the researchers, this form covers the socio-demographic characteristics of the participants, including their experiences and opinions about COVID-19.

Depression anxiety stress scale-21 (DASS-21)

This instrument evaluates HCPs' psychological symptoms in the past week. DASS-21 consists of 21 questions on a 4-point Likert-type scale (0-3) with its depression, anxiety, and stress subscales having seven questions each. Higher scores indicate a higher level of symptoms (17). DASS-21 has already been translated into Turkish and validated (18).

Multidimensional scale of perceived social support (MSPSS)

This item measures one's subjective assessment of support from different sources. A total of 12 questions cover its three subscales (i.e., family, friends, and significant others) and are scored from 1 to 7 points (19). MSPSS has already been translated into Turkish and validated (20).

Data analysis

The chi-squared and Fisher's exact tests were performed on the qualitative variables, while the Mann-Whitney U test was used for paired quantitative variable and the Kruskal-Wallis test for more than two quantitative variables with non-parametric distributions. Bonferroni corrections were used for subgroup analyses. Spearman correlation analysis was used to determine the relationships among variables. A Spearman coefficient >0.25 and p value <0.05 were considered correlated. Linear regression analysis was used to identify related factors regarding the odds ratios at a 95% confidence interval with a p-value less than 0.05 being considered significant. SPSS 15.0 for Windows was used for the statistical analyses.

RESULTS

A total of 967 responses were received from the online survey, with the majority being from private centers (n=941; 97.3%). The response/refusal rate could not be calculated due to the survey's distribution path. After excluding the incomplete responses, a total of 953 participants were included in the study. Due to the significant difference between the response numbers from private and public centers, the analyses were conducted over only the group of dialysis HCPs from private centers.

The study group consists mainly of nurses (n=465; 48.8%), followed by healthcare workers (n=402; 42.2%) and physicians (n=86; 9%). The median age of the participants is 38 years old (Range=27-43), most of whom (n=596; 62.5%) are female. The nurses predominantly have an education level of university/postgraduate (51.4%).

The number of married or cohabitant participants (n=652; 68.7%) are significantly higher than either single (n=238; 25.1%) or divorced/widowed participants (n=59; 6.2%; p<0.001). The number of participants with children (n=638; 67.9%) is higher than those without children (n=301; 32.1%; p<0.001). Of the participants, 88.4% live with family members and 67.0% have worked in dialysis centers for more than five years. Table 1 presents the demographic variables. Table 2 shows the changes in work practices as well as the psychosocial features associated with COVID-19. Of the participants, 60.6% declared no change in their weekly work hours, 89.9% declared no difficulty in accessing PPE, and 60.3% declared not worrying about finding PPE in the future. Nurses led the number of those concerned about PPE shortages (52.9%). Of the participants, 62.5% had no dialysis patient with a COVID-19 diagnosis, 75.2% had no colleague with a COVID-19 diagnosis, and 77.3% had no family member with a COVID-19 diagnosis. When considering all the participants, the significant concerns were getting infected with COVID-19 (90.1%) and transmitting the disease to their loved ones (92.5%). Nurses have the highest number of those who declared having worries most of the time. Overall, 67.8% of the participants (primarily physicians) stated the information level concerning COVID-19 to have been sufficient.

Most respondents do not use, decreased or did not change the amount of their alcohol (99.4%) /tobacco (97.6%) consumption after the emergence of COVID-19.

Nearly half of the participants (48.7%) suffered a new onset of sleep problems (e.g., decrease/increase in duration, difficulty falling asleep, or interrupted sleep). Of those who reported sleep problems, nurses ranked highest (62.2%). The majority declared no new onset of any appetite problems (77.4%). Of the participants, 44.8% suffered a new onset or increase in somatic symptoms associated with the pandemic, with these again being higher in nurses (58.4%).

MSPSS scores showed no statistical difference among the participants', regarding sources of support (i.e., fam-

Table 1: Demographic characteristics of the participants

		Total (n=953)	Physician (n=86)	Nurse (n=465)	Healthcare worker (n=402)	p
Age (years, median ± IQR)		38 (27-43)	49 (45-52)	32 (26-41)	38 (31-43)	<0.001
Gender (n, %)	Female	596 (62.9)	28 (32.6)	421 (90.7)	147 (37.0)	<0.001
	Male	351 (37.1)	58 (67.4)	43 (9.3)	250 (63.0)	
Education (n, %)	Elementary school	168 (17.7)	0 (0.0)	0 (0.0)	168 (42.2)	
	High school	293 (30.9)	0 (0.0)	164 (35.3)	129 (32.4)	<0.001
	University/post-graduate	488 (51.4)	86 (100)	301 (64.7)	101 (25.4)	
Marital Status (n, %)	Single	238 (25.1)	6 (7.0)	153 (32.9)	79 (19.8)	
	Married/partnered	652 (68.7)	73 (84.9)	286 (61.5)	293 (73.6)	<0.001
	Divorced/widow	59 (6.2)	7 (8.1)	26 (5.6)	26 (6.5)	
Having children (n, %)	No	301 (32.1)	11 (12.9)	193 (42.2)	97 (24.4)	<0.001
	Yes	638 (67.9)	74 (87.1)	264 (57.8)	300 (75.6)	
Cohabitation (n, %)	Living by oneself	75 (7.9)	7 (8.1)	44 (9.5)	24 (6.1)	
	With housemate	34 (3.6)	1 (1.2)	22 (4.8)	11 (2.8)	
	With elementary family	170 (18.0)	2 (2.3)	101 (21.9)	67 (16.9)	<0.001
	With parents	591 (62.6)	73 (84.9)	255 (55.2)	263 (66.4)	
	With large family	74 (7.8)	3 (3.5)	40 (8.7)	31 (7.8)	
Work duration in dialysis center (n, %)	0-6 months	52 (5.5)	1 (1.2)	20 (4.3)	31 (7.8)	
	6 months-5 years	260 (27.5)	7 (8.1)	117 (25.2)	136 (34.4)	<0.001
	> 5 years	633 (67.0)	78 (90.7)	327 (70.5)	228 (57.7)	

IQR; Interquartile range

ily, friend, others) or work position (i.e., physician, nurse, healthcare worker) (p=0.43, 0.469, 0.695, and 0.832 respectively) However, nurses had significantly higher overall and subscale scores on DASS-21 (n=13 with scores between 5-21) compared to physicians (n=9 with scores between 3-15) and healthcare workers (n=5 with scores between 0-12; p<0.001).

Females scored significantly higher on every subscale (depression, anxiety, stress) of DASS-21 compared to males (p<0.001). Single participants; those with no children; those experiencing difficulty finding PPE; those worried about finding PPE in the future; those in contact with COVID-19 (+) patients, coworkers, or family members; those who stated their tobacco and alcohol use to have increased during the pandemic; and those who declared a new onset of sleep, appetite, and/or somatic problems scored higher in all subscales of DASS-21 (Table 3). The duration of employment in a dialysis center (r=0.034, p=0.291) and knowledge about COVID-19 (r=0.02, p=0.537) did not correlate with the DASS-21 total score; however, worry about getting infected (r=0.348, p<0.001) and transmitting the disease to loved ones (r=0.298, p<0.001) did positively correlate with the DASS-21. Meanwhile, perceived total social support showed no strong correlation with depression, anxiety, or stress levels (r=-0.218, p<0.001; r=-0.196, p<0.001; r=-0.187, p<0.001, respectively) (Table 4).

The regression analyses indicate the variables associated with psychological outcomes. Depression was found to be associated with being female, not having children, cohabiting with family/housemates, having a longer work experience worrying about finding PPE in the future, worrying about getting infected, and knowing an acquaintance with COVID-19. Similar associations were found regarding anxiety for all these factors except work duration and with stress for all these factors except cohabitation status. Interestingly, worrying about transmitting the disease to loved ones was only associated with stress scores. In addition, lower levels of perceived social support were determined to be associated with anxiety and stress scores (Table 5).

Table 2: Lifestyle changes, opinions, and psychological parameters of the participants related to COVID-19

		Total	Physician	Nurse	Healthcare worker	p
Changes in working hours (n, %)	Decreased	214 (22.6)	17 (19.8)	84 (18.1)	113 (28.5)	0.004
	Not changed	574 (60.6)	58 (67.4)	293 (63.1)	223 (56.2)	
	Increased	159 (16.8)	11 (12.8)	87 (18.8)	61 (15.4)	
The difficulty of finding PPE (n, %)	No	850 (89.9)	78 (90.7)	409 (88.3)	363 (91.4)	0.313
	Yes	96 (10.1)	8 (9.3)	54 (11.7)	34 (8.6)	
Worry about finding PPE in the future (n, %)	No	570 (60.3)	60 (70.6)	219 (47.1)	291 (73.7)	<0.001
	Yes	375 (39.7)	25 (29.4)	246 (52.9)	104 (26.3)	
COVID-19 (+) patient in the center (n, %)	No	591 (62.5)	50 (58.1)	291 (62.7)	250 (63.1)	0.002
	Yes	171 (18.1)	27 (31.4)	86 (18.5)	58 (14.6)	
	Do not know	184 (19.5)	9 (10.5)	87 (18.8)	88 (22.2)	
COVID-19 (+) coworker in the center (n, %)	No	712 (75.2)	59 (68.6)	346 (74.6)	307 (77.3)	0.082
	Yes	103 (10.9)	16 (18.6)	54 (11.6)	33 (8.3)	
	Do not know	132 (13.9)	11 (12.8)	64 (13.8)	57 (14.4)	
COVID-19 (+) people, in relationship (n, %)	No	733 (77.3)	53 (61.6)	340 (73.3)	340 (85.4)	<0.001
	Yes	215 (22.7)	33 (38.4)	124 (26.7)	58 (14.6)	
Worry about getting infected (n, %)	no	94 (9.9)	8 (9.4)	25 (5.4)	61 (15.3)	<0.001
	sometimes	266 (28.1)	42 (49.4)	96 (20.6)	128 (32.2)	
	most of the shift	310 (32.7)	22 (25.9)	193 (41.5)	95 (23.9)	
	nearly all day, every day	278 (29.3)	13 (15.3)	151 (32.5)	114 (28.6)	
Worry about transmitting the disease to beloveds (n, %)	no	71 (7.5)	9 (10.5)	15 (3.2)	47 (11.8)	<0.001
	sometimes	186 (19.6)	29 (33.7)	68 (14.6)	89 (22.4)	
	most of the shift	130 (13.7)	18 (20.9)	63 (13.5)	49 (12.3)	
	nearly all day, every day	562 (59.2)	30 (34.9)	319 (68.6)	213 (53.5)	
Knowledge about COVID-19 (n, %)	not much	305 (32.2)	11 (12.8)	123 (26.5)	171 (43.1)	<0.001
	enough/ very much	643 (67.8)	75 (87.2)	342 (73.5)	226 (56.9)	
Tobacco use (n, %)	Not using	573 (60.4)	58 (67.4)	285 (61.3)	230 (57.9)	0.158
	Decreased	119 (12.6)	11 (12.8)	46 (9.9)	62 (15.6)	
	no change	233 (24.6)	16 (18.6)	121 (26.0)	96 (24.2)	
	Increased	23 (2.4)	1 (1.2)	13 (2.8)	9 (2.3)	
Alcohol use (n, %)	Not using	790 (83.8)	49 (57.6)	390 (84.2)	351 (88.9)	<0.001
	Decreased	64 (6.8)	17 (20.0)	20 (4.3)	27 (6.8)	
	no change	83 (8.8)	17 (20.0)	50 (10.8)	16 (4.1)	
	Increased	6 (0.6)	2 (2.4)	3 (0.6)	1 (0.3)	
New-onset sleep problems (n, %)	No	486 (51.3)	48 (56.5)	176 (37.8)	262 (66.0)	<0.001
	Yes	461 (48.7)	37 (43.5)	289 (62.2)	135 (34.0)	

Table 2: Continue

		Total	Physician	Nurse	Healthcare worker	p
New-onset appetite problems (n, %)	No	734 (77.4)	64 (74.4)	329 (70.9)	341 (85.7)	<0.001
	Yes	214 (22.6)	22 (25.6)	135 (29.1)	57 (14.3)	
Somatic Symptoms (n, %)	No/ decreased	441 (55.2)	37 (50.0)	160 (41.6)	244 (71.8)	<0.001
	New-onset/ increased	358 (44.8)	37 (50.0)	225 (58.4)	96 (28.2)	
MSPSS (years, median ± IQR)	Family	27 (20-28)	26 (20-28)	27 (21-28)	28 (19-28)	0.43
	Friend	22 (15-28)	22 (16-25)	22 (16-26)	22 (15-28)	0.469
	Significant Other	26 (16-28)	25 (18-28)	26 (16-28)	26 (15-28)	0.695
	Total	72 (54-82)	73 (56-79)	71 (54-81)	72 (54-83)	0.832
DASS-21 (years, median ± IQR)	Depression	3 (0-6)	3 (0-6)	4 (2-8)	1 (0-4)	<0.001
	Anxiety	2 (0-5)	2 (0-4)	3 (1-6)	1 (0-3)	<0.001
	Stress	4 (1-6)	4 (1-6)	5 (2-8)	2 (0-5)	<0.001
	Total	9 (2-17)	9 (3-15)	13 (5-21)	5 (0-12)	<0.001

PPE; Personal protective equipment, MSPSS; Multidimensional Scale of Perceived Social Support, DASS-21; Depression, anxiety, stress scale-21, IQR; Interquartile range

Table 3: Demographic and social features, working practice patterns, and psychosomatic problems associated with DASS-21

		Depression (median ± IQR)	Anxiety (median ± IQR)	Stress (median ± IQR)	Total
Gender	Female	4 (1-7.4)	3 (1-6)	5 (2-7.1)	12 (4.6-21)
	Male	1 (0-4)	1 (0-3)	2 (0-4.7)	4 (0-11)
	p	<0.001	<0.001	<0.001	<0.001
Marital status	Single	3 (1-8)	3 (1-5)	4.5 (1-8)	10.4 (3-21.3)
	Married/partnered	3 (0-6)	2 (0-4.5)	4 (0.9-6)	9 (1.6-16)
	Divorced/widow	2 (0-6.6)	1 (0-5)	3 (0.6-6)	6 (1-15)
	p	0.006	0.019	0.007	0.005
Having children	No	4 (1-8)	3 (1-6)	4.6 (1-8)	11 (4-21.4)
	Yes	2.5 (0-5.2)	2 (0-4)	3 (0-6)	8 (1-15)
	p	<0.001	<0.001	<0.001	<0.001
Cohabitation	by one-self	2 (0-6)	2 (0-4)	3 (1-7)	7 (2-18)
	housemate	4.8 (0-8)	3.5 (0-5)	4.6 (1.6-7.25)	12.2 (1.6-20)
	elementary family	3 (1-7)	2.8 (0.4-5)	4 (1-7.6)	10 (3-20)
	parents	3 (0-6)	2 (0-4.2)	3.5 (0.8-6)	8.5 (1.5-16)
	large family	3 (1-7)	2 (1-5)	4.9 (2-7.4)	11 (4.5-16.6)
	p	0.132	0.124	0.089	0.101
Difficulty finding PPE	No	3 (0-6)	2 (0-4)	3.1 (1-6)	8.1 (2-16.1)
	Yes	5 (2-9.75)	3.9 (1-7)	5 (2.2-8)	13 (6.25-22.75)
	p	<0.001	0.001	<0.001	<0.001

Table 3: Continue

		Depression (median ± IQR)	Anxiety (median ± IQR)	Stress (median ± IQR)	Total
Worry about finding PPE in the future	No	2 (0-5)	1 (0-4)	2 (0-5)	6 (1-14)
	Yes	4.6 (2-8.9)	3.2 (1-6)	5 (2-8)	13 (7-22)
	p	<0.001	<0.001	<0.001	<0.001
COVID-19 (+) patient in the center	No	2 (0-6)	2 (0-4)	3 (0-6)	8 (1-16)
	Yes	4 (1-7)	3 (1-5)	4 (1-7)	11 (4-19)
	Do not know	4 (1-7)	3 (1-6)	4.4 (1-7)	11 (3-21.3)
	p	<0.001	<0.001	<0.001	<0.001
COVID-19 (+) coworker in the center	No	2 (0-6)	2 (0-4)	3 (0-6)	8 (1-15.6)
	Yes	4 (1-7)	3 (1-7)	4.8 (2-8)	12.1 (5-21)
	Do not know	4.1 (1-8)	3.2 (1-6)	5 (2-8)	13 (5-22)
	p	<0.001	<0.001	<0.001	<0.001
COVID-19 (+) people in the relationship	No	2.5 (0-6)	2 (0-4)	3 (0-6)	8 (1-16)
	Yes	4 (1-7)	3 (1-6)	5 (2-7)	11.7 (5-20)
	p	<0.001	<0.001	<0.001	<0.001
Tobacco use	Not using	3 (0-6)	2 (0-4.4)	4 (1-6)	9 (1.4-16)
	Decreased	2 (0-5)	2 (0-4)	2 (0-6)	6 (1-16)
	no change	3 (1-6.7)	2 (0-5)	4 (1-7)	9.2 (3-18)
	Increased	8 (2-16)	5 (3-13)	9 (4-14)	27 (11-44)
	p	<0.001	<0.001	<0.001	<0.001
Alcohol use	Not using	2.8 (0-6)	2 (0-4)	3 (1-6)	8 (1-16)
	Decreased	4 (0-7)	3 (0-4.75)	5 (2-7.75)	12.3 (4-18)
	no change	4 (2-9.5)	3.2 (1-8)	5 (2.6-10)	13 (5-27)
	Increased	5.5 (1-17.25)	7.5 (0-12.75)	8 (4.5-15.75)	20.5 (5.75-46.25)
	p	0.001	0.003	<0.001	<0.001
New-onset sleep problems	No	1 (0-3.7)	1 (0-3)	1 (0-4)	3 (0-10.8)
	Yes	5 (2-9)	4 (2-7)	6 (3-9)	14.5 (8-24)
	p	<0.001	<0.001	<0.001	<0.001
New-onset appetite problems	No	2 (0-5)	2 (0-4)	3 (0-5.5)	7 (1-15)
	Yes	5 (2-10)	5 (2-8)	6 (4-10)	15.5 (9.5-28)
	p	<0.001	<0.001	<0.001	<0.001
Somatic Symptoms	No/ decreased	1 (0-3)	0 (0-2)	1 (0-4)	3 (0-10)
	New-onset/ increased	5 (2-9)	4 (2-7)	6 (3-9)	14 (8-24.25)
	p	<0.001	<0.001	<0.001	<0.001

PPE; Personal protective equipment, DASS-21; Depression, anxiety, stress scale-21, IQR; Interquartile range

Table 4: Correlation between perceived social support and depression, anxiety, and stress levels.

		DASS-21			
		Depression	**Anxiety**	**Stress**	**Total**
Age	rho	-0.094	-0.106	-0.108	-0.111
	p	0.004	0.001	0.001	0.001
Duration of work in the dialysis center	rho	0.027	0.031	0.039	0.034
	p	0.404	0.347	0.227	0.291
Worry about getting infected	rho	0.318	0.347	0.335	0.348
	p	<0.001	<0.001	<0.001	<0.001
Worry about transmitting the disease to beloveds	rho	0.266	0.276	0.293	0.298
	p	<0.001	<0.001	<0.001	<0.001
Knowledge about COVID-19	rho	0.018	0.015	0.015	0.02
	p	0.589	0.653	0.633	0.537
MSPSS- Family	rho	-0.18	-0.187	-0.157	-0.183
	p	<0.001	<0.001	<0.001	<0.001
MSPSS- Friend	rho	-0.203	-0.202	-0.193	-0.211
	p	<0.001	<0.001	<0.001	<0.001
MSPSS - Significant other	rho	-0.137	-0.101	-0.094	-0.117
	p	<0.001	0.002	0.004	<0.001
MSPSS Total	rho	-0.218	-0.196	-0.187	-0.212
	p	<0.001	<0.001	<0.001	<0.001

MSPSS; Multidimensional Scale of Perceived Social Support, DASS-21; Depression, anxiety, stress scale-21

DISCUSSION

The COVID-19 pandemic has caused many physical and psychosocial problems in HCPs. The present paper aims to evaluate the psychological impact of COVID-19 on dialysis HCPs, as they are known as one of the most vulnerable groups in health care (10, 11, 13).

The study found most participants, especially nurses, to have declared being worried about getting infected and transmitting the disease, and these concerns also showed correlations with psychological symptoms. As a result, lower levels of perceived social support were associated with higher anxiety and stress levels. In addition, nearly half of the group reported either a new onset or increased levels of sleep and somatic problems. In addition, increased tobacco and alcohol use was associated with increased psychological difficulties.

Nurses and women were the two prominent groups demonstrating elevated adverse psychological outcomes. This result is not surprising, as being female and the occupational pressure nurses experience have been reported as major risk factors for negative mental consequences during the pandemic (7, 21-23).

Being in close physical contact with the patients' specimens more frequently, working in high-risk units, and reporting more workload are the prominent risk factors associated with higher rates of psychological problems in nurses (23, 24). In addition, the higher rate of females in the nurse group according to the gender distribution in the current study's sample may explain the occurrence of elevated mental symptoms among females here, as mental health disorders are known to show a higher prevalence in female HCPs compared to male HCPs (25).

The study's results emphasize the occupational position of being an HCP, especially working as a nurse, to be an important risk factor for developing mental health problems during crises. Therefore, institutions are recommended to focus on the workload, risks, and needs of this vulnerable group of HCPs.

Most of the study's sample reported their knowledge about COVID-19 as being somewhere between "enough" and "very much." The participants' knowledge levels did not correlate with elevated depression, anxiety, or stress levels. Unlike this study's sample, several studies have shown a relationship to exist between lower occupational competence and mental health problems. Elbay et al. (21) reported lower feelings of occupational compe-

Table 5: Factors associated with DASS-21 scores

	DASS-21 depression score					DASS-21 anxiety score					DASS-21 stress score				
	B	Beta	p	95% CI Lower bound	95% CI Upper bound	B	Beta	p	95% CI Lower bound	95% CI Upper bound	B	Beta	p	95% CI Lower bound	95% CI Upper bound
Constant	5.484			3.034	7.934	4.019			2.048	5.990	5.233	1.149		2.979	7.488
Working position	-0.539	-0.074	**0.024**	-1.006	-0.072	-0.266	-0.045	0.166	-0.641	0.110	-0.582	0.219	**0.008**	-1.012	-0.152
Age	0.000	0.008	0.801	-0.002	0.003	0.001	0.002	0.939	-0.002	0.002	0.000	0.001	0.795	-0.003	0.002
Gender	-1.458	-0.153	**0.000**	-2.059	-0.856	-1.420	-0.184	**0.000**	-1.904	-0.937	-1.362	0.282	**0.000**	-1.916	-0.808
Marital status	-0.131	-0.015	0.734	-0.883	0.622	0.174	0.024	0.573	-0.431	0.779	-0.330	0.353	0.350	-1.022	0.363
Having children	-1.567	-0.159	**0.001**	-2.494	-0.640	-1.352	-0.170	**0.000**	-2.098	-0.607	-1.194	0.435	**0.006**	-2.047	-0.341
Cohabitation	0.403	0.084	**0.030**	0.038	0.767	0.447	0.115	**0.003**	0.154	0.740	0.321	0.171	0.061	-0.015	0.656
Duration of work in the dialysis center	0.562	0.072	**0.045**	0.013	1.111	0.272	0.043	0.228	-0.170	0.713	0.610	0.257	**0.018**	0.104	1.115
Change in a weekly working hour	0.406	0.055	0.074	-0.040	0.851	-0.001	0.000	0.994	-0.360	0.357	0.383	0.209	0.067	-0.027	0.792
Difficulty in finding PPE	0.794	0.051	0.118	-0.203	1.790	0.309	0.024	0.450	-0.493	1.110	0.737	0.467	0.115	-0.180	1.653
Worry about finding PPE in the future	1.207	0.128	**0.000**	0.565	1.849	0.660	0.086	**0.012**	0.143	1.177	0.867	0.301	**0.004**	0.276	1.458
COVID-19 (+) patient in the center	-0.070	-0.012	0.728	-0.466	0.325	0.002	0.000	0.992	-0.317	0.320	0.087	0.186	0.640	-0.277	0.451
COVID-19 (+) coworker in the center	0.354	0.055	0.119	-0.092	0.800	0.449	0.085	**0.014**	0.090	0.808	0.233	0.209	0.265	-0.177	0.643
COVID-19 (+) people in relationship	0.849	0.077	**0.012**	0.185	1.512	0.825	0.093	**0.002**	0.292	1.359	0.922	0.311	**0.003**	0.312	1.532

Table 5: Continue

	DASS-21 depression score					DASS-21 anxiety score					DASS-21 stress score				
	B	Beta	p	95% CI Lower bound	95% CI Upper bound	B	Beta	p	95% CI Lower bound	95% CI Upper bound	B	Beta	p	95% CI Lower bound	95% CI Upper bound
Worry about getting infected	0.746	0.157	**0.000**	0.391	1.101	0.783	0.203	**0.000**	0.497	1.069	0.677	0.167	**0.000**	0.350	1.004
Worry about transmitting the disease to beloveds	0.186	0.041	0.283	-0.154	0.527	0.168	0.046	0.229	-0.106	0.442	0.361	0.160	**0.024**	0.048	0.674
Knowledge about COVID-19	0.025	0.002	0.936	-0.576	0.625	-0.092	-0.011	0.708	-0.575	0.391	-0.127	0.282	0.652	-0.680	0.426
MSPSS Family	-0.045	-0.069	0.145	-0.105	0.016	-0.061	-0.116	**0.014**	-0.109	-0.012	-0.042	0.028	0.138	-0.098	0.014
MSPSS Friend	-0.048	-0.079	0.073	-0.101	0.004	-0.056	-0.113	0.009	-0.099	-0.014	-0.072	0.025	**0.004**	-0.121	-0.024
MSPSS Significant other	-0.023	-0.040	0.378	-0.073	0.028	0.038	0.083	0.067	-0.003	0.079	0.017	0.024	0.470	-0.029	0.064

MSPSS; Multidimensional Scale of Perceived Social Support, DASS-21; Depression, anxiety, stress scale-21, CI; Confidence interval, p values below 0.05 are marked as bold.

tence during COVID-19-related tasks to be associated with higher DASS scores. Du et al. reported insufficient knowledge about COVID-19, insufficient psychological preparedness, and perceived self-efficacy among HCPs to be associated with depression and anxiety symptoms (26). The data from the current study have shown having sufficient knowledge about the present conditions and being prepared to be positive factors against distress. When considering both this study's results alongside those from other studies, facilities are recommended to provide adequate training on the causes and course of a current disease, the methods of protection, and detailed treatment guides to enhance HCPs' professional competencies.

The study's data indicate that having COVID-19 (+) patients in the dialysis center or among acquaintances and worrying about contracting/transmitting the disease to loved ones to be the prominent issues correlated to depression, anxiety, and stress levels. HCPs are observed to fear being infected or infecting others during infection outbreaks, and these concerns happen to be greater if they experience symptoms of the disease (27, 28). Studies have revealed results similar to the current study's findings where an increased number of diagnosed patients with a disease and having higher levels of concern about a disease are associated with higher stress levels; also, being tested for COVID-19 has been associated with anxiety, insomnia, and distress (21, 23, 29). Karataş et al.'s (30) study on dialysis center employees in particular also found anxiety and depression symptoms to be positively correlated with having/treating COVID-19 patients at the center. In addition, HCPs with confirmed cases in their living community show greater anxiety, with those who have confirmed cases among their relatives, friends, and colleagues representing even greater depression and stress symptoms (23, 31). Lastly, the findings show that special attention is needed for HCPs who encounter and work with more patients who've been diagnosed with COVID-19, as well as HCPs who declare gradually increasing concerns for themselves and others, as this may indicate higher levels of psychological proneness.

The results show that having difficulty finding PPE and being concerned about being able to access PPE in the future correlate to higher levels of depression, anxiety, and stress. The proper and regular usage of PPE is known to provide sufficient protection from COVID-19 (32). The inadequate availability of PPE experienced in the early phase of the pandemic, as well as dissatisfaction with institutions' responses such as feeling not at all or poorly protected by administrations both regarding physical safety (i.e., hygiene) and aspects of emotional support, have been associated with higher perceptions of risk, higher anxiety levels, greater sleep disturbance, and more psychosomatic symptoms (21, 33). In addition, an association of trust in equipment and infection control

procedures with lower emotional exhaustion was also reported during the previous SARS outbreak (34). Previous findings along with this study's data reveal HCPs' confidence in their ability to protect and maintain their health is essential to their positive mental status.

Perceived social support did not differ significantly among all three groups. However, the regression analyses revealed lower levels of support from family members and friends to be associated with higher anxiety and stress. Support obtained from others has been associated with HCPs' psychological status during both previous and current epidemics (35). A systematic review covering 59 studies during COVID-19 showed social support to be associated with fewer mental problems and HCPs to be more interested in social support than professional psychological help (36). Kılınç and Sis Çelik's (37) study on nurses during the pandemic reported an increase in perceived social support to increase nurses' psychological resilience. Alongside the studies mentioned above, the data from the current study suggest that providing sufficient social support to HCPs may have an essential role in decreasing depression, anxiety, and stress. Talking about the challenges and concerns about the current crisis, discussing physical and emotional experiences, and sharing individual needs with others can help reduce negative feelings. Lastly, this study recommends that local administrators create a warm work environment with a positive peer-and-supervisor feedback system to help maintain HCPs' psychological well-being.

Nearly half the participants in the current study (primarily nurses) stated experiencing a new onset of sleep problems, with the participants who experienced sleep problems showing higher levels of depression, anxiety, and stress. HCPs experience sleep disturbances during health crises (7). Preti et al.'s (38) review on the current pandemic and previous outbreaks found the rate of insomnia symptoms among HCPs to be between 34%-36.1%. The close relationship between anxiety and sleep disturbances is well known; thus, significant sources of anxiety such as occupational stress during the pandemic, uncertainty regarding the nature of the disease and controlling its spread, worries about getting infected or contracting the virus, and the stress of keeping oneself and loved ones healthy can be noted as significant sources of HCPs' sleep problems during the outbreak (7, 39-40). Likewise, studies have shown poor sleep quality in nurses during COVID-19 to be related to their occupational stress (7, 26, 39).

Although appetite problems were not as prevalent as expected in this study's sample, a new onset of problems correlated with DASS-21 and changes in populations' eating behaviors during the pandemic have been widely reported and mainly attributed to COVID-19-induced stress, negative emotions, and lockdown restrictions (41). Studies with HCP also state poor appetite to be related to distress (8).

Of the participants in the current study, 44.8% expressed either a new onset of or increase in somatic problems. The relationship between the deterioration of emotional well-being and somatic symptoms is well-known (8, 42). Likewise, the current study's results indicate higher levels of depression, anxiety, and stress in participants who express somatic problems. Studies on HCPs have revealed occupational pressure, emotional exhaustion, inadequate institutional support perception, and insufficient safety and hygiene regulations to be correlated with psychosomatic symptoms (33).

A new onset of or increase in sleep, appetite, and/or somatic problems in the current study's sample as well as in the studies mentioned above indicate that HCPs experience not only emotional difficulties but also physical problems related to their negative psychological states. These disturbances may also appear as earlier signs of mental problems. Therefore, paying attention to these problems in HCPs may assist in recognizing and addressing their psychological needs.

This study has associated participants' increased tobacco and alcohol use with their depression, anxiety, and stress levels. Studies have revealed COVID-19-related psychological distress, social distancing, lockdown restrictions, uncertainty, and hopelessness to be closely associated with tobacco and alcohol consumption as coping behaviors (43, 44). Compatible with other studies, the current study's findings emphasize the importance of evaluating HCPs' tobacco, alcohol, and even substance use habits in order to bring deeper psychological problems to light. For example, a new onset of or increase in usage may be an earlier sign of developing mental disorders that would require exceptional support.

This study has several limitations. First of all, the study was performed using convenience sampling without any power analysis, and the majority of the sample was obtained from private dialysis centers. Private centers usually offer better work conditions and sufficient supplies. Therefore, the study's findings are not generalizable to most Turkish dialysis HCPs, and Type I and II errors may have occurred. Secondly, the study's sample does involve a control group (i.e., HCPs from other medical specialties or intervention centers). Therefore, the results cannot be interpreted precisely for dialysis HCPs. In addition, the data collection was based on self-report inventories instead of a diagnostic evaluation performed by a mental health professional. This may have resulted in biased results due to assessing only the participants' subjective perceptions. Lastly, this cross-sectional study was performed in regard to the early period of the pan-

demic. Since then, several new waves have occurred, and COVID-19 remains a global health problem in many aspects (45). The current study shows the immediate psychological response to the crisis; however, the long-term consequences remain unknown.

CONCLUSION

This study has examined the early psychological response to the COVID-19 pandemic and its relevant factors with regard to dialysis HCPs in Turkiye. The study has indicated the worries and lifestyle changes associated with the outbreak to be associated with depression, anxiety, and stress. In addition, the dialysis HCPs' reported having adequate levels of knowledge about the disease and sufficient self-protection to be essential issues for their confidence. In addition, employers should take into account the safety of their medical care teams, provide reassurance about their staff's protection, and fulfill their needs regarding PPE and hygiene in the best possible way.

The findings have also shown nurses and women to have a high risk of developing psychological problems. When considering the vulnerability these two groups have regarding the healthcare system, more attention and closer monitoring should be given to the psychological difficulties they experience.

Healthcare providers mental well-being is a critical factor in the optimal sustainability of the healthcare system, especially during crises such as COVID-19. The study's results have shown HCPs' social support and their perceptions of it to be associated with mental well-being. Thus, HCPs are recommended to express their worries about COVID-19 with each other and their loved ones, as well as to their supervisors and/or institution representatives. Government institutions, local administrations, and employers should focus on the psychological status of their staffs and provide immediate interventions as needed. Firstly, close contact with and observation of staff members' physical and psychosocial aspects are essential. This is achievable by communicating empathetically, asking how they feel, talking about more than work, and sharing their uneasiness. Administrators are recommended to plan appropriate actions (i.e., establish informal or formal support groups, provide training and applications on how reducing stress, activating functional coping mechanisms, and learning relaxation techniques can be beneficial) in accordance with HCPs' needs; institutions may also facilitate contact between HCPs and mental health professionals for advanced evaluations or assistance.

Prospective studies are needed for the period ahead in order to research the long-term consequences of this outbreak as well as the emerging needs of dialysis HCPs.

Acknowledgments: The study would like to thank Fresenius Medical Care Türkiye for helping reach the most dialysis physicians, nurses, and other personnel.

Ethics Committee Approval: This study was approved by Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 08.05.2020, No: 09).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- I.P., M.Ş.S., M.Ö., S.K.K., R.P., H.Y.; Data Acquisition- I.P., S.K.K., R.P.; Data Analysis/Interpretation- I.P., H.U., M.Ş.S., M.Ö.; Drafting Manuscript- I.P., E.D.; Critical Revision of Manuscript- M.Ş.S., M.Ö.; Final Approval and Accountability- M.Ş.S.; Supervision- M.Ş.S., M.Ö.

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

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

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RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1153370
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

PREVALENCE AND ASSOCIATED FACTORS OF ADVERSE EFFECTS IN CHILDREN AND ADOLESCENTS TREATED WITH SELECTIVE SEROTONIN REUPTAKE INHIBITORS: A CHART REVIEW STUDY*

SEÇİCİ SEROTONİN GERİ ALIM İNHİBİTÖRLERİYLE TEDAVİ EDİLEN ÇOCUK VE ERGENLERDE YAN ETKİLERİN SIKLIĞI VE İLİŞKİLİ FAKTÖRLER: BİR DOSYA TARAMA ÇALIŞMASI

Hanım Hülya ALINAY[1], Ali KARAYAĞMURLU[2], Murat COŞKUN[2]

[1]Sirnak State Hospital, Child and Adolescent Psychiatry Department, Sirnak, Turkiye
[2]Istanbul University, Istanbul Faculty of Medicine, Child and Adolescent Psychiatry Department, Istanbul, Turkiye

ORCID IDs of the authors: H.H.A. 0000-0003-3029-5439; A.K. 0000-0001-5464-2891; M.C. 0000-0002-4808-5870

Cite this article as: Alinay HH, Karayagmurlu A, Coskun M. Prevalence and associated factors of adverse effects in children and adolescents treated with selective serotonin reuptake inhibitors: a chart review study. J Ist Faculty Med 2023;86(1):28-36. doi: 10.26650/IUITFD.1153370

ABSTRACT

Objective: This study aims to examine the prevalence and associated clinical and sociodemographic factors of adverse effects in medication among naïve young subjects who received selective serotonin reuptake inhibitor (SSRI) monotherapy.

Material and Methods: The medical records of 85 patients who had received SSRI monotherapy in a university hospital's child and adolescent psychiatry clinic were reviewed. The subjects who met the inclusion criteria were included in the study.

Results: A total of 67 subjects (10.82±3.63 years) were included. More than half (n=39, 58.9%) developed at least one adverse effect possibly associated with SSRI treatment, with psychic (n=25, 37.3%) and autonomic (n=20, 29.9%) adverse effects as well as behavioral activation (n=13, 19.4%) being the most frequently reported. Medication was discontinued in 13 subjects (19.4%) due to adverse effects, with behavioral activation (6 out of 13 subjects) being the most frequent reason for discontinuation. The development of behavioral activation was significantly associated with younger age, diagnosis of obsessive compulsive disorder in the subjects, and psychiatric history in the subjects' fathers (p value<0.05).

Conclusions: Despite the fact that SSRIs are generally safe and well-tolerated in young subjects, adverse effects may be fre-

ÖZET

Amaç: Bu çalışma, seçici serotonin geri alım inhibitörü (SSRI) monoterapisi alan genç hastalar arasında ilaç yan etki sıklığını ve bu yan etkilerle ilişkili klinik ve sosyodemografik faktörleri incelemeyi amaçlamaktadır.

Gereç ve Yöntem: Bir üniversite hastanesinin çocuk ve ergen psikiyatrisi kliniklerinde SSRI monoterapisi alan 85 hastanın tıbbi kayıtları incelendi. Dahil edilme kriterlerini karşılayan hastalar çalışmaya dahil edildi.

Bulgular: Toplam 67 hasta (10,82±3,63 yıl) dahil edildi. Hastaların yarısından fazlası (n=39, %58,9) muhtemelen SSRI tedavisi ile ilişkili en az bir yan etki geliştirdi. En sık bildirilen yan etkiler; psişik (n=25, %37,3) ve otonomik yan etkilerin (n=20, %29,9) yanısıra davranışsal aktivasyondu (n=13, %19,4). Yan etkiler nedeniyle 13 hastada (%19,4) ilaç tedavisi sonlandırıldı ve en sık ilaç kesme nedeni davranışsal aktivasyondu (13 hastanın 6'sı). Davranış aktivasyonunun ortaya çıkması; daha küçük yaş, hastada obsesif kompulsif bozukluk tanısı varlığı ve babada psikiyatrik hastalık varlığı ile anlamlı olarak ilişkiliydi (p değeri<0,05).

Sonuç: SSRI'lar genç hastalarda genellikle güvenli ve iyi tolere edilmesine rağmen, yan etkiler sık olabilir ve bazı durumlarda ilacın kesilmesi gerekebilir. Bu nedenle, genç hastaları tedavi eden klinisyenler, özellikle davranışsal aktivasyonun gelişimi konusun-

*This study was presented as an oral research presentation at the 12th International Congress on Psychopharmacology (Antalya) between 17-20 November 2021. This article has been modified from this study.

Corresponding author/İletişim kurulacak yazar: Hanım Hülya ALINAY – hnmozkan@gmail.com

Submitted/Başvuru: 02.08.2022 • **Revision Requested/Revizyon Talebi:** 04.08.2022 •
Last Revision Received/Son Revizyon: 02.11.2022 • **Accepted/Kabul:** 24.11.2022 • **Published Online/Online Yayın:** 27.01.2023

quent, and medication discontinuation may be required under some conditions. Thus, clinicians treating young subjects should be cautious, particularly about the development of behavioral activation. They should also be mindful of the clinical and sociodemographic factors associated with the adverse effects that may arise during SSRI treatment.

Keywords: Adolescents, Adverse Effects, Children, Side Effects, Selective Serotonin Reuptake Inhibitors, Behavioral Activation

da dikkatli olmalıdır. Ayrıca SSRI tedavisi sırasında ortaya çıkabilecek yan etkilerle ilişkili klinik ve sosyodemografik faktörleri de dikkate almalıdırlar.

Anahtar Kelimeler: Ergen, Yan Etki, Çocuk, Seçici Seratonin Geri Alım İnhibitörü, Davranışsal Aktivasyon

INTRODUCTION

Selective serotonin reuptake inhibitor (SSRI) drugs have been widely used among the young population to treat psychiatric disorders with or without FDA-approved indications (1, 2). In clinical practice, major depressive and obsessive compulsive disorders (OCD) are FDA-approved indications, and SSRIs have also been used with several other indications, including anxiety and post-traumatic stress disorders. CDC data show that, between 2011 and 2014, 12.7% of young people aged 12 years and older received antidepressant treatment in the USA (1-4). Although the overall efficacy and safety of SSRIs are supported by large controlled multicenter studies, the adverse effects of SSRI treatment in children and adolescents may be different from adults mainly due to the pharmacodynamic and pharmacokinetic developmental differences between these age groups (5, 6). Indeed, many studies have reported that the adverse effects associated with SSRIs show significant differences across age groups, and children may be more susceptible to such adverse effects of SSRIs, which may be partially explained by their biological immaturity (2, 7, 8). In a review of the side effects of SSRIs in children and adolescents, the commonly reported side effects were behavioral activation, insomnia, somnolence, and gastrointestinal system (GIS)-related symptoms; some of these side effects were reported to be more common in younger children (2). Physical adverse effects due to SSRIs were more commonly reported than psychiatric adverse effects. However, the severity of the latter is significant, as these symptoms result in higher rates of treatment discontinuation (9). Such adverse psychiatric effects can be categorized as follows: signs of behavioral activation (increased activity, impulsivity, and behavioral disinhibition without manic symptoms), manic symptoms (mania, hypomania, and elevated moods), depressive symptoms (worsening depression, crying, irritability, anger, and hypersensitivity), agitative symptoms (agitation, akathisia, restlessness, and irritability), anxiety and panic symptoms, apathy, tremor, and feelings of emptiness (9, 10). Psychiatric adverse effects of SSRIs, especially bipolar disorder, behavioral activation, apathy, and suicidality, can have serious impacts on the patient and his/her family. However, the majority of studies concerning this age group have focused on the efficacy of SSRIs, and relatively few studies have examined the safety profile of SSRI treatment in children and adolescents. To date, no satisfactory data have been presented that recognize adverse effects and how adverse effects relate to SSRI dosages and types as well as the clinical and sociodemographic characteristics of subjects (9, 10). Additionally, data regarding the safety and adverse effects of SSRIs in the young population may sometimes come from studies in which participants received multiple psychotropic drugs during the study period or had experienced previous psychotropic drug use (8). Therefore, it may be important to assess the adverse effects of SSRI treatment and the potential relationship between sociodemographic and clinical variables in medication among naïve young subjects undergoing SSRI monotherapy.

In this retrospective chart review study, we aimed to investigate the prevalence and nature of SSRI-related adverse effects among young subjects receiving SSRI monotherapy and to evaluate the possible relationships between several clinical (i.e., psychiatric diagnosis) and sociodemographic variables (i.e., age and gender).

MATERIAL AND METHODS

Patient selection and procedure

This study was conducted in the Child and Adolescent Psychiatry Department of the Istanbul Faculty of Medicine. The subjects were among the children and adolescents referred to the outpatient clinic from January to June 2018 who had received SSRI monotherapy for any psychiatric diagnosis. The study protocol was approved by the Istanbul University, Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 04.05.2018, Decision No:09). All patients provided written informed consent prior to the study procedures. During the initial clinical interview, the subjects were assessed in terms of reason(s) for referral, provisional diagnosis, and the need for psychopharmacological treatment. The subjects then underwent a detailed clinical assessment, which included a diagnostic interview, if they were lined up to start treatment with SSRIs. The inclusion criteria were as follows: participants must a) be receiving SSRI monotherapy, b) undergo a complete and detailed

psychiatric examination and assessment using routine clinical instruments for the efficacy and safety of SSRI monotherapy, and c) have been followed up for at least three months following the initiation of SSRI monotherapy. Among the 85 subjects, a total of 67 met the inclusion criteria and were subsequently included in the study.

Before SSRI treatment was started, each subject was assessed by an experienced child psychiatry fellow and supervisor for a complete psychiatric diagnostic evaluation using the DSM-5 criteria. Upon receiving a psychiatric diagnosis, each subject was asked to fill out relevant instruments to measure symptom severity, such as those for anxiety and depression. Next, every subject was given SSRI treatment on a routine clinical basis, which included fluoxetine and sertraline as the most commonly prescribed SSRIs in the clinic. As a routine clinical practice, each subject who started SSRI monotherapy was assessed after two or three weeks. The efficacy and safety of SSRI treatment were evaluated using the Clinical Global Impression Scale (CGI), the Screen for Child Anxiety and Related Disorders (SCARED), the Children's Depression Inventory (CDI), the Udvalg for Kliniske Undersogelser (UKU) Side Effect Rating Scale, and a screening tool for investigating SSRI-associated behavioral activation, mania, apathy, and suicidality (SABAMAS). These scales were used in most of the clinical visits. Evaluations in this study occurred subsequent to three months of medication treatment.

Measures

Sociodemographic data

Several parameters, including gender, age, education status, medical history, psychiatric diagnoses, and family characteristics, as well as the type, dosage, and duration of SSRI treatment, were coded depending on the information in the patients' charts.

Clinical Global Impression (CGI) Scale

CGI has three subscales, which assess symptom severity (CGI-S), global improvement (CGI-I), and side effects. The CGI scale has been widely used in clinical studies for all age groups (11). In the present study, the CGI-S (with scores from 1 to 7, with 1= normal or not at all ill and 7= extremely ill) and the CGI-I (with scores from 1 to 7, with 1= very much improved and 7= very much worse) subscales were used to measure symptom severity and improvement, respectively.

Udvalg for Kliniske Undersogelser Side Effect Rating Scale (UKU)

The UKU is a frequently used 48-item scale that measures adverse effects and tolerability related to psychotropic medication (12). The internal consistency coefficients (Cronbach's alpha) were 0.76 for females and 0.82 for males (13). It investigates the side effects among four domains (psychic, neurological, autonomic, and other) for the prior three days, with severity scores ranging from 0 (no, or doubtful, side effects) to 3 (severe side effects).

SSRI-Associated Behavioral Activation, Mania, Apathy, and Suicidality (SABAMAS)

SABAMAS was previously developed by the author (M.C.) and used in clinical practice to investigate the four domains of adverse effects, including behavioral activation (8 items), mania (3 items), apathy (4 items), and suicidality (3 items), which may emerge during treatment with SSRIs. It consists of a total of 18 items, with scores ranging from 0 (no adverse effect) to 3 (a severe adverse effect that requires medication discontinuation). The rationale and the need to develop SABAMAS was that despite there being particularly important adverse effects that may emerge during treatment with SSRIs in young subjects, the UKU did not include the majority of these adverse effects. This scale was developed with respect to the present literature (14) and to accumulated clinical experience. If subjects experienced any symptoms in any of these four domains, they were considered to have developed a particular adverse effect. For example, if the subject had even one symptom in the behavioral activation domain, it was considered an emergence of behavioral activation. The internal consistency was measured using Cronbach's α coefficient; the Cronbach's α coefficient of the SABAMAS in the current study was 0.79.

Statistical analysis

The SPSS 21.0 software program was used for the statistical analysis. The mean, percentage, and standard deviations were used to evaluate the descriptive and clinical characteristics of the subjects. A chi-squared test was employed to compare qualitative data between groups. Binary logistic regression analyses were used to predict the development of behavioral activation as well as psychic and autonomic adverse/side effects. For behavioral activation, the independent variables (age, body mass index, comorbidity, diagnosis of OCD, and psychiatric diagnosis in the father) were used in the regression model.

RESULTS

A total of 67 subjects aged 5–17 years (10.82± 3.63 years) were included in the study. Almost half of the subjects were male (n=33, 49.3%), and slightly more than half of the subjects were under 10 years of age (n=35, 52.2%). The most frequent diagnoses were social anxiety disorder (SAD) (n=48, 71.6%), generalized anxiety disorder (GAD) (n=29, 43.3%), attention deficit hyperactivity disorder (ADHD) (n=28, 41.8%), and specific phobia (SP) (n=17, 25.4%). The most frequent diagnoses for SSRI treatments were anxiety disorders (n=50, 74.6%) and OCD (n=10, 14.9%). The most frequently prescribed SSRIs were

sertraline (n=43, 64.2%) and fluoxetine (n=20, 29.9%). There was a history of diagnosed psychiatric disorder(s) in the first-degree relatives in the majority of the subjects (n=50, 74.6%). The rates of diagnosed psychiatric disorder(s) in mothers and fathers were 41.8% (n=28) and 23.9% (n=16), respectively. Table 1 shows the clinical and sociodemographic characteristics of the subjects.

At the end of the 12th week of SSRI treatment, 23.8% of the subjects (n=16) had at least one adverse effect according to SABAMAS and 70.1% (n=47) had at least one side effect according to the UKU scale. While irritability was the most frequently reported adverse effect (n=13, 19.4%) in SABAMAS, nausea/vomiting was the most frequent side effect for the UKU scale (n=15, 22.4%). According to the UKU scale, none of the subjects developed neurological side effects during treatment. Tables 2 and 3 show the details of the SABAMAS and UKU scales, respectively.

Binary logistic regression was employed to assess the variables that predicted behavioral activation as well as autonomic and psychic adverse/side effects. The regression model for behavioral activation (R2=0.338, p=0.007) showed statistical significance. In particular, behavioral activation was associated with the age of the subject (B=6.4, CI: 1.05; 39.3, p=0.044), a diagnosis of OCD in the subject (B=6.3, CI: 1.05; 39.3, p=0.034), and psychiatric disorder(s) in the father (B=4.9, CI: 1.1; 22.7, p=0.036). There was no significant difference

Table 1: Clinical and sociodemographic characteristics of the subjects

	n	%
Age	**10.82±3.63 years**	
Gender		
Female	34	50.7
Male	33	49.3
Psychiatric disorders		
Social anxiety disorder	48	71.6
Generalized anxiety disorder	29	43.3
Attention deficit hyperactivity disorder	28	41.8
Predominantly inattentive	19	28.3
Combined	9	13.5
Specific phobia	17	25.4
Obsessive-compulsive disorder	12	17.9
Separation anxiety disorder	11	16.4
Tic disorders	8	11.9
Major depressive disorder	6	9
Enuresis	4	6
Post-traumatic stress disorder	2	3
Panic disorder	1	1.5
Encopresis	1	1.5
Oppositional defiant disorder	1	1.5
Indications of SSRI initiation		
Anxiety disorders	50	74.6
Obsessive-compulsive disorder	10	14.9
Major depressive disorder	6	9
Post-traumatic stress disorder	1	1.5
Type of SSRI		
Sertraline	43	64.2
Fluoxetine	20	29.9
Paroxetine	2	3
Escitalopram	2	3
Mean doses of SSRIs/mg		
Sertraline	41.2±18.6	
Fluoxetin	16.9±7.9	
Paroxetin	10.0±0.0	
Escitalopram	3.5±0.7	

Note: SSRI: Selective serotonin reuptake inhibitor

Table 2: SSRI Associated Behavioral Activation, Mania, Apathy and Suicidality (SABAMAS)

	Severity			n (%)
	Mild	Moderate	Severe	
Behavioral activation				13 (19.4)
Irritability	8	4	1	13 (19.4)
Oppositional behaviors	5	4	-	9 (13.4)
Excessive talking	3	4	-	7 (10.4)
Aggressive behaviors	2	3	2	7 (10.4)
Hyperactivity	4	2	-	6 (9)
Risky behaviors	1	2	-	3 (4.5)
Talking with unfamiliar people	1	-	-	1 (1.5)
Apathy				2 (3)
Laziness	-	2	-	2 (3)
Not to care anything	-	2	-	2 (3)
Suicidality				2 (3)
Suicidal ideation	-	2	-	2 (3)
Suicidal behavior	-	-	1	1 (1.5)
Suicide attempt	-	-	1	1 (1.5)

Table 3: UKU side effects in the subjects

	Severity			n (%)
	Mild	Moderate	Severe	
Psychic				25 (37.3)
Concentration difficulties	2	5	3	10 (14.9)
Asthenia/lassitude/increased fatigability	3	5	2	10 (14.9)
Sleepiness/sedation	2	2	-	4 (6)
Failing memory	1	2	-	3 (4.5)
Tension/inner unrest	-	5	1	6 (9)
Increased duration of sleep	4	-	-	4 (6)
Reduced duration of sleep	6	-	1	7 (10.4)
Increased dream activity	2	1	-	3 (4.5)
Emotional indifference	1	1	-	2 (3)
Autonomic				20 (29.9)
Nausea/vomiting	7	5	3	15 (22.4)
Diarrhea	1	1	-	2 (3)
Constipation	1	-	-	1 (1.5)
Polyuria/polydipsia	-	1	-	1 (1.5)
Orthostatic dizziness	6	1	-	7 (10.4)
Palpitations/tachycardia	1	-	-	1 (1.5)
Other				6 (9)
Rash	2	-	-	2 (3)
Headache	2	2	-	4 (6)

Table 4: Results of binary logistic regression models for the variables predicting behavioral activation

Model 1: Binary logistic regression analysis for predictors of behavioral activation adverse/side effects (R^2=0.338, p=0.007)		
Variables	**OR (95%CI)**	**p value**
BMI (Weight/Height^2)	1.04 (0.88-1.22)	0.612
Comorbidity (Absence Presence)	1.73 (0.24-12.16)	0.578
Age (Above 10 → Below 10)	6.44 (1.05-39.38)	**0.044**
Diagnosis of OCD (Absence →Presence)	6.35 (1.15-35.04)	**0.034**
Psychiatric diagnosis in the father (Absence →Presence)	4.95 (1.10-22.17)	**0.036**

Note: Bold data, p<0.05 (significance). CI: Confidence interval. OR: Odd ratio. BMI: Body Mass Index. OCD: Obsessive Compulsive Disorder.

Table 5: Details of Medication Discontinuation in the Subjects

Case	Age (years)/ Gender	Indication for SSRI treatment	SSRI Medication and dosage that adverse(s) effect emerged	Adverse effect(s) led to discontinuation
1	7/F	Separation anxiety disorder	Fluoxetine 10 mg/day	Apathy
2	9/F	Separation anxiety disorder	Sertraline 50 mg/day	Rash
3	10/F	Social anxiety disorder	Paroxetine 10 mg/day	Behavioral activation
4	15/M	Obsessive-compulsive disorder	Sertraline 50 mg/day	Sedation
5	16/F	Generalized anxiety disorder	Sertraline 50 mg /day	Tooth grinding
6	13/F	Social anxiety disorder	Sertraline 50 mg/day	Headache
7	5/F	Obsessive-compulsive disorder	Fluoxetine 5 mg/day	Behavioral activation
8	8/M	Generalized anxiety disorder	Sertraline 25 mg/day	Behavioral activation
9	8/F	Obsessive-compulsive disorder	Sertraline 25 mg/day	Behavioral activation
10	10/M	Generalized anxiety disorder	Sertraline 25 mg/day	Suicidal ideation
11	8/M	Social anxiety disorder	Sertraline 25 mg/day	Behavioral activation
12	15/F	Major depressive disorder	Fluoxetine 20 mg/day	Suicide attempt
13	6/M	Obsessive-compulsive disorder	Fluoxetine 10 mg/day	Behavioral activation

Note: F: Female, M: Male

between fluoxetine (n=20) and sertraline (n=43) in terms of the frequency of behavioral activation (p=0.732). The results of the binary regression model for the variables predicting behavioral activation are presented in table 4.

In our study, none of the cases developed bipolar shift and neurological adverse/side effects. The frequency of apathy and suicidal behavior was lower than the frequency of behavioral activation as well as the frequencies of autonomic and psychological adverse/side effects. Medication was discontinued in 13 subjects (19.4%) due to adverse/side effects. The most frequent adverse effect that led to medication discontinuation was behavioral activation which occurred in six subjects. Table 5 presents the details of medication discontinuation.

DISCUSSION

This study investigated the various extents of adverse effects seen in children and adolescents being treated with SSRI drugs; these included autonomic, psychic, and neurological side effects as well as SABAMAS. Additionally, cases in which SSRI treatment was discontinued due to adverse effects were analyzed with respect to their clinical characteristics. Adverse effects were observed in 58.2% (n=39) of our study cases, with the most common effects being autonomic and psychic problems as well as behavioral activation.

In the present study, behavioral activation was observed in 19.4% (n=13) of children and adolescents. This finding is consistent with those of other studies. For example,

in a study of 82 children and adolescents, Wilens et al. determined that the frequency of behavioral activation was 22% (15). Similarly, a current retrospective study that involved 139 children and adolescents undergoing SSRI therapy reported that adverse effects related to behavioral activation were present in 20.9% of cases (8). Coskun et al. reported high rates of symptoms of behavioral activation in preschool children treated with fluoxetine for distressing OCD symptoms (83%), and with escitalopram for anxiety disorders (45%) (16-17). They concluded that younger age and the presence of high comorbidity particularly ADHD may be associated with high rates of symptoms of behavioral activation. While these symptoms were managed by medication discontinuation or dosage reduction in some subjects, risperidone had been used for pre-existing behavioral problems or to manage symptoms of behavioral activation in some of them (18). In some other reports, Coskun (2017) reported an escitalopram-induced manic switch, and Coskun and Karayagmurlu (2020) reported fluoxetine-induced behavioral activation in two preschool subjects who were treated for distressing symptoms of OCD both having comorbid diagnoses of ADHD and anxiety disorders (19-21).

In our study, the factors associated with the emergence of behavioral activation included younger age, psychiatric disorder(s) in the father, and an OCD diagnosis. Our study results are similar to the findings of other studies when comparing activation and age (16, 17). For instance, Carlson et al. reported that the incidence of medication-induced behavioral activation was more frequent in younger people compared to older people (22). In addition, a systematic review revealed an 11% frequency rate of SSRI-induced behavioral activation in children, with rates ranging from 2% to 4% in teenagers and adults (5). The serotonergic system's maturity at a young age is widely considered to be the mechanism behind the high prevalence of behavioral activation in children compared to adults (2, 8, 23). This theory supports the concept that although SSRI therapy has a high efficacy in children and adolescents, it is also more likely to lead to behavioral activation in children in comparison to adults (24).

Another factor associated with the frequency of behavioral activation is the history of psychiatric disorder(s) in the father. This finding is consistent with the research of Strawn, who reported that children who had family members with medical histories of bipolar disorder were at an increased risk of behavioral activation with SSRI use (25). In addition, children diagnosed with OCD have been shown to be at an increased risk of experiencing behavioral activation. Coskun et al. reported higher rates of behavioral activation with fluoxetine in preschool children for the treatment of OCD than with escitalopram treatment for anxiety disorders (16, 17). However, it is unclear whether this difference is due mainly to medication or diagnosis. Moreover, the literature reveals that the signs of activation syndrome intensify as the SSRI dose increases (26, 27). These results are thought to be related to the poor response of OCD to SSRI drugs compared to the response of other psychiatric disorders, thus requiring higher doses for treatment.

The most common adverse effects associated with SSRI use were psychic side effects (37.3%). Of this group, the most frequently observed adverse effects were concentration problems, sedation, and insomnia. The findings observed in this study mirror those of previous studies that have also examined SSRI-related adverse effects (10, 28, 29). The Treatment for Adolescents with Depression Study (TADS) showed that sedation was among the most common side effects experienced by children using SSRIs (10). Two randomized controlled studies were conducted on the efficacy of sertraline in treating children and adolescents, and the most commonly observed adverse effects were concentration problems, sedation, and insomnia (22, 23).

In the present study, 29.9% of children and adolescents using SSRIs experienced autonomic side effects, with GIS problems and orthostatic hypotension being the most common. Two systematic reviews evaluated the efficacy and safety of SSRI treatment in children and adolescents, and the results showed that GIS side effects, such as nausea, vomiting, and dry mouth, were common in patients (30, 31). In addition, the TADS found that nausea, vomiting, and abdominal pain were among the most frequent side effects (10).

In our study, SSRI-induced neurological side effects or manic shifts were not observed in any of the cases, and apathy and suicidality were observed in 3% of the patients. These findings resemble the results of other studies in the literature. In particular, a systematic review regarding the use of SSRIs in children and adolescents showed that the neurological side effects had a very low rate of occurrence (32). A randomized controlled study that evaluated the risk of manic shift during SSRI treatment revealed that the highest risk was during the peripubertal period and that children and adolescents had a less than 2% risk of experiencing manic shift (33). To the best of the researchers' knowledge, the literature does not contain any data regarding the prevalence of SSRI-induced apathy in this age group, although it alternatively includes case series (34). As mentioned above, 3% of the cases in our study experienced apathy. Data regarding SSRI-induced suicidal behavior in children and adolescents were obtained from multicentered, randomized double-blind placebo control studies, including TADS, TORDIA, TASA, and ADAPT. Although no suicides were realized following SSRI treatment, the

rate of suicidal behavior ranged from 10% to 17% (35). While the samples in the previously mentioned studies consisted mostly of children and adolescents diagnosed with major depressive disorder (MDD), our study mainly included patients with anxiety disorders. The lower occurrence of suicide or suicidality in our study could be explained by the differences in the diagnoses of the samples. In a contemporary meta-analysis study, Sørensen et al. evaluated the risk of suicidality in children and adolescents receiving SSRI treatment, and their results showed that suicide risk was associated with older age and the diagnosis of depression (36).

Although SSRIs have been known to be safe and tolerated well by children and adolescents, two meta-analysis studies reported that 12%–26% of patients using SSRIs discontinued treatment due to adverse effects (37,38). In our study, 58.2% (n=39) of cases experienced adverse effects, and 19.4% (n=13) discontinued SSRI treatment as a result. The most common reason for discontinuing treatment in our study was behavioral activation (n=6). Even though behavioral activation was present in 13 patients, various methods were applied to reduce activation; of these 13 patients, 6 were managed through discontinued treatment, 4 through lowered doses, 1 through the addition of an anti-psychotic dose, and the remaining 2 were monitored in the clinic, as their signs of behavioral activation were mild. Although the literature does not present precise, collective data on how to reduce activation signs, most of the studies recommend lowering the dose as the first approach (8, 39-40). In our study, many methods were applied to reduce signs of activation; however, discontinuing treatment was the initial approach in six cases due to the severity of these symptoms. In conclusion, longitudinal studies must be conducted to further illuminate SSRI-induced adverse effects and their predictive clinical and sociodemographic characteristics.

CONCLUSION

In this study, children and adolescents undergoing SSRI monotherapy were extensively evaluated for potential adverse effects over a period of 12 weeks. The results of this study indicated that children using SSRIs commonly experienced adverse effects, with behavioral activation being the most common reason for discontinuing treatment. Therefore, clinicians treating young subjects should weigh the potential benefits and risks associated with SSRI treatment and be aware of the possible factors associated with the development of adverse effects during SSRI treatment for better compliance and success in children and adolescents.

The current study has certain limitations, such as having a retrospective design, not including a control group, being conducted in a single center, and consisting of a relatively small sample. Additionally, the number of subjects using paroxetine and escitalopram was very low compared to sertraline and fluoxetine which may affect the results of the study.

Ethics Committee Approval: This study was approved by Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 21.05.2018, No: 696).

Peer Review: Externally peer-reviewed.

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

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

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

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ISTANBUL UNIVERSITY PRESS

RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1202881
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

ISOLATED ABERRANT RIGHT SUBCLAVIAN ARTERY: SHOULD INVASIVE INTERVENTION BE RECOMMENDED IN THE ERA OF NONINVASIVE PRENATAL TESTS?

İZOLE ABERAN SAĞ SUBKLAVYAN ARTER: NONİNVAZİV PRENATAL TESTLERİN VARLIĞINDA PRENATAL TANI İÇİN İNVAZİV GİRİŞİM ÖNERİLMELİ Mİ?

Tuğba SARAÇ SİVRİKOZ[1], Selen GÜRSOY ERZİNCAN[2], Lütfiye SELÇUK UYGUR[3], Çiğdem KUNT İŞGÜDER[4], Savcı Bekir TELEK[1], Recep HAS[1], İbrahim Halil KALELİOĞLU[1]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Obstetrics and Gynecology, Istanbul, Turkiye
[2]Bakirkoy Dr. Sadi Konuk Training and Research Hospital, Department of Obstetrics and Gynecology, Istanbul, Turkiye
[3]Zeynep Kamil Women and Children Health Education and Research Hospital, Department of Obstetrics and Gynecology, Istanbul, Turkiye
[4]Prof. Dr. Ilhan Varank Education and Research Hospital, Department of Obstetrics and Gynecology, Istanbul, Turkiye

ORCID IDs of the authors: T.S.S. 0000-0001-5482-9429; S.G.E. 0000-0003-1764-0285; L.S.U. 0000-0002-6325-1910; Ç.K.İ. 0000-0002-0420-1913; S.B.T. 0000-0003-3833-9109; R.H. 0000-0002-1372-8506; İ.H.K. 0000-0003-1349-2561

Cite this article as: Sarac Sivrikoz T, Gursoy Erzincan S, Selcuk Uygur L, Kunt Isguder C, Telek SB, Has R, et al. Isolated aberrant right subclavian artery: Should invasive intervention be recommended in the era of noninvasive prenatal tests? J Ist Faculty Med 2023;86(1):37-43. doi: 10.26650/IUITFD.1202881

ABSTRACT

Objective: An aberrant right subclavian artery (ARSA) is an aortic arch anomaly isolated or associated with other ultrasound markers and/or congenital anomalies. This study aimed to evaluate the necessity of invasive prenatal tests (PIT) in cases with isolated ARSA (iARSA) in prenatal sonography.

Materials and Methods: The presence of ARSA was evaluated retrospectively in 7690 fetuses who underwent a second-trimester ultrasonography evaluation between March 2015 and February 2021. PIT was recommended for patients with non-iARSA. cfDNA test (including 22q11.2 microdeletion/duplication syndrome (MMS) or PIT was suggested for patients with iARSA.

Results: The mean week of gestation was 20.26±3.93 in 95 fetuses diagnosed with ARSA. Of the fetuses, fourty-two (44%) had iARSA, and 53 (56%) had additional findings. No chromosomal abnormality was found in any of the isolated cases. Trisomy 21 in 14, Trisomy 18 in one, 47,XX,+i(9)(p10) in one of 53 were found in non-isolated cases. Additional abnormalities and/or soft ultrasound markers were accompanied in all fetuses with chromosomal abnormalities.

Conclusion: When iARSA is detected in prenatal ultrasonography, cfDNA testing may be sufficient, including 22q11.2 MMS. However, PIT should be recommended in the presence of struc-

ÖZET

Amaç: Aberan sağ subklavyen arter (ASSA), izole veya diğer ultrason belirteçleri ve/veya konjenital anomalilere eşlik eden bir aortik ark anomalisidir. Bu çalışmada, prenatal sonografide izole ASSA saptanan olgularda prenatal invaziv test (PIT) gerekliliğinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: Mart 2015 ile Şubat 2021 arasında ikinci üçay ultrasonografi değerlendirilmesi yapılan 7690 fetüsten oluşan popülasyonda, ASSA varlığı retrospektif olarak değerlendirildi. ASSA ile birlikte ek konjenital anomalisi olan hastalara PIT önerilirken, ASSA'nın izole olduğu olgularda 22q11.2 mikrodelesyon/dublikasyon (MMS) dahil hücre dışı DNA (cfDNA) testi veya PIT önerilmiştir.

Bulgular: ASSA bulunan 95 fetüste ortalama gebelik haftası 20,26±3,93 olarak saptanmıştır. Bunlardan 42'sinde izole ASSA, 53'ünde ise ASSA dışı ek bulgular mevcuttu. İzole olguların hiçbirinde kromozom anomalisi saptanmazken, izole olmayan 53 olgudan, 14'ünde Trizomi 21, birinde Trizomi 18, birinde ise 47, XX,+i(9)(p10) saptanmıştır. Kromozom anomalisi saptanan fetüslerin tamamında ek anomali ve/veya minor belirteçler eşlik etmekteydi.

Sonuç: Prenatal ultrasonografide izole ASSA saptanan olgularda, 22q11.2 MMS da dahil olmak üzere noninvaziv cfDNA testinin

Corresponding author/İletişim kurulacak yazar: Tuğba SARAÇ SİVRİKOZ – tugbasrc@gmail.com

Submitted/Başvuru: 11.11.2022 • **Revision Requested/Revizyon Talebi:** 08.12.2022 •
Last Revision Received/Son Revizyon: 16.12.2022 • **Accepted/Kabul:** 16.12.2022 • **Published Online/Online Yayın:** 24.01.2023

tural abnormalities, soft ultrasound markers, or increased risk in the antenatal screening test.

Keywords: Aberrant right subclavian artery, Cell-Free DNA, Down syndrome, 22q11.2 microdeletion, prenatal diagnosis, ultrasound

yapılması yeterli olabilir. Ancak, ek majör anomali, minör belirteç veya tarama testinde risk artışı varlığında PIT önerilmelidir.

Anahtar Kelimeler: Aberran sağ subklavyen arter, hücre dışı DNA, Down sendromu, 22q11 mikrodelesyonu, prenatal tanı, ultrason

INTRODUCTION

An aberrant right subclavian artery (ARSA) is the most common aortic branching abnormality and occurs either in isolation or in association with other soft markers and congenital anomalies (1-4). Fetuses with ARSA are at risk of having chromosomal aberrations such as Trisomy 21 (Down syndrome; DS) and 22q11.2 microdeletion syndrome (DiGeorge Syndrome; DGS) (1). While the incidence of ARSA is about 1% to 2% in fetuses with normal karyotype, the incidence is reported to be about 28% to 37.5% in fetuses with DS diagnosed in the second trimester (2-4). In a recent systematic review covering 12 studies, ARSA established an important marker for DS, with a likelihood ratio (LR) of 26.9 (5). However, in a meta-analysis evaluating the performance of sonographic soft markers detected in the second trimester, it was suggested that the risk of aneuploidy in the prediction of Trisomy 21 risk was mainly derived from first trimester findings (6). Based on this data, in the absence of all other markers, the positive LR was found to be 3.94 in the presence of isolated ARSA (iARSA). Hence, according to local guidelines during the second trimester evaluation, some authors recommend that pregnant women be classified as low-intermediate and high-risk (6, 7). The published data has limited evidence to describe the value of microdeletion/duplication syndromes (MMSs) in fetuses with ARSA (8). In some countries, prenatal invasive testing, including chromosomal microarray, is recommended for fetal structural anomalies, including ARSA (1). Consequently, although an association between DS/DGS and ARSA has been reported in preliminary studies, this association still needs to be investigated, especially in isolated ARSA cases.

Cell-free DNA (cfDNA) in maternal circulation was first reported by Lo et al. in 1997, and this discovery brought up the development of a noninvasive prenatal approach as a screening test for fetal chromosomal abnormalities (9). Detection rates in a recent meta-analysis evaluating cfDNA screening were higher than 99% for trisomy 21, 98% for trisomy 18, and 99% for trisomy 13, with a combined false-positive rate of 0.13% (10). Conventional aneuploidy screening is not designed to detect MMSs, and fetal ultrasonographic assessment may be limited as prenatal findings associated with MMSs may not be obvious (11). Most of the MMSs associated with clinically significant copy number variations (CNVs) and the pathogenic CNVs are diagnosed by chromosomal microarray analysis (11, 12). It was reported that most cases of DGS, including both classical and nested deletions that are >500 kb, are identified with single-nucleotide polymorphism (SNP) based cfDNA screening (12). A more recent study presented that a targeted cfDNA test for DGS detects the common nested deletions with a low false-positive rate (12-14). Although there are long-held reservations about using prenatal cfDNA screening tests for microdeletion syndromes, recent studies have reported a sensitivity of 86.7% and a specificity higher than 99% for DGS, despite low positive predictive values (15). However, in more recent studies, the predictive values of cfDNA testing for DGS were higher (14). Hence, it is known that preventing unnecessary prenatal invasive testing by using cfDNA in border cases is still a controversial issue.

The study aimed to investigate whether there is a need for invasive intervention when cfDNA testing is used as a prenatal aneuploidy screening test for iARSA cases.

MATERIALS AND METHODS

This unselected population-based retrospective study was performed in a prenatal diagnosis clinic between March 2015 and February 2021. All data were obtained during detailed fetal midtrimester ultrasounds. Previous antenatal aneuploidy screening tests and cfDNA test results were evaluated and recorded in all patients who were admitted to our outpatient clinic for the routine mid-trimester fetal ultrasound scan. The records of the patients were kept in Medikbase's electronic medical record system, known as Gynobserve. After informing each patient about the success and limitations of the ultrasound to be performed and obtaining their consent, a detailed sonographic examination was performed using the checklists by the same operator. All fetal organ systems and soft markers were evaluated in detail with high-frequency transabdominal transducers (Voluson E8 Expert system, RAB6-D; GE Healthcare, Zipf, Austria). The study was conducted in accordance with the Declaration of Helsinki and was approved by the local Ethics Committee of the Istanbul Faculty of Medicine (Date:21.10.2022, No:19).

The ARSA assessment was carried out with the technique Chaoui et al. previously described (2). After the fetal three-vessel and tracheal views were obtained, Doppler velocity was reduced to 15 to 30 cm/s. An ARSA was diagnosed as a separate artery originating from the junction of the aortic and ductal arches and running between the trachea and vertebra. The thymus was also evaluated in all fetuses detected with ARSA. Cases with ARSA were considered isolated when there were no associated sys-

temic structural anomalies and/or soft markers during the midtrimester sonography. In the case of related abnormalities and/or soft markers, patients were categorized as non-isolated. Soft markers included increased nuchal translucency (NT)>95th percentile detected at first trimester ultrasonography, echogenic intracardiac focus, short femur <5th percentile, short humerus <5th percentile, choroid plexus cyst, thickened nuchal fold, pyelectasis, echogenic intestine, hypoplastic and/or absent nasal bone.

We offered either cfDNA testing or an invasive procedure to detect DS and DGS to the patients whose fetuses were found to have ARSA with no associated structural anomaly (for fetuses with isolated ARSA or fetuses with ARSA plus soft markers and/or screening test positivity). The invasive intervention was primarily recommended to pregnant women whose fetuses had concomitant cardiac or structural abnormalities other than ARSA. After counseling for prenatal invasive procedures, amniocentesis (AC) was performed by standard procedure. AC samples were investigated by cell-culture techniques with fluorescent in-situ hybridization (FISH). If the cfDNA test was positive for MMSs, microarray analysis was planned. Neonatal echocardiography was offered to all fetuses detected with ARSA after delivery to exclude other possible cardiac defects. Postnatal karyotyping was considered normal in newborns who did not undergo invasive prenatal testing. Postnatal karyotyping was offered if the newborn had any abnormal physical appearance or structural abnormality.

RESULTS

A detailed fetal systemic midtrimester ultrasound examination was performed on 7690 singleton pregnancies within the specified time period. Among them, ARSA was detected in 95 of them (1.23%) (Figure 1). In the study group, ARSA (isolated or non-isolated) was detected, the mean maternal age was 31.9±5.6, and the mean gestational age was 20.26±3.93 weeks at diagnosis. Hypoplasia or aplasia of the thymus was not detected in fetuses with ARSA.

Isolated ARSA as a sonographic finding was detected in 42 (44%) cases; antenatal screening tests were also positive in six cases. Prenatal invasive intervention or cfDNA testing was offered for all cases in the isolated ARSA group. Ten of the 42 cases had already undergone cfDNA testing. Five of the cases in this group chose the invasive procedure, and 10 chose the cfDNA testing. The remaining cases in this group accepted neither cfDNA testing nor an invasive procedure. No chromosomal abnormality was detected in this group.

The remaining cases (53/95; 56%) were categorized as non-isolated. Twenty-five cases (25/53; 47%) had at least one of the soft markers, 20 of the remaining cases (20/53; 38%) had cardiovascular abnormalities, and 8 (8/53; 15%) had extracardiac abnormalities (Table 1, 2). The prenatal invasive intervention was offered to all cases with any systemic structural abnormalities. In 25 fetuses with additional soft markers, four cases had cfDNA testing before ultrasonography, and the test results were negative. Six-

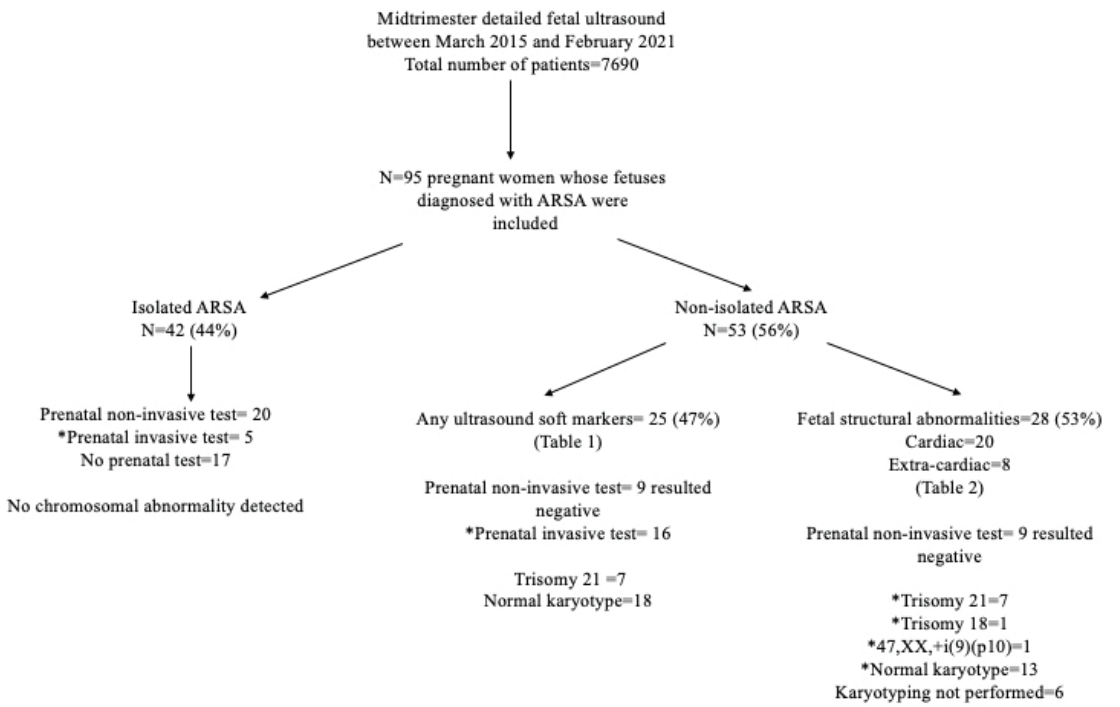

Figure 1: Diagnostic flow of all prenatal cases included in the current study (*Amniocentesis was performed in these prenatal cases)

Table 1: Prenatal ultrasonographic characteristics and outcomes in the cases of ARSA associated with soft markers

	Age	Screening test result	iNT	EIF	SH/SF	CPC	tNF	P	EI	NB	cfDNA testing	Fetal karyotype	Outcome
1	41	High risk	+	+								T21	TOP
2	32	High risk		+	+							T21	TOP
3	35	High risk		+				+				T21	Alive *
4	33	High risk		+							Low risk	Normal	Alive
5	39	Low risk		+		+						T21	TOP
6	30	Low risk					+					Normal	Alive
7	35	Low risk							+		Low risk	Normal	Alive
8	31	Low risk		+			+					Normal	Alive
9	34	Low risk						+			Low risk	Normal	Alive
10	37	Low risk					+					Normal	Alive
11	25	Low risk		+							Low risk	Normal	Alive
12	25	Low risk		+					+			T21	TOP
13	29	High risk	+									Normal	ID, CHARGE syndrome
14	28	Low risk		+		+			+		Low risk	Normal	Alive
15	33	Low risk						+			Low risk	Normal	Alive
16	33	Low risk					+	+				Normal	Alive
17	28	Low risk				+					Low risk	Normal	Alive
18	31	High risk	+							+		Normal	Alive
19	32	Low risk				+					Low risk	Normal	Alive
20	38	High risk	+									Normal	Alive
21	31	Low risk						+		+		T21	TOP
22	40	Low risk					+					Normal	Alive
23	27	Low risk		+				+				Normal	Alive
24	34	High risk	+	+								T21	TOP
25	23	Low risk		+							Low risk	Normal	Alive

cfDNA: cell free DNA, CPC: choroid plexus cyst, EI: echogenic intestine, EIF: echogenic intracardiac focus, NB: hypoplastic and/or absent nasal bone, iNT: increased nuchal translucency, tNF: thickened nuchal fold, SH: short humerus, SF: short femur, P: pyelectasis, T21: Trisomy 21, TOP: termination of pregnancy, ID: Infant death
**This case was diagnosed trisomy 21 postnatally.*

teen cases in this group chose the invasive procedure, and 5 chose the cfDNA testing. In this group, seven fetuses, one postnatal, were diagnosed with DS. A procedure-related abortion occurred in one case who had no chromosomal abnormality.

Prenatal invasive diagnostic testing was offered to all cases (28/53; 53%) which had an additional structural (cardiac or extracardiac) abnormality (Table 2). Although six denied prenatal invasive intervention, the remaining 22 cases opted to have karyotype. Pregnancies were terminated due to multiple anomalies in 2 of the 6 cases that underwent no invasive procedure. One case (case 9, table 2), which was associated with multiple abnormalities, including polyhydramnios, resulted in abortion after premature rupture of membranes. In the other case (case 12, table 2), the pregnancy was terminated due to maternal Mirror syndrome. The remaining 2 cases are currently alive and are under echocardiographic follow-up. The prenatal cardiac findings (case 10 and 14, table 2) were confirmed postnatally.

DS was diagnosed in 7 of 28 fetuses. Trisomy 18 and 47,XX-,+i(9)(p10) were diagnosed in another 2 cases (case 19 and 20; table 2). All parents with fetal chromosomal abnormalities opted to terminate the pregnancy, except for one diagnosed with DS. The remaining 2 of the 13 cases died postnatally. One was diagnosed with Cornelia de Lange syndrome after birth, while the other died after cardiac surgery. Five of 6 live births are being followed up due to cardiac anomalies, and one case was operated on for a portosystemic shunt.

Table 2: Prenatal ultrasound findings and outcomes in ARSA cases with additional congenital anomalies

Case	Maternal age	CVS	CNS	Face/ Neck	GUS	GIS	Skeletal System	Number of associated soft markers	Karyotype	Outcome
1	38	AVSD			EK				T21	TOP
2	37	VSD	BBV		UDK	iGB		3	NP	TOP
3	30	VSD				DBS		2	T21	TOP
4	29	AVSD						3	T21	Alive
5	39	None	HC					1	Normal	TOP
6	41	VSD					Talipes	3	Normal	Alive
7	22	AVSD						2	T21	TOP
8	26	aDV						1	Normal	Alive (PSS surgery)
9	32	None	IHC, C			EA, poly-hydramniosis			NP	TOP
10	36	VSD							NP	Alive (surgery)
11	32	VSD						2	Normal	CdLS, ID
12	34	None		CH	BRA				NP	TOP (MMS)
13	31	RAA							Normal	Alive
14	29	PLSVC							NP	Alive
15	34	None				iGB			Normal (BA)	TOP
16	27	None		CH				1	Normal	TOP
17	23	VSD							Normal	Alive
18	34	None					SB		NP	TOP
19	34	AVSD			EK	Omphalocele		2	T18	TOP
20	31	DORV							47,XX,+i(9)(p10)	TOP
21	40	None	BBV	CLP					Normal	TOP
22	25	CoaAo				EA		1	Normal	Alive (surgery)
23	27	None				Omphalocele		2	T21	TOP
24	28	AVSD						1	T21	TOP
25	20	DORV						1	Normal	ID (perop)
26	42	RAA				DBS		1	T21	TOP
27	26	aDV	BBV				Talipes, DRD		Normal	TOP
28	29	AVSD		CLP	BRA		Talipes	1	Normal	TOP

aDV: Agenesis of Ductus venosus, AVSD: Atrioventricular septal defect, BBV: Bilateral borderline ventriculomegaly, BA: Biliary atresia, BRA: Bilateral renal agenesis, C: Cephalocele, CVS: Cardiovascular system, CNS: Central nervous system, CLP: Cleft lip-palate, CoaAo: Coarctation of aorta, CdLS: Cornelia de Lange syndrome, CH: Cystic hygroma, DRD: Distal reduction defect, DBS: Double-bubble sign, DORV: Double outlet right ventricle, EK: Echogenic kidneys, EA: Esophageal atresia, GIS: Gastrointestinal system, GUS: Genitourinary system, HC: Hydrocephaly, iGB: Invisible gall bladder, ID: Infant death, IHC: Interhemispheric cyst, MMS: Maternal Mirror syndrome, NP: not performed, PLSVC: Persistent left superior vena cava, perop: peroperative, PSS: Porto-systemic shunt, RAA: Right aortic arch, SB: Spina bifida, T21: Trisomy 21, T18: Trisomy 18, TOP: Termination of pregnancy, UDK: Unilateral dysplastic kidneys, VSD: Ventricular septal defect

DISCUSSION

ARSA is known to be a clinically useful prenatal ultrasound marker of DS. Regarding its association with DS, Paladini et al. demonstrated that ARSA was the third most important second trimester marker for Down syndrome after hypoplastic nasal bone and cardiac abnormalities (16). In our unselected population, the prevalence of ARSA was 1.23%, similar to the other studies. DS was not detected in any of the iARSA cases. Fourteen cases with ARSA were diagnosed with DS (14/53; 26%), and all were in the non-isolated ARSA group. Half of these DS fetuses had associated congenital anomalies, and the remaining seven had ultrasound soft markers. In the literature, some studies revealed ARSA as the only ultrasonographic marker in fetuses with Down syndrome (4,17,18). However, in these studies, study populations comprised mostly high-risk patients for chromosomal abnormalities (4,17,18). The meta-analysis by Agathokleous et al. demonstrated that ARSA is a significant marker for Down syndrome (positive likelihood ratio, LR+=21.48), whereas its normal course is a protective marker (negative likelihood ratio, LR−=0.7) (6).

However, in most recent studies, iARSA is found to be benign and not associated with Down syndrome or 22q11 microdeletion syndrome (19-21). Similarly, the meta-analysis by De León-Luis et al. showed no association between isolated ARSA and DS (22). They detected the LR+ as 0 in iARSA cases, whereas for non-isolated cases, it was 199 (23). They highlighted that the presence of high background risk, associated abnormalities, and/or soft markers should guide the management of karyotyping. In the current study, iARSA was not detected in any cases diagnosed with DS. Moreover, in a meta-analysis for DS, detection rate (DR) and false positive rates in singleton pregnancies were 99.2% (95% CI, 98.5 – 99.6%) and 0.09% (95% CI, 0.05 – 0.14%), respectively (23). From this point of view, it raises doubts that non-invasive prenatal tests should be included in the management steps in isolated ARSA cases.

In accordance with the recent literature, all fetuses with ARSA and genetic abnormalities had additional ultrasound findings in our cohort (6,20). Our previous study detected a weak association between ARSA and DS in an unselected population (24). With additional malformations, soft ultrasound markers, and high background risk, the risk of chromosomal abnormalities in a fetus with ARSA may be increased. As in the current study, 16 of the 53 fetuses (30.18%) in non- isolated ARSA group had chromosomal abnormalities. In this group, 14 fetuses were trisomy 21 (26%), one case was trisomy 18, and the other was 47,XX,+i(9)(p10). In a large case series in the Turkish population, it was reported that 18.9% of the cases with non-isolated ARSA were diagnosed with a chromosomal abnormality (25). It should be kept in mind that ARSA alone may not create a sufficient indication for invasive testing; instead, it may be managed with noninvasive prenatal testing.

Besides DS, the association between ARSA and DGS has also been reported in the literature (17). Although most guidelines do not recommend cfDNA testing as a routine screening test for microdeletions, recent studies report greater clinical performance of the test for DGS and suggest using cfDNA testing for pregnancies at risk for DGS to avoid maternal anxiety and unnecessary invasive procedures (14,15). Maya et al. demonstrated that ARSA was associated with DGS, especially in the presence of increased nuchal translucency (>4 mm), ventricular septal defect, clubfoot, right aortic arch, echogenic intracardiac focus, and increased risk for trisomy 21 at maternal serum screening (26). In the Sagi-Dain study, no 22q11.2 deletion was detected among 246 isolated ARSA cases (8). Although there are different results in the literature, no cases with DGS were detected in the current study, which was associated with ARSA with/without cardiac anomalies.

The main limitations of our study were its retrospective design and limited sample size of isolated cases. This may have affected the detection rate of chromosomal abnormalities in the current study, although several isolated cases were similar to those in other studies (22). Our study's strengths are that the same operator examined many fetuses, and all of these examinations were performed using the checklist. The number of patients in the current study was limited to generalize about both DS and DGS. Although no chromosomal/non-chromosomal abnormality was detected in isolated cases with ARSA, larger case series are needed to guide the literature. In addition, we think that the result of the current study may be noteworthy since it does not contradict the data published so far.

In summary, it has been shown that iARSA cases may not be associated with DS and DGS, as reported in the current study's results. The detection rate of cfDNA testing for DS has been reported as 99.7%, with a false positive rate of 0.04% (10). Hence, the current study is compatible with the literature about screening DS associated with soft ultrasound markers by non-invasive prenatal tests. Therefore, it is suggested that isolated cases of ARSA may be managed with non-invasive cfDNA testing, including analysis for 22q11 microdeletion. Moreover, karyotyping should be recommended in patients with additional major anomalies, associated soft markers, and/or high-risk results at screening tests.

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

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- T.S.S., İ.H.K., S.G.E.; Data Acquisition- Ç.K.İ., L.S.U., S.B.T.; Data Analysis/Interpretation- İ.H.K., T.S.S., R.H.; Drafting Manuscript- T.S.S.,

İ.H.K., S.G.E., L.S.U., Ç.K.İ., S.B.T.; Critical Revision of Manuscript- İ.H.K., R.H.; Final Approval and Accountability- T.S.S., İ.H.K., S.G.E.; Material or Technical Support- S.B.T., L.S.U., Ç.K.İ.; Supervision- İ.H.K., R.H.

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

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

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İSTANBUL UNIVERSITY PRESS

RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1193997
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

DETERMINING THE IMPORTANCE OF GLYCEMIC VARIABILITY IN GESTATIONAL DIABETES MELLITUS USING VARIOUS TECHNIQUES

GESTASYONEL DİABETES MELLİTUSTA GLİSEMİK DEĞİŞKENLİKLERİN ÖNEMİ VE FARKLI YÖNTEMLERLE ARAŞTIRILMASI

Nida ÖZTOP[1], Ayşe KUBAT ÜZÜM[2], Selda ÇELİK[3], Cemile İDİZ[2], Yıldız TÜTÜNCÜ[4], Elif BAĞDEMİR[2], Nevin DİNÇÇAĞ[2]

[1]Istanbul Başakşehir Çam ve Sakura City Hospital, Department of Adult Allergy and Clinical Immunology, Istanbul, Turkiye
[2]Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine, Division of Endocrinology and Metabolism, Istanbul, Turkiye
[3]University of Health Sciences, Hamidiye Faculty of Nursing, Istanbul, Turkiye
[4]Koç University, Faculty of Medicine, Department of Immunology, Istanbul, Turkiye

ORCID IDs of the authors: N.Ö. 0000-0003-2607-3833; A.K. 0000-0003-0478-1193; S.Ç. 0000-0003-4328-3189; C.İ. 0000-0001-6635-5996; Y.T. 0000-0002-3905-6429; E.B. 0000-0002-0035-6360; N.D. 0000-0003-3986-4546

Cite this article as: Oztop N, Kubat Uzum A, Celik S, Idiz C, Tutuncu Y, Bagdemir E, et al. Determining the importance of glycemic variability in gestational diabetes mellitus using various techniques. J Ist Faculty Med 2023;86(1):44-51.
doi: 10.26650/IUITFD.1193997

ABSTRACT

Objective: The study aims to determine glycemic variation in patients with gestational diabetes mellitus (GDM) and to evaluate the effect on the fetal growth using a continuous glucose monitoring system (CGMS) and to investigate the correlation between glucose variation through biomarkers including HbA1c, fructosamine (FRM), and 1.5-Anhydroglucitol (1.5-AG).

Materials and Methods: The study involves 31 women with GDM at gestational week ≥35 who'd only had diet therapy. Blood glucose levels were monitored for three consecutive days using CGMS to evaluate mean blood glucose levels and mean absolute difference (MAD). Self-monitoring of blood glucose (SMBG) was required from the patients while having the CMGS on their body. Blood samples were collected to measure serum 1.5-AG, HbA1c, and FRM.

Results: The mean levels were HbA1c=5.0±0.3%, FRM=2.1±0.2 µmol/L, 1.5-AG=17.0±4.9 ng/ml, and 3-day average max-min glucose range=131.1±22.5 and 54.7±11.6 mg/dl (MAD=6.7±3.1%). The mean glucose levels measured using SMBG and CGMS were similar (82.9±10.2 vs 86.1±10.3 mg/dL). No correlation occurred between CMGS and biomarkers. The baby weight at birth and head circumference was determined to be lower for patients with glucose fluctuations.

Conclusion: Biomarkers do not reflect glycemic fluctuation, and regular SMBG is required to achieve the desired glucose

ÖZET

Amaç: Gestasyonel Diabetes Mellitus (GDM)'da gün içi glukoz dalgalanmaları ve bunun bebek üzerine etkisini belirlemek; ayrıca, glukozdaki dalgalanmaların HbA1c, fruktozamin (FRM) ve 1,5-Anhidroglucitol (1,5-AG) ile korelasyonunu saptamaktır.

Gereç ve Yöntem: Sadece diyetle takip edilen GDM tanısı alan ve ≥35 gebelik haftasındaki 31 hastada devamlı glukoz ölçüm sistemi (CGMS) ile 72 saatlik glisemik değişkenlikler (ortalama mutlak değer %MAD ve ortalama glukoz değeri) ölçüldü, ayrıca hastalardan, CGMS takılı olduğu günler, kendi kendine glukoz ölçüm sistemi (SMBG) her öğün öncesinde ve birinci saat sonrasında parmak ucundan kan glukoz düzeylerini ölçmeleri istendi. 1,5 AG, Hba1c ve FRM düzeyleri CGMS çıkarıldığı üçüncü gün hastalardan alındı.

Bulgular: Hastaların ortalama HbA1c, FRM ve 1,5-AG sırasıyla %5,0±0,3, 2,1±0,2 µmol/L, ve 17,0±4,9 ng/mL idi. Üç günlük izlemde maximum-minimum glukoz düzeyi ortalaması 131,1±22,5 ve 54,7±11,6 mg/dL iken %MAD değeri %6,7±3,1 idi. SMBG ve CGMS ile ölçülen ortalama glukoz değeri birbiri ile koreleyken (82,9±10,2 ve 86,1±10,3 mg/dL); glukoz dalgalanması ile FRM, HbA1c ve 1,5-AG arasında anlamlı korelasyon yoktu. Hastaların glikoz dalgalanmaları varsa doğumdaki bebek ağırlığının ve baş çevresinin düşük olduğu belirlendi.

Sonuç: Çalışmamızda biyobelirteçlerin glisemik dalgalanmayı yansıtmadığı; istenilen glukoz seviyesinin sağlanması için, diyet-

Corresponding author/İletişim kurulacak yazar: Nida ÖZTOP – nida_oztop@hotmail.com

Submitted/Başvuru: 24.10.2022 • **Revision Requested/Revizyon Talebi:** 16.11.2022 •
Last Revision Received/Son Revizyon: 16.11.2022 • **Accepted/Kabul:** 29.12.2022 • **Published Online/Online Yayın:** 26.01.2023

level, even in diet-regulated GDM. Lower head circumference and birth weight were determined in GDM mothers with high glycemic fluctuations, and CGMS may be an alternative method despite its cost and application difficulties.

Keywords: Gestational diabetes, 1.5-anhydroglucitol, glycemic variability, HbA1c, fructosamine

le regüle GDM de bile, SMBG'un sık, düzenli olarak yapılmasının gerekliliği saptanmıştır; ancak glisemik dalgalanmaları fazla olan GDM'li annenin bebeğinde baş çevresi ve doğum kilosu daha düşük saptanmıştır ve glisemik dalgalanmayı daha yakından gösteren CGMS' in her ne kadar maliyet ve uygulama zorluğu olsa da, SMBG' ye alternatif yöntem olabileceği gösterilmiştir.

Anahtar Kelimeler: Gestasyonel diabetes, 1.5 anhidroglucitol, glisemik değişkenlik, HbA1c, fruktozamin

INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as glucose intolerance resulting in maternal and fetal complications that first begin in pregnancy (1). Pancreatic beta cell defect and chronic insulin resistance are thought to be present in GDM pathophysiology (2). Multiple maternal and fetal complications can occur such as hydramnios, preeclampsia, macrosomia, hypertension, and neonatal respiratory problems in GDM (3). The oral glucose tolerance test (OGTT) is recommended between the 24th-28th week of pregnancy due to GDM's high risk of complications (4).

Poorly controlled blood glucose levels are known to correlate with microvascular complications, with post-prandial hyperglycemia being a significant risk factor for macrovascular complications in GDM (5). In the third trimester of pregnancy, the insulin effect decreases by 50-70%, with the insulin concentrations being twice as high compared to non-pregnant women. Additionally, total gluconeogenesis increases in late pregnancy (6). Blood glucose levels may fluctuate during the day, and fluctuations can define a glycemic variability that can cause an increase in free radicals through oxidative stress. Free radicals are well known to trigger tissue damage by causing endothelial dysfunction due to a wide variety of pathological pathways and different mechanisms (7). Although the correlation between glycemic variability and maternal/fetal complications is known, not enough information is found on this subject. Furthermore, no indicator is present that directly reflects glycemic variability (5). Therefore, markers that show glycemic variability are needed in clinical practice (8). Self-monitoring of blood glucose (SBMG) can be used for glycemic control but can only be used to identify symptomatic hypoglycemia. Most hyperglycemia variabilities should be known to be asymptomatic, and this situation can be overlooked when using SBMG (4, 9). The most ideal method for determining the amplitude of glycemic variability is a continuous glucose monitoring system (CGMS), which can determine the percentage of mean absolute difference (MAD%) and be used to evaluate glycemic variability in patients (10). However, the uses of CGMS in clinical practice are limited due to its expensive and invasive nature. In clinical practice, glycemic control monitoring occurs by Hemoglobin A1c (HbA1c) and fructosamine (FRM) in GDM. Although HbA1c reflects three-month glycemic control, it may test normal even in the presence of significant glycemic variability, as hypoglycemic episodes compensate for hyperglycemia. Studies have shown fetal complications to develop frequently, even at normal HbA1c levels (11). FRM is another marker that reflects the average glucose level over a 1-3week period and has been recommended due to its ability to reflect short-term glucose changes in cases where rapid therapy change may be required during pregnancy (12). Neither HbA1c nor FRM are sensitive indicators of glycemic variability in GDM. A more sensitive marker is needed in addition to these markers for evaluating variability. The literature has shown 1.5 Anhydroglucitol (1.5-AG) to reflect short-term glycemic control, variability, and postprandial hyperglycemia (13).

In line with this information, this study aims to investigate the fluctuations in daily glucose levels in the third trimester of pregnancy with GMD using CGMS and SMBG and to evaluate the correlation among the HbA1c, FRM, and 1.5 AG markers.

MATERIALS AND METHODS

Study design and patient selection

The study involves 33 pregnant women with GDM undergoing follow-up in Istanbul University Adult Endocrinology Clinic after the 35th week of pregnancy. GDM was diagnosed in accordance with the Turkish Society of Endocrinology and Metabolism Diabetes Mellitus guidelines (4). The patients' demographic and clinical characteristics include questions about family history, gestational week, pre-gestational weight, weight during pregnancy, pre-gestational body mass index (BMI), comorbidity, previous abortion history, stillbirth, or large baby history. A physical examination of each patient was performed by the physician conducting the study, and each patient consulted with the gynecology clinic in terms of the fetal exam. Pregnant women who'd been diagnosed with GDM in previous pregnancies or have GDM and receive insulin therapy were excluded from the study in order to prevent iatrogenic glycemic variability. In addition, those with GDM and hemoglobin levels <12 gr/dL were also excluded from the study to prevent false low readings regarding HbA1c.

This study was approved by the local institution's ethics committee (Date: 21.02.2014, No: 04) and was conducted with the funding through the Istanbul University Scientific Research Project (Project No. 44800). Written informed consent was obtained from all study participants.

Evaluating glycemic variability

In order to evaluate the daily glucose level in each patient, a 72-hr CMGS (brand name Enlite Glucose Sensor®) was applied to all patients' extensor side of the upper arm, and all glucose measurements over the 72 hours were recorded on the Medtronic recorder®. At the end of the 72 hours, the patients were called in to have the sensor removed. The data from the CGMS was uploaded over the Internet to the software program CareLink iPro® specially organized by Medtronic®. To correlate the CMGS measurement with the SMBG, patients were asked to measure and keep a dairy of their blood glucose levels using their own blood glucose meters six times daily (pre- and 1-hr post-breakfast, pre- and 1-hr post-lunch, and pre- and 1-hr post-dinner). The patients' total number of glucose measurements during the 72 hours with CGMS; the highest, lowest, and mean glucose levels over the three days; standard deviation; daily absolute mean variability in glucose (MAD%); and number of high, low, and total fluctuations in glucose level were determined using the CGMS. In addition, the time interval below, between, and above the limit glucose values of 70-140 mg/dL, as well as the areas under the curves above the limit glucose values of 140 mg/dL (AUC -above limit) and below 70 mg/dL (AUC-below limit) were detected by the CGMS. The MAD% value was used for the 72-hr glucose variability in patients with GDM.

To determine the correlations between CMGS and SMBG using the 1.5-AG, HbA1c, and FRM, a blood sample was taken from the patients on the day of CGMS insertion. For the 1.5-AG level, the plasma was separated from the blood samples and kept at -80°C.The 1.5-AG measurements were studied collectively once all samples had been collected using the ELISA technique (Cusabio, Wuhan, China). The reference value for 1.5-AG is 14.4-30.2 mg/L in healthy subjects (14).

To evaluate the correlation 1.5-AG has with HbA1c and FRM, the HbA1C levels were measured using cation-exchange high performance liquid chromatography (HPLC; Bio-Rad, Richmond, California, USA), and FRM levels were determined using the colorimetric method within the Roche modular system (Roche, Mannheim, Germany).

Evaluating the effect of mothers' glycemic levels during pregnancy on their babies postpartum

To determine the effect of mother's glucose level on their baby when labor occurred, the patients were contacted by the physician conducting the study to record information about the date of birth; gestational week at the time of birth; delivery type; baby's birth weight, height, and head circumference; neonatal complications; and birth complications.

Statistical analysis

The data were analyzed and figures obtained using the program Statistical Package for Social Sciences (SPSS Inc., Armonk, NY, USA) v25.0. Demographic and clinical features were shown as percentages and means (±SD) or as a median with min-max levels in accordance with the data distribution. Spearman and Pearson correlation analyses were used for intercorrelations in accordance with the data distribution, with $p<0.05$ being considered significant for all analyses.

RESULTS

Results from the patients' demographics and clinical characteristics

The study involved 33 patient; however, due to data missing in the CGMS for two patients, the study had to dismiss these two and finish with data from 31 patients with GDM. The patients' mean age was 31.9±6.9 years, with a mean gestation period of 35.8±0.7 weeks. Additionally, the mean pre-pregnancy BMI was 26.2±5.9 kg/m^2, with a mean weight increase during pregnancy of 12.2±3.5 kg. When examining the patients' obstetric histories, 16.1% were seen to have a history of stillbirth, 67.7% a history of miscarriage, and 3.2% a history of large babies regarding the previous pregnancy duration. Patients' demographics and clinical characteristics are summarized in table 1.

Results from the patients' laboratory findings

While the patients' mean HbA1c level was 5.0±0.3%, their mean level of FRM was 2.1±0.2 µmol/L. Additionally, their mean 1.5-AG level was 17.0±4.9 ng/mL. All of the patients' laboratory findings are summarized in table 2.

Results from the patients' CGMS and SMBG

While the mean number of patients' glucose measurements during the 72-hr CGMS was 788.8±46.5, their mean highest glucose level during that span was 131.1±22.5 mg/dL and their mean lowest glucose level during that span was 54.7±11.6 mg/dL. Additionally, the mean glucose level for the 72-hr CGMS was 86.1±10.3 mg/dL. The 72-hr mean MAD%, which is the marker of daily glucose variation, was 6.7±3.1%. The mean glucose value measured by SMBG six times daily over the 72 hours was 82.9±10.2 mg/dL. The patients' CGMS and SGMS values are summarized in table 3.

A significant positive correlation was found between the mean glucose level for the 72-hr CGMS (86.1±10.3 mg/dL), and the mean glucose level as measured by SMBG six times a day for 72 hours (82.9±10.2 mg/dL; $r=0.767$, $p<0.001$) (figure 1).

Table 1: Demographics and clinical characteristics of the patients

Features	Median (min-max)	mean±SD	n, (%)
Age (years)	32 (20-47)	31.9±6.9	
DM history in the family			20 (64.5%)
Pregnancy duration (weeks)	36 (35-38)	35.8±0.7	
BMI (kg/m²)	26.4 (17.3-44.1)	26.2±5.9	
Weight before pregnancy (kg)	66 (46-110)	67.0±15.0	
Increase in the weight during pregnancy (kg)	13 (5-19)	12.2±3.5	
Obstetric examination findings during pregnancy			
Polyhydraamnios			2 (6.4%)
Oligohydroamnios			1 (3.2%)
Suspicion of trizomy 21			2 (6.4%)
Cleft palate-lib			1 (3.2%)
Number of pregnancy			
I			5 (16.1%)
II			10 (32.3%)
III			8 (25.8%)
IV			3 (9.7%)
V			4 (12.9%)
VI			1 (3.2%)
History of having stillbirth			5 (16.1%)
History of having miscarriage			10 (32.3%)
History of having large baby			1 (3.2%)
Concomitant disease			4 (12.9%)

BMI: Body mass index, DM: Diabetes mellitus, n: patient number; kg: kilogram, SD: standard deviation, min: minimum, max: maximum

Table 2: Laboratory findings of the patients

Laboratory features	
HbA1c (%) (mean±SD)	5.0±0.3
FRM (µmol/L) (mean±SD)	2.1±0.2
1,5-Anhydroglucitol (ng/mL) (mean±SD)	17.0±4.9
Triglyceride (mg/dL) (mean±SD)	204.3±72.3
HDL (mg/dL) (mean±SD)	65.5±17.3
LDL (mg/dL) (mean±SD)	151.5±38.7

HbA1c: Hemoglobin A1c, HDL: High density lipoprotein, LDL: Low density lipoprotein, SD: standard deviation, FRM: Fructosamine

At the end of the study, the glucose level of three of the patients whose CGMS data were examined was found to have been above 140 mg/dL during the 1st hour, and insulin therapy was requested. While one patient did not accept the use of insulin and continued diet therapy, basal-bolus insulin therapy was started in these other two patients. Existing diet therapy was continued in the remaining 28 patients.

Correlation analysis of CGMS measurements and laboratory parameters

Upon performing the correlation analyses of the CGMS measurements and laboratory findings for 1.5-AG, HbA1c, and FRM, no significant correlation was determined to exist between the CGMS measurements and laboratory findings (table 4). Additionally, no significant correlation was found among the laboratory measurements regarding 1.5-AG, HbA1c, and FRM.

Results regarding labor and newborns

Fourteen patients (45.2%) delivered vaginally, and 17 patients (54.8%) delivered by cesarean section. While the babies' mean birth weight was 3,142.9±366.2 gr, the babies' mean birth length (height) was 47.7±2.6 cm and mean head circumference was 34.3±1.3 cm. Thirteen infants had neonatal jaundice, with one having prolonged jaundice. Postnatal respiratory distress occurred in two babies. One of these babies had a cleft palate (lip defect). Neonatal hypoglycemia was not detected in any of the babies.

Table 3: Results of CGMS and SGMS values of the patients

Features	
Number of the glucose measurements in patients during 72 hours (mean±SD)	788.8±46.5
The highest glucose level for 72 hours with CGMS (mg/dL) (mean±SD)	131.1±22.5
The lowest glucose level for 72 hours with CGMS (mg/dL) (mean±SD)	54.7±11.6
Mean glucose level for 72 days with CGMS (mg/dL) (mean±SD)	86.1±10.3
MAD % (mean±SD)	6.7±3.1
AUC Above-140 mg/dL (median/min-max)	0.0 (0-1.5)
AUC Below-70 mg/dL (median/min-max)	1.0 (0-3.7)
Mean glucose level as measured by SMBG (mg/dL) (mean±SD)	82.9±10.2

AUC: Area under the curve, MAD%: Percentage of mean absolute differences, CGMS: Continuous glucose monitoring system, SMBG: Self-monitoring of blood glucose; SD: Standard deviation, min: minimum, max: maximum

Table 4: Correlation analysis between CGMS measurements and laboratory findings including HbA1c, FRM and 1,5 AG

CGMS Measurements		HbA1c	FRM	1,5- Anhydroglucitol
The highest glucose level for 72 hours with CGMS	r	-0.188	0.108	0.088
	p	0.310	0.565	0.640
The lowest glucose level for 72 hours with CGMS	r	0.096	-0.193	-0.059
	p	0.609	0.298	0.754
Mean glucose level for 72 days with CGMS	r	-0.154	0.153	-0.038
	p	0.409	0.413	0.839
MAD%	r	-0.036	-0.009	0.023
	p	0.849	0.961	0.904
AUC Above-140 mg/dL	r	-0.185	-0.078	0.113
	p	0.318	0.676	0.545
AUC Below-70mg/dL	r	-0.071	0.121	0.121
	p	0.703	0.517	0.518

CGMS: Continuous glucose monitoring system, AUC: Area under curve, MAD%: Percentage of mean absolute differences, HbA1c: Hemoglobin A1c, FRM: Fructosamine

A negative correlation was found between the AUC>140 mg/dL and mean birth weight (r=-0.428, p=0.016). A negative correlation was also determined between MAD% and babies' mean head circumference (r=-0.459, p=0.009). The babies of three patients (9.7%) who had post-prandial glucose levels > 140 mg/dL as measured by CGMS had lower birth weights (2,949.9±316.1 gr) and head circumferences (30.3±1.1 cm) compared to the other mothers' babies' birth weights (3,042.9±326.2 gr) and head circumferences (32.1±1.2 cm). However, these weight differences were not statistically significant (p>0.05).

Subgroup analysis of the patients

When dividing the patients into two groups according to the presence of a family history of DM, while 20 patients had a family history of diabetes, 11 patients had no family history. While the CGMS measurements and laboratory findings did not different between these two groups, infants' heights and weights were found to be significantly lower in the group with a family history (p=0.043 and p=0.029, respectively).

Patients were additionally divided into two different groups: those with a bad obstetric history and those without a bad obstetric history in terms of having a history of miscarriage, large baby, or stillbirth. While 12 patients had a history of large baby, stillbirth, or abortion in their previous pregnancies, 19 patients had no history. Upon considering both groups, their data regarding HbA1c, FRM, 1.5-AG, baby birth height, baby birth weight and head circumference were found to be similar.

The mean MAD% of the current study's group is the glycemic fluctuation parameter and was found to be 6.7%.

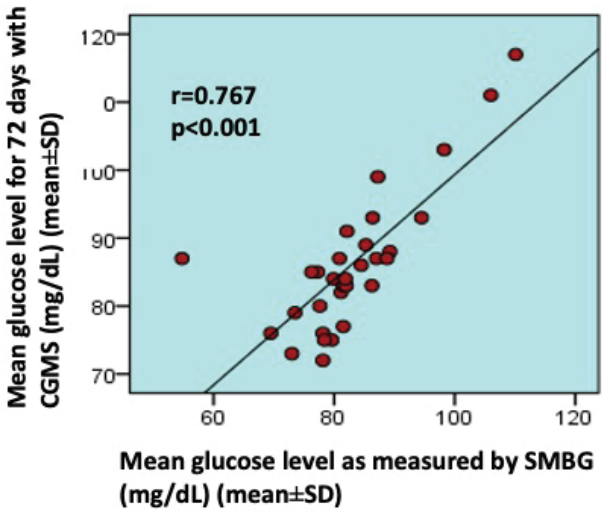

Figure 1: Correlation analysis between mean glukoz level for 72 days with CGMS and mean glucose level as measured by SMBG.

Upon separating patients into those with a MAD%>6.7 and those with MAD%<6.7, while 17 patients had a MAD%<6.7, 14 had a MAD%≥6.7. No significant difference was determined to occur between these two groups in terms of their demographics or clinical and laboratory findings, nor in terms of their babies' weight, height, or head circumference.

DISCUSSION

This prospective study has demonstrated that biomarkers HbA1c, FRM, and 1.5 AG do not determine the glycemic variability in patients with GDM during pregnancy. As a result of CGMS measurements, the patients were additionally observed to be able to experience glycemic variability throughout the day, even when their biomarkers were normal. Furthermore, the mean glucose level calculated as a result of the SBMG measurements showed a correlation with those calculated using the CGMS. When considering how simple, inexpensive, and easily applicable the SBMG measurement is compared to CGMS, this study can speculate that GDM complications can be minimized if the importance of self-monitoring glucose during pregnancy is emphasized.

Glycemic variability is important in pregnant women because complications can develop in both the pregnant woman and her fetus, and this means experiencing more episodes of hyperglycemia and hypoglycemia throughout the day (15). Furthermore, postprandial glycemia level is the biggest contributor to this glycemic fluctuation (16). Although the effect of glycemic variability on fetal complications is known, no indicator has been found yet that directly reflects these fluctuations (5). Neither A1C nor FRM are sensitive indicators of glycemic variability, and a more sensitive indicator is needed in addition to these (5). Studies have shown 1.5-AG to better reflect short-term glycemic control and postprandial hyperglycemia (16-18). With regard to the literature, Nowak et al. reported 1.5-AG to be a better marker than HbA1c for reflecting the glucose profiles of patients with gestational Type 1 DM and 1.5-AG to decrease in the third trimester (19); they argued it to be a highly predictive indicator for the development of macrosomia and thus the 1.5-AG measurement should be used in the clinic for pregnant women with Type 1 DM. However, in contrast to Nowak et al.'s study, the current study observed no correlation between glycemic variability determined using CGMS and the 1.5-AG levels of patients with GDM. The reason for the difference in two studies' result may be that the current study's patient group was made up of diet-regulated patients with GDM, while Nowak et al.'s study group involved pregnant women with Type 1 DM. Glycemic variability may be seen more frequently in patients with Type 1 DM due to the use of insulin and therefore they may have found lower 1.5-AG levels in Type 1 DM patients (19).

Another study determined glycemic variability with CGMS in patients with Type 2 DM, investigated the relationship this variability has with 1.5-AG, and also aimed to determine the correlations among 1.5-AG, A1C, and FRM (20). They found 1.5-AG to be negatively correlated with MAD% and to not be correlated with HbA1c and FRM. As a result, that study concluded 1.5-AG to better reflect glycemic fluctuations, especially during the postprandial period, compared to A1C and FRM. The current study also accordingly was unable to determine a correlation among the HbA1c, FRM, or 1.5-AG biomarkers. Also similar to the literature, no correlation was able to be observed between MAD% and 1.5-AG. Meanwhile, two studies were designed, one around pregnant women with Type 1 DM and the other around pregnant women with Type 2 DM; however, the current study was designed around pregnant women whose GDM was diet regulated (19-20). One can speculate that the reason no correlation was able to be detected was due to the current study's patient population being different, with a lower number of patients compared to other studies.

CGMS is well-known as a technique that is useful for managing patients with DM and especially for determining glycemic variability. Its use is even advocated for children and teens with Type 1 DM in accordance with the American Diabetes Association recommendations; however, no strong recommendations are found for this usage in people with Type 2 DM without insulin therapy or for women with GDM (21, 22). Although CGMS is an expensive and invasive technique for DM management and care, it can detect glycemic variabilities in patients with DM more sensitively due to the measurement frequency (21). SMBG has been published many times as

being a much cheaper method compared to CGMS, and daily blood glucose monitoring can be done correctly with SBMG once users are properly educated about it (23, 24). The current study found a significant correlation between the mean daily glucose level determined by CGMS and the mean glucose level determined by SBMG. In line with this result, one can consider SBMG to be an effective and sufficient method for blood glucose monitoring in pregnant women with GDM due to being both inexpensive and easily accessible. Meanwhile, this study also determined three patients (9.7%) who'd undergone CGMS to have had normal HbA1c and FRM levels and, despite having no abnormal measurement from the SBMG, their average postprandial blood glucose level was greater than 140 mg/dL after the CGMS measurement. Additionally, the study determined the patients with glucose levels>140 mg/dL as measured by CGSM to have a baby with lower birth weight and head circumference compared to the others. No other reason was found to explain low birth weight or small head circumferences in these patients. With these findings, one can speculate that, although SBMG is inexpensive, easy to use, and measures mean glucose levels similar to CGMS, fetuses suffering from growth retardation (e.g., low weight, small head circumference) whose mother may have high blood glucose levels may want to consider CGMS over SBMG for monitoring their glucose more closely.

When separating the patients into two subgroups according to having a bad obstetric history regarding their previous pregnancy or not, no difference was observed between the groups with regard to the laboratory findings or fetal/maternal complications. While approximately half of this study's patients had previously bad obstetric histories (n=12), these patients did not experience any complications during their current pregnancy. Maybe these patients also had GMD in their previous pregnancies; still, they may have had complications because the diagnosis and follow-up had not been done closely in their previous pregnancies. These findings from the current study are thought to show how the diagnosis and close monitoring of GDM can significantly prevent pregnancy and fetal complications.

This study has some limitations. Unfortunately, no healthy control group occurred for comparing the glucose variability and biomarkers. Also, this study's patients had GMD that was regulated by diet. Glucose variability is known to be more common in people who use insulin therapy or have Type 1 DM. The reason why glucose variability and biomarkers were not significant in the patients here may be due to the study having been conducted with a patient group that was considered to be well-monitored. Therefore, further studies can include a larger number of patients, and checks should be done to support this thesis.

In conclusion, the biomarkers in this study did not reflect glycemic fluctuation. The study did find frequent and regular SMBG to be required to achieve the desired glucose level, even in diet-regulated GDM. Meanwhile, head circumference and weight were found to be lower in the babies of mothers with GDM and high glycemic fluctuations; this shows that CGMS, which measures glycemic fluctuations more closely, may be an alternative method despite its cost and application difficulties.

Ethics Committee Approval: This study was approved by Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 21.02.2014, No: 04).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- N.Ö., A.K.Ü., N.D.; Data Acquisition- N.Ö., Y.T., S.Ç., E.B., C.İ.; Data Analysis/Interpretation- N.Ö., A.K.Ü., N.D.; Drafting Manuscript- N.Ö., Y.T., S.Ç., E.B., C.İ.; Critical Revision of Manuscript- A.K.Ü., N.D.; Final Approval and Accountability- N.Ö., Y.T., S.Ç., E.B., C.İ., A.K.Ü., N.D.; Material or Technical Support- N.Ö., Y.T., S.Ç., E.B., C.İ., A.K.Ü., N.D.; Supervision- A.K.Ü., N.D.

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

Financial Disclosure: This study was funded by Scientific Research Projects Coordination Unit of Istanbul University (Project No: 44800)

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RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1178319
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

CHARACTERISTICS AND SURVIVAL OF BRAIN METASTASIS FROM TWO RADIORESISTANT TUMORS, MALIGNANT MELANOMA AND RENAL CELL CARCINOMA: A SINGLE RADIOTHERAPY CENTER STUDY

İKİ RADYOREZİSTAN TÜMÖR OLAN MALİGN MELANOM VE RENAL HÜCRELİ KARSİNOMUN BEYİN METASTAZLARININ ÖZELLİKLERİ VE SAĞKALIMLA İLİŞKİLERİ: BİR RADYOTERAPİ MERKEZİ ÇALIŞMASI

Zümrüt BAHAT[1], Özlem AYNACI[1], Vildan ALTUNAYOĞLU ÇAKMAK[2], Ertuğrul ÇAKIR[3], Mustafa KANDAZ[4], Serdar ÖZKÖK[5]

[1]Karadeniz Technical University, Faculty of Medicine, Department of Radiation Oncology Trabzon, Turkiye
[2]Karadeniz Technical University, Faculty of Medicine, Department of Neurology, Trabzon, Turkiye
[3]Karadeniz Technical University, Faculty of Medicine, Department of Neurosurgery Trabzon, Turkiye
[4]Karadeniz Technical University, Faculty of Medicine, Department of Radiation Oncology, Trabzon, Turkiye
[5]Hatay Training and Research Hospital, Department of Internal Medicine, Division of Geriatrics Hatay, Turkiye

ORCID IDs of the authors: Z.B. 0000-0002-1636-9393; Ö.A. 0000-0002-1799-5521; V.A.Ç. 0000-0003-2828-2583; E.Ç. 0000-0003-3164-8574; M.K. 0000-0003-1106-6227; S.Ö. 0000-0002-0994-1152

Cite this article as: Bahat Z, Aynaci O, Altunayoglu Cakmak V, Cakir E, Kandaz M, Ozkok S. Characteristics and survival of brain metastasis from two radioresistant tumors, malignant melanoma and renal cell carcinoma: a single radiotherapy center study. J Ist Faculty Med 2023;86(1):52-8. doi: 10.26650/IUITFD.1178319

ABSTRACT

Objective: Malignant melanoma (MM) and renal cell carcinoma (RCC) are rare radioresistant tumors that often metastasize to the brain. Because of their rarity, studies on brain metastatic RCC and MM are limited. We aimed to outline the characteristics of brain metastasis (BM) patients from RCC and MM and analyze the potential prognostic factors for survival.

Materials and Methods: This is a retrospective-observational study using data from patients admitted to a radiotherapy (RT) center of a university hospital between 1998-2020. Clinicopathological characteristics, treatment details, and outcome results were analyzed. Univariate and multivariate survival analyses were performed.

Results: Among a total of 14,603 patients treated in our center in the study period, only 52 (0.004%) were BM cases from MM or RCC. Forty patients had complete data (median age at diagnosis of MM or RCC-related BM: 57.7; females: 25%; MM in 52.5% and RCC in 47.5%). The time between primary diagnosis and first extracranial metastases was weakly correlated with the time

ÖZET

Amaç: Malign melanom (MM) ve renal hücreli karsinom (RCC), beyne sıklıkla metastaz yapan nadir ve radyorezistan tümörlerdir. Nadir olmaları nedeniyle beyin metastatik MM ve RCC ile ilgili çalışmalar sınırlıdır. MM ve RCC kaynaklı beyin metastazı (BM) gelişen hastaların karakteristik özelliklerini ve sağkalım için potansiyel prognostik faktörleri analiz etmeyi amaçladık.

Gereç ve Yöntem: Bu çalışma, 1998-2020 yılları arasında bir üniversite hastanesinin radyoterapi (RT) merkezine başvuran hastaların verilerini kullanan geriye dönük-gözlemsel bir çalışmadır. Klinikopatolojik özellikler, tedavi detayları ve sonlanım verileri analiz edildi. Tek değişkenli analizler ve çok değişkenli sağkalım analizleri yapıldı.

Bulgular: Çalışma döneminde merkezimizde tedavi edilen toplam 14,603 hastadan sadece 52'si (%0,004) MM veya RCC ilişkili BM vakasıydı. Çalışma popülasyonunu verileri eksiksiz olan 40 hasta oluşturmaktaydı. MM veya RCC ilişkili BM tanısında medyan yaş 57,7 olup, hastaların %25'i kadındı. MM sıklığı %52,5 ve RCC sıklığı %47,5 idi. "Primer tanı ile ilk ekstrakraniyal metastazlar arasındaki

Corresponding author/İletişim kurulacak yazar: Zümrüt BAHAT – zbahat@hotmail.com
Submitted/Başvuru: 03.10.2022 • **Accepted/Kabul:** 29.11.2022 • **Published Online/Online Yayın:** 27.01.2023

between diagnosis of extracranial metastasis and BM (r=0.405, p=0.021). Among the potential prognostic factors on survival [age, sex, older vs younger age group, primary diagnosis (MM vs RC), presence of extracranial metastasis, number of BM, location of BM, presence of gross total resection, dose of RT, completion of prescribed RT, field of RT], none were independently associated with survival.

Conclusion: Our findings suggest that when MM or RCC patients develop brain metastasis, survival is limited without any favorable prognostic factor belonging to the patient, the tumor, or the preference of the treatment.

Keywords: Brain metastasis, malignant melanoma, radioresistant tumor, renal cell cancer, survival

süre" ile "ekstrakraniyal metastaz tanısı ile BM arasındaki süre" arasında zayıf bir korelasyon vardı (r=0,405, p=0,021). Sağkalıma yönelik potansiyel prognostik faktörler olan "yaş, cinsiyet, yaşlı veya genç olma, primer tanı (MM veya RCC olması), ekstrakraniyal metastaz varlığı, BM sayısı, BM'nin yeri, tam yada tama yakın rezeksiyon, RT dozu, planlanan RT'nin tamamlanması, RT alanı" gibi parametrelerin hiçbiri sağkalım ile bağımsız ilişkili değildi.

Sonuç: Bulgularımız, MM veya RCC hastalarında beyin metastazı geliştirdiğinde hastaya, tümöre veya tedavi tercihine ait herhangi bir olumlu prognostik faktör olmadan sağkalımın sınırlı olduğunu göstermektedir.

Anahtar Kelimeler: Beyin metastazı, malign melanom, radyorezistan tümör, renal hücreli karsinom, sağkalım

INTRODUCTION

Brain metastasis (BM) is the most common brain tumor and about 20-40% of cancer patients develop BM eventually (1). Morbidity and mortality of BM are very high (2). BM is most seen in malignant melanoma (MM) and renal cell carcinoma (RCC) followed by lung cancer and breast cancer (3).

MM and RCC are well known for their radioresistant characteristics. As an overview, in a study that analyzed the effectiveness of whole brain radiation therapy (WBRT) of metastatic brain lesions, complete response was observed in about 40% of small cell lung cancer, 25% of squamous cell carcinoma (SCC), 15% of non-breast adenocarcinoma and 3% of breast cancer, while there was no complete response in RCC and MM cases (4).

MM and RCC have low prevalence but a high rate of BM. The prevalence of MM is about 5%, and the prevalence of BM from MM is strikingly high, being about 40-60% and 75% in the autopsy series (5, 6). If MM with BM would be left untreated, the expected survival is less than three months (5) while when treated with WBRT survival has been suggested to increase to up to eight months (7, 8). On the other hand, the prevalence of RCC has been reported as 1-2% (9, 10). Yet, the prevalence of BM from RCC is as high as %2-17 (11). If BM from RCC would be left untreated, the survival is limited to three months. If it would be treated with WBRT, survival has been suggested to improve to up to nine months (8). In case BM is operable and standard WBRT is applied, survival is suggested to be extended up to 15.5 months (9).

The prevalence of MM and RCC doubled in the last 25 years requiring increased attention to these two tumors and their associated brain metastases (7). The major treatment modality in the management of brain metastasis is still WBRT (7, 8, 12).

Clinicians need information on the characteristics of patients suffering from BM associated with MM and RCC given the increasing prevalence of both tumors and high rates of BM associated with these tumors. The analysis of various prognostic clinical factors which may aid in selecting patients for applicable treatment modalities is required as well. However, given their low prevalence so far, this information is very limited. As such, we aimed to outline the demographic and clinical characteristics of the BM patients from RCC and MM treated in our radiotherapy center over a period of 22 years and analyze the potential prognostic factors for survival.

MATERIALS AND METHODS

Population and setting

This study is a retrospective, observational study that followed the report Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) (13). We analyzed the data of the patients who applied to the radiation oncology center and received radiotherapy for BM from MM or RCC (according tonternational Commission on Radiation Units and Measurements [ICRU] 83 definitions) (14) at Radiation Therapy Clinics of a tertiary health center, between January 1998 and December 2020 in 22 years. The patients with unavailable data were excluded, otherwise, the data of all patients were included. Forty [40] participants composed the study population.

All patients were applied cranial irradiation and concomitant dexamethasone treatment. WBRT was performed with 6-10 MV photon beams from a linear accelerator or cobalt 60, via parallel opposed fields (90° and 270°) with a commercial thermoplastic mask fixation. All the metastases were treated with a fractionation dose of 3Gy or 4Gy.

All procedures performed in studies involving human participants were following the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol was approved by the local ethics committee (Date: 05.02.2018, Number: 2012/04). Informed consent was obtained from all of the participants included in the study.

Measurements and definitions

Medical charts were reviewed systematically regarding the demographic and clinical characteristics. Age at primary diagnosis, sex, histopathology of the tumor, presence of extracranial disease at primary diagnosis, age at extracranial metastasis, age at the time of diagnosis of BM, localization of metastases, presence of single or multiple metastases, application of metastatic brain lesion surgery, type of surgery, a dose of RT for BM, implementation of the prescribed dose, the time between the initial diagnosis and BM, the time between the initial diagnosis and extracranial metastasis, the time between extracranial metastasis and BM, survival time following diagnosis, following extracranial metastasis, and BM were recorded. If the time between the primary lesion and metastasis was less than one month, these metastases were accepted *synchronous* tumors. Brain overall survival (B-OS) was defined as the time between the diagnosis of BM and death.

Outcomes

The primary outcomes were prognostic factors for MM and RCC that developed BM and received RT. We considered age, sex, age group (age>=65 vs <65), primary diagnosis (MM vs RCC), presence of extracranial metastasis, number of BM (solitary vs multiple), location of BM (cerebral vs cerebral+cerebellar), presence of gross total resection (GTR), a dose of RT, completion of prescribed RT, the field of RT (all over cranium vs all over cranium+additional RT over the specific BM location).

The secondary outcomes were clinicopathological characteristics, treatment details, and correlations between survival periods after primary diagnoses, first extracranial metastases and, brain metastases.

Statistical analysis

We examined the normality of the parameters with Shapiro Wilk test and visual histograms considering relatively low number of participants. Accordingly, non-parametric tests and parametric tests were used as appropriate. Descriptive statistics were given as percentages for categorical variables and mean, standard deviation or median, minimum-maximum for numerical data. Independent sample two test or Mann-Whitney U test was used to compare numerical variables between the group and Pearson or Spearman's correlation tests were used in correlation analyses. When the correlation was detected as significant, it was regarded strong if correlation coefficient (r) was> 0.7, moderate if between 0.5-0.7, low if between 0.3-0.5 and negligible if< 0.3. The survival analyses were performed by Log-Rank test with Kaplan–Meier survival analyses. IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. $p<0.05$ was defined as statistical significance.

RESULTS

Demographics and Baseline Characteristics (table 1)

A total of 14,603 patients were treated in our center during the study period. Among them, 52 (0.004%) patients were diagnosed as BM from MM or RCC. Twelve (12) patients' data were unavailable and therefore excluded. A flow chart on the number of patients included and excluded at different steps can be found in figure 1.

Table 1. The demographics and baseline clinical characteristic of MM and RCC patients with brain metastasis (n=40)

Parameter	
Age at primary diagnosis (years) [a]	55.9 (22.7-80.1)
Median age at the time of CNS metastasis, years[a]	57.7 (22.8-80.7)
Age group[b]	
<65	31 (77.5%)
≥65	9 (22.5%)
Primary cancer[b]	
RCC	19 (47.5%)
MM	21 (52.5%)
Sex[b]	
Women	10 (25%)
Men	30 (75%)
Extracranial metastasis[b]	
Present	32 (80%)
Absent	8 (20%)
Age at brain metastasis (years) [b]	57.7 (22.8-80.7)

MM, multiple myeloma; RCC, renal cell carcinoma
[a]Data are given as median (range, minimum-maximum)
[b]Data are given as number (percentage)

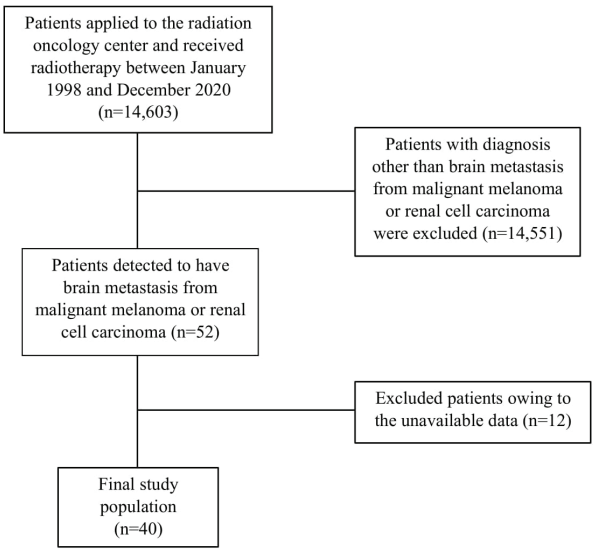

Figure 1: Flow chart on the number of patients included and excluded during the study period

The median age at diagnosis of MM or RCC was 55.9 (22.7-80.1). The females constituted 25% (n=10) of the study population. In the total study population, 52.5% (n=21) were MM and 47.5% (n=19) were RCC. The majority of patients (80%) had extracranial metastatic disease at a median age of 56.6 (23-77). The median age at the time of BM was 57.7 years (22.9 to 80.7 years).

A total of 27.5% (n=11) patients were diagnosed with distant metastasis with BM simultaneously while 52.5% (n=21) had developed distant metastases before BM. Only 20% (n=8) of the study population were free from extracranial metastasis when BM was diagnosed. We outlined the demographics and baseline clinical characteristics of the study participants in table 1.

Data at diagnosis of metastases and treatments

Tumor histopathology was established by pathologic analysis, with biopsy either from the primary site or metastases. All patients who had BM were investigated with brain computerized tomography (CT) or magnetic resonance imaging (MRI). The time from diagnosis of the primary tumor to first extracranial metastasis was 11.3 months (0-121.7 months); the time between the diagnosis of the primary tumor to BM was 21.2 months (0-123.3 months); the time between extracranial metastases to BM was 10.1 months (0-76.9 months) (table 2). The time between primary diagnosis and first extracranial metastases correlated with the time between diagnosis of extracranial metastasis and BM (r=0.405, p=0.021).

whole Brain Radiotherapy consisted of a 30 Gy in 10 fractions in two weeks in 18 patients. A 20 Gy WBRT in five fractions in one week was applied to 15 patients. 15Gy boost irradiation for 30Gy schedule was performed up to a total dose 45Gy to single BM to four patients. Three patients were unable to receive the prescribed dose of RT.

Among the study group, 50% (n=20) had solitary brain metastasis, 45% (n=18) had multiple metastases and 5% (n=2) of patients had an unknown number of metastases. In the study population, 72.5 % (n=29) had metastasis at the cerebrum, 17.5 % (n=7) at both cerebrum and cerebellum, and 2.5% (n=1) at the cerebellum [location of metastases was unknown in 5% (n=3)]. Five patients (12.5%) had undergone gross total resection of BM (all were single metastatic), 25 % had a partial resection and 10% (n=4) had only a biopsy. A total of 30 patients (75%) had no surgery (table 3).

Survival data

Survival data of two patients was not available. All other 38 patients died within the study period. The median survival time from diagnosis of the primary tumor was 16.3 months (1.0-125.4 months). The survival time from the first extracranial metastasis was 10.2 months (1.0-87.7 months); after diagnosis of BM (Brain overall survival, B-OS) was 6.8 months (1.0-22.3 months) (table 4).

Correlation between survival periods after primary diagnoses, first extracranial metastases and brain metastases

Time from primary diagnosis to BM was weakly correlated with the time from first extracranial metastasis to death (r=0.47, p=0.007). Time from first extracranial metastasis to death and time from BM to death (B-OS) was weakly correlated as well (r=0.44, p=0.013). On the other hand, time from primary diagnosis to BM was not correlated with time from first extracranial metastasis to death, and

Table 2. Times between diagnoses of primary tumor-first extracranial metastasis and between diagnosis of extracranial metastases -brain metastasis

	Median (months)	Range
Primary diagnosis-extracranial met*	11.3	0-121.7
Primary diagnosis-BM	21.2	0-123.3
Extracranial metastasis-BM*	10.1	0-76.9

Primary tumors (renal cell carcinoma or malignant melanoma)
Met: Metastases; BM: Brain metastasis, *The data from 32 patients that developed extracranial metastases before BM

Table 3. The characteristics of brain metastasis of MM and RCC (n=40)

Characteristic	
Site of brain metastasis[a]	
Only cerebrum	29 (72.5%)
Only cerebellum	1 (2.5%)
Cerebrum and cerebellum	7 (17.5%)
Not recorded	3 (7.5%)
Number of brain metastasis[a]	
Single	20 (52.6%)
Multiple	18 (47.4%)
Not recorded	2 (5.0%)
Surgery[a]	
Gross total resection	5 (12.5%)
Partial resection	1 (2.5%)
Only biopsy	4 (10%)
No surgery	30 (75%)
RT[a]	
<prescribed RT dose	3 (7.5%)
20Gy	15 (37.5%)
30Gy	18 (45%)
45Gy	4 (10%)

CNS: Central nervous system, MM: Multiple myeloma, RCC: Renal cell carcinoma, RT: Radiotherapy
[a]Data are given as number (percentage)

Table 4. Survival data of the patients with MM and RCC related brain metastasis (n=38)

	Median (months)	Range (months)
Survival time after primary diagnosis (OS)	16.3	1.0-125.4
Survival after first extracranial metastasis	10.2	1.0-87.7
Survival after brain metastasis (B-OS)	6.8	1.0-22.3

B-OS: Brain overall survival, MM: Multiple myeloma, OS: Overall survival, RCC: Renal cell carcinoma

time from primary diagnosis to BM was not correlated with time from BM to death (brain overall survival, B-OS).

B-OS was not correlated with the completion of the prescribed RT dose in the study population. However, when the patients were grouped as older (aged >=65) vs younger (aged<65) patients, in the younger group, B-OS was significantly correlated (r=0,42, p=0,02) with failure in receiving the prescribed RT dose while in an older group, it was not.

Examining the potential prognostic factors associated with brain overall survival (B-OS)

We grouped the patients as those that had B-OS time equal to or longer vs shorter than the median B-OS (6.8 months). Consequently, we examined the association between B-OS and potential prognostic factors. The studied prognostic factors were as follows: age (p=0.31), sex (p=0.82), age group (>=65 years vs <65 years of age) (p=0.67), primary diagnosis (RCC vs MM) (p=0.29), presence of extracranial metastasis (p=0.91), number of BM (solitary vs multiple) (p=0.23), location of BM (cerebral vs cerebral+cerebellar) (p=0.76), presence of gross total resection (GTR) (p=0.82), a dose of RT (p=0.69), completion of prescribed RT (p=0.40), the field of RT (all over cranium vs all over cranium+additional RT over the specific BM location) (p=0.62); hence, neither was associated with B-OS. We examined the relationship between B-OS and potential prognostic factors with multivariate analyses as well. We studied different models by including different independent parameters. In *model 1*, we included age at BM, sex, completion of RT, and presence of brain surgery in *model 2*, we included age at BM, sex, completion of RT, and time between primary diagnosis and first extracranial metastasis, in *model 3* we included age, sex, primary diagnosis, number of BM, completion of prescribed RT and in *model 4*, we included age group, sex, primary diagnosis, number of BM, completion of prescribed RT. In none of the models, there was an association between the B-OS and the potential prognostic factors.

DISCUSSION

In this study, we have reported the experience of a single tertiary radiotherapy center on BM of two most common radioresistant tumors, MM and RCC collected over a period of 22 years. We considered a wide range of potential prognostic factors on survival including age, sex, older vs younger age group, primary diagnosis (MM vs RC), presence of extracranial metastasis, number of BM, location of BM, presence of gross total resection, a dose of RT, completion of prescribed RT, the field of RT and found that none of these parameters were independently associated with B-OS. Hence, our findings suggest that when a MM or RCC patient develops brain metastasis, survival is limited without any favorable prognostic factor belonging to the patient, the tumor, or the preference of the treatment. Another explanation may be that competing factors for mortality (other than the tumor itself) might have been effective in the death of older tumor patients, which is a frequent issue and practice in older patients (15, 16).

Ferrel et al. reported that a higher number of brain metastases (>5) and lower performance scores were statistically significant predictors of a lower B-OS prognosis (8). A correlation between B-OS and gross total resection (GTR) has been reported in some studies (17). In our series, we were not able to reach this correlation. Although the exact data about the performance status of the participants was not available in our study, an assumption of the study group demonstrating a poor performance status would not be wrong, since we have been applying a lower dose of radiation to the patients with poor performance. Our findings showed that half of them got a radiation dose of ≤30 Gy. Hence, a factor underlying the lack of such association might be the lower performance status of the patients in our study group. However, we cannot comment more on this point.

When we consider the overall characteristics of the patients, the median age at primary diagnosis was 56 which is compatible with the literature reporting median age of 55-66 years (12, 18, 19). In the study population, 78.4% had metastasis at the demographic and clinical characteristics of the BM patients from RCC and MM treated in our radiotherapy center over 22 years period and analyze the potential prognostic factors for survival. cerebrum, 2.7% at the cerebellum, and 17.5% at both the cerebrum and cerebellum pointing out a significant predilection of metastases at the cerebrum. This feature is also in line with the literature findings reporting a predominance of cerebral metastases in BM cases (5).

Stereotactic radiotherapy (SRT) has become a standard of care for patients with a limited number of brain metastases (18). However, during the study period, we lacked an SRT facility in our center. Hence we applied WBRT and consequently a total of 15 Gy boost irradiation to the solitary BM reaching a total dose of 45Gy. Also in our series, 47.4% of the patients had multiple metastases. For extensive BM, WBRT was the gold standard in line with our application of WBRT in the study patients (12).

The time between radioresistant cancer diagnosis and first extracranial metastasis was correlated with the time between diagnosis of extracranial metastasis and BM (p=0.021). The sooner an extracranial metastasis occurred, the sooner BM occurred after extracranial metastasis. This is somewhat an expected finding as it shows clinical aggressiveness and metastatic capacity of the primary tumor but we did not find any article researching this point. Our results suggest that if there is an extracranial metastasis, it is logical to screen for a BM with cranial magnetic resonance imaging.

This study has its limitations and strengths. First, this is a retrospective study suffering from the shortcomings of such studies. Confounding factors which have not potentially been considered might be related to the survival of the patients. Although SRT is used as a standard treatment in patients with limited metastases, SRT was not performed as an RT technique in our study. This is a single-center study; however, the center was a tertiary referral serving a population of about 1,600,000 people pointing out the quality of data derived from the present data. Our other strengths are we reported our data on BM of the two most common radioresistant tumors and considered several potential prognostic factors on survival over a considerably long period. Our patient number was somewhat limited to 38 and 40 patients, however, these are rare tumors, and it is difficult to have extensive related data. Similarly, a significant study included only 122 MM and RCC BM cases, which includes about three times the present patients but is still limited (8). Another such study included only 27 such patients which is lower than the number in this study (7). Overall, these reports suggest that meta-analyses type studies are needed on this issue.

In conclusion, our findings suggest that when a MM or RCC patient develops brain metastasis, survival is limited without any favorable prognostic factor belonging to the patient, the tumor, or the preference for the treatment. Due to limited data in the literature, meta-analysis-type studies are needed to make more comments on this subject.

Ethics Committee Approval: This study was approved by Karadeniz Technical University Faculty of Medicine Clinical Research Ethics Committee (Date: 05.02.2018, No: 24237859).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- Z.B., Ö.A., V.A.Ç., E.Ç.; Data Acquisition- Ö.A., M.K.; Data Analysis/Interp-

retation- Z.B., S.Ö.; Drafting Manuscript- Z.B., Ö.A., M.K.; Critical Revision of Manuscript- V.A.Ç., E.Ç., S.Ö.; Final Approval and Accountability- Z.B., Ö.A., V.A.Ç., E.Ç., M.K., S.Ö.

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

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

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RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1168793
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

THE EFFECT OF miR-34a-5p ON OVEREXPRESSED AML ASSOCIATED GENES

MiR-34a-5p'NİN AŞIRI İFADE EDİLEN AML İLİŞKİLİ GENLER ÜZERİNDEKİ ETKİSİ

Murat KAYA[1], İlknur SUER[1]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Internal Medicine, Division of Medical Genetics, Istanbul, Turkiye

ORCID IDs of the authors: M.K. 0000-0003-2241-7088; İ.S. 0000-0003-1954-4190

Cite this article as: Kaya M, Suer I. The effect of miR-34a-5p on overexpressed AML associated genes. J Ist Faculty Med 2023;86(1):59-68. doi: 10.26650/IUITFD.1168793

ABSTRACT

Objective: Acute myeloid leukemia (AML) is a deadly type of leukemia. The expression of AML-related genes may be altered not only by genetic changes but also by various epigenetic factors such as microRNAs (miRNAs). The expression levels of many genes can be altered by miRNAs. The detection of miRNA's target genes is critical for an understanding of the disease's molecular mechanism. In this study possible target genes of miR-34a-5p in AML were determined and the effect of the relationship between miR-34a-5p and target genes on the cancer process was investigated.

Materials and Methods: Leukemia Gene and Literature Database web tool (http://soft.bioinfo-minzhao.org/lgl/) includes a useful leukemia gene and literature da. There are more than 600 AML-related genes on this database. In the present study, in order to define the potential target genes of miR-34a-5p on the database, we used miRDB tool and then confirmed the findings using miRWalk, miRTarbase, Tarbase and miRNet tools. Defined miR-34a-5p AML related genes were verified by the DisGeNET platform. A Protein-Protein Interaction (PPI) network analysis of the genes was conducted using several bioinformatics tools. The effect of miR-34a-5p on cell proliferation was investigated by transfecting mimic miR-34a-5p into HL60 and NB4 cells. The mRNA expressions of *NOTCH2, IGF1R, SKP2* and *CDC25A* genes were investigated in miR-34a-5p transfected NB4 and HL60 cells and control groups.

Results: Using bioinformatics tools we determined 44 AML-related genes that could be targeted by miR-34a-5p. According to our in vitro study results statistically significant suppression of proliferation was observed in miR-34a-5p transfected cells (48h HL60 cells p=0.00011; NB4 cells p=0.0031 and 96h HL60 cells p=0.00013; NB4 p=0.00018). It was also found that *NOTCH2, IGF1R, SKP2* and *CDC25A* mRNA expressions were down-

ÖZET

Amaç: Akut miyeloid lösemi (AML) ölümcül bir lösemi türüdür. AML ilişkili genlerin ekspresyonu sadece genetik değişikliklerle değil aynı zamanda mikroRNA'lar (miRNA'lar) gibi çeşitli epigenetik faktörlerle de değiştirilebilir. MiRNA'lar birçok genin ifade seviyesini değiştirerek hücrede oldukça kritik görevler yapabilmektedir. miRNA ve hedef genleri arasındaki etkileşimin tespit edilmesi, hastalığın moleküler mekanizmasının aydınlatılması açısından oldukça önemlidir. Çalışmamızda AML dahil birçok kanserde tümör baskılayıcı role sahip miR-34a-5p'nin AML hücre proliferasyonu üzerindeki etkisi ve AML ilişkili genlerin ifade değişimindeki rolü araştırılmıştır.

Gereç ve Yöntem: Leukemia Gene and Literature Database web sitesi (http://soft.bioinfo-minzhao.org/lgl/)'de, lösemi ile ilişkili genleri içeren 600'den fazla AML ile ilgili gen bulunmaktadır. Bu web sayfasında yer alan miR-34a-5p'nin potansiyel hedef genlerini tanımlamak için yaptığımız bu çalışmada miRDB veri tabanı kullanılmıştır. Sonrasında miRWalk, miRTarbase, Tarbase ve miRNet araçlarıyla doğrulanmıştır. PPI etkileşimleri, yolak analizi, çeşitli biyoinformatik araçlar kullanılarak tanımlanmıştır. İn vitro olarak miR-34a-5p'nin AML hücreleri üzerindeki etkisi belirlenip *NOTCH2, IGF1R, SKP2* ve *CDC25A* genlerinin mimik miR-34a-5p ile transfekte edilmiş NB4 ve HL60 hücrelerinde ekspresyonu araştırılmıştır.

Bulgular: Çeşitli biyoinformatik araçlar kullanılarak miR-34a-5p tarafından hedeflenebilecek 44 AML ilişkili gen belirlenmiştir. Sonrasında yapılan in vitro çalışmada miR-34a-5p ile transfekte edilen hücrelerde proliferasyonun istatistiksel olarak anlamlı şekilde baskılandığı gözlenmiştir (48 saat HL60 hücreleri p=0,00011; NB4 hücreleri p=0,0031 ve 96 saat HL60 hücreleri p=0,00013; NB4 p=0,00018). miR-34a-5p mimik transfekte edilen NB4 ve HL60 hücrelerinde *NOTCH2, IGF1R, SKP2* ve *CDC25A* mRNA ifade seviyelerinin kontrol gruplarına göre anlamlı şekilde

Corresponding author/İletişim kurulacak yazar: Murat KAYA – kmurat@istanbul.edu.tr

Submitted/Başvuru: 31.08.2022 • **Revision Requested/Revizyon Talebi:** 05.09.2022 •
Last Revision Received/Son Revizyon: 22.11.2022 • **Accepted/Kabul:** 24.11.2022 • **Published Online/Online Yayın:** 27.01.2023

regulated in miR-34a-5p mimic-transfected HL60 cells (p=0.003; p=0.02; p=0.01; p=0.0009 respectively) and NB4 cells (p=0.02; p=0.02; p=0.01; p=0.0007 respectively) compared to the control groups.

Conclusion: miR-34a-5p may inhibit AML cell proliferation by targeting many genes like *NOTCH2, IGF1R, SKP2* and *CDC25A*. The results of our study indicate that appropriate bioinformatics tools and in vitro methods can successfully be used together when investigating the relationship between miRNAs and target genes. Further studies are required to determine the detailed relationship between these genes and miR-34a-5p.

Keywords: miR-34a-5p, AML, NB4, HL60

azaldığı tespit edilmiştir (HL60 hücrelerinde sırasıyla (p=0,003; p=0,02; p=0,01; p=0,0009) ve NB4 hücrelerinde sırasıyla p=0,02; p=0,02; p=0,01; p=0,0007).

Sonuç: miR-34a-5p; *NOTCH2, IGF1R, SKP2* ve *CDC25A* gibi birçok geni hedefleyerek AML hücre proliferasyonunu inhibe edebilir. Bu genler ile miR-34a-5p arasındaki ilişkiyi net bir şekilde belirleyebilmek için daha ileri tekniklerle farklı çalışmaların yapılmasına ihtiyaç vardır. Çalışma sonuçlarımız, miRNA-hedef gen ilişkisi araştırılırken uygun biyoinformatik araçlarla in vitro yöntemlerin birlikte başarıyla kullanılabileceğini göstermektedir.

Anahtar Kelimeler: miR-34a-5p, AML, NB4, HL60

INTRODUCTION

Acute myeloid leukemia (AML) is the most prevalent type of leukemia in adults (1). AML develops when blast cells expand clonally and invade the peripheral blood and bone marrow. Patients frequently suffer as a result of immature and ineffective erythropoiesis and bone marrow failure. Parallel to recent developments, treatment success rates have increased to 15% in individuals over 60 years of age and to around 40% in patients under the age of 60. Despite this, the prognosis remains very poor, particularly for elderly patients (2). Therefore, there is an urgent need for new research into the diagnosis, treatment, and prognosis monitoring of AML (3). MicroRNAs (miRNAs) are non-protein-coding small RNAs that are about 20 nucleotides in length (4). These small RNAs bind to the mRNAs of target genes and regulate their expression levels in the cell (5). There are over 2,000 human miRNAs and approximately 20,000 protein-coding genes (6, 7). Furthermore a miRNA may play a critical role in the regulation of numerous genes and the expression of a gene can be regulated by multiple miRNAs (8). To address this major issue various free and useful online programs based on base pairing have been developed for the detection of interactions between miRNAs and genes. Although these programs are extremely useful in miRNA-target gene research, the results of various miRNA-target gene web programs are frequently inconsistent (9, 10). As a result, when determining miRNA-target genes, it is critical to first select the genes by combining different bioinformatics tools, and then validate the findings using in vitro studies on cell lines. Therefore, we aimed to determine the miR-34a-5p target genes relationship in AML by combining the power of bioinformatics applications and in vitro methods. In the current study several bioinformatics tools were used to determine and verify the deeper associations of selected genes with AML and miR-34a-5p. The effect of miR-34a-5p on cell proliferation in HL60 and NB4 (AML cancer cell lines) was first evaluated. Then we attempted to determine the miR-34a-5p related genes involved in cancer cell proliferation.

MATERIAL AND METHODS

Bioinformatics assessment

Hundreds of AML-associated genes have been reported in the literature. A search using the Leukemia Gene and Literature Database web tool (http://soft.bioinfominzhao.org/lgl/) revealed that more than 600 genes are associated with adult AML. Potential target AML genes of miR-34a-5p in the database were identified using the miRDB tool. To verify AML-related genes, the DisGeNET platform was used. Protein-protein interaction (PPI) was demonstrated using the String tool (version 11.5) (https://string-db.org/). KEGG and GO enrichment analyses were performed using the Enrichr web server, after which miRTarbase, Tarbase, miRNet and miRWalk databases were used in order to define stronger candidate miR-34a-5p related genes. In this way, Neurogenic Locus Notch Homolog Protein 2 (*NOTCH2*), Insulin Like Growth Factor 1 Receptor (*IGF1R*), S-Phase Kinase Associated Protein 2 (*SKP2*), and Cell Division Cycle 25A (*CDC25A*) genes were selected. The GEPIA2 tool was used to define survival analysis. To uncover the relationship between the four genes and miR-34a-5p in cancers, especially in AML, the literature was searched using PubMed.

Cell line culture and miRNA mimic transfection

HL60 and NB4 AML cell lines were seeded into RPMI-1640 medium supplemented with 10% FBS and 1% penicillin and the cells were incubated (with 37°C-5% CO_2). After 24 hours of incubation the cells were transfected with mimic miR-34a-5p for 48 hours and for 96 hours with assays. Lipofectamine 2000 reagent (Thermo Fisher Scientific Inc.) was used to transfect the cells with miR-34a-5p and the non-targeting control (nt control) with mimics (Ambion mirVana, Applied Biosystems). Transfection reagent was prepared by mixing 300 µL Opti-MEM medium with 3 µL from 10 µM stock miRNA mimic reagent and 9 µL of Lipofectamine 2000 reagent following the manufacturer's protocol. In brief, 30 pmol miR-34a-5p mimics were added to each well, and the same amount of nt control mimic was added to the control group (11).

Determination of the HL60 and NB4 cells' proliferation
Cell proliferation was defined by colorimetric cell viability (WST-8) assay using a CVDK-8 kit (EcoTech Biotechnology). In a 96-well plate, HL-60 and NB4 cell lines were seeded at around $5x10^3$ cells per well and cultured. To evaluate the effect of miR-34a-5p mimic transfection on cell proliferation, measurements were taken at 48 and 96 hours. 10 µL of CVDK-8 reagent was supplied to each well for measurement and incubated for three hours. Finally, a microplate reader (Thermo) capable of measuring absorbance at 450 nm was used to assess cell viability.

Total RNA isolation from HL60 and NB4 cells and qRT-PCR
Total RNAs from miRNA mimic transfected HL60 and NB4 cells were isolated with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA concentration was evaluated by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). cDNA synthesis and qRT-PCR processes were performed using a total of 1000 ng RNA obtained from both the study group and control group cells. The SCRIPT kit (Jena Bioscience) was used for cDNA synthesis and SybrMaster (Jena Bioscience) was used for qRT-PCR processes. All test steps were performed according to the kit manufacturers' protocols. *NOTCH2, IGF1R, SKP2,* and *CDC25A* gene expression levels were determined in miR-34a-5p transfected HL60 and NB4 cells as well as in the control groups. Table 1 shows the primer sequences for the genes studied.

Verification of the transfection process
TaqMan probes were used in qRT-PCR to detect the expression level of miR-34a-5p in the transfected cells. The cDNA of miRNA was constructed using 30 ng of total RNA and performed using the TaqMan miRNA reverse transcription kit (Applied Bio., Foster City, CA, USA) and miRNA RT primers according to the manufacturer's protocol. qRT-PCR was performed using TaqMan miR-34a-5p probes (Thermo Fisher Scientific Inc.), *RNU43* (control miRNA) probes (Thermo Fisher Scientific Inc.), and the TaqMan Universal Master Mix (Thermo Fisher Scientific Inc.) kit. The assay was carried out in duplicate, and the $2^{-\Delta\Delta Ct}$ method was applied for the analysis of relative quantitation. To normalize the expression of genes whose expression was investigated in the in vitro study step, *ACTB* primers were used as an internal control. Experiments were performed in duplicate. Relative quantification analysis was performed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis
Analyses were performed using Student's t-test and the data were presented as mean±standard deviation. The data were considered statistically significant if the p-value was less than 0.05. Graphs were created with the GraphPad Prism 5 program and SPSS software ver.21(IBM Corp., Armonk, NY, USA). Overall survival analysis was generated using the Kaplan-Meier test via the GEPIA 2 tool. Points to consider when analyzing: Group cutoff: Median, Cutoff-High(%): 50, Cutoff-Low(%): 50, Hazards Ratio (HR): Yes, 95% Confidence Interval: Yes, Axis Units: Months

RESULTS

Bioinformatics analysis results

Identification of potential AML-related candidate genes
The miRDB database predicts 899 genes as potential targets of hsa-miR-34a-5p. We found that 44 genes overlap among 600 AML-related genes in http://soft.bioinfo-minzhao.org/lgl/ and that 899 genes are possible targets of miR-34a-5p in miRDB. The relationship between miR-34a-5p and the 44 selected genes was confirmed by miRTarbase, Tarbase, and miRNet, as presented in figure 1.

Table 1: Primers' List, that target unique sequences

Primer	Sequence	Reference
NOTCH2-F	5′-GGGACCCTGTCATACCCTCT-3′	(49)
NOTCH2-R	5′-GAGCCATGCTTACGCTTTCG-3′	
IGF1R-F	5′-TTTCCCACAGCAGTCCACCTC-3′	(50)
IGF1R-R	5′-AGCATCCTAGCCTTCTCACCC-3′	
SKP2-F	5′-ATGCCCCAATCTTGTCCATCT-3′	(51)
SKP2-R	5′-CACCGACTGAGTGATAGGTGT-3′	
CDC25A-F	5′-ATGAGGATGATGGCTTCG-3′	(52)
CDC25A-R	5′-AACACTGACCGAGTGCTG-3′	
ACTB-F	5′-GCCTCGCCTTTGCCGATC-3′	(53)
ACTB-R	5′-CCCACGATGGAGGGGAAG-3′	

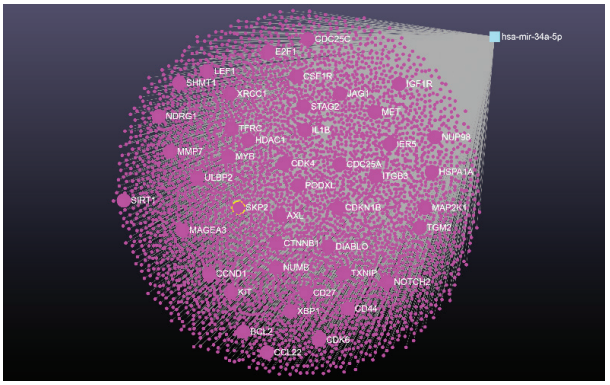

Figure 1: Forty-four AML-associated genes that are possible targets of miR-34a-5p. 5075 genes that may be targets of miR-34a-5p have been reported in the miRTarbase V8 and Tarbase V8 databases. The names of 44 genes studied in our study are shown in the figure as a larger circle. It was constructed using the miRNet tool (https://www.mirnet.ca/).

Confirmation of the selected potential AML-related genes

The association of 44 genes with AML and other cancers is shown in figure 2. Apart from 4 genes (*TGM2, ITGB3, HSPA1A1 and PODXL*), other genes were also associated with AML in the DisGeNET database. These 4 genes, which are not found in DisGeNET, were included in the study because they were found to be related to AML after the literature review (12-15). The fact that the vast majority of the 44 genes chosen from http://soft.bioinfo-minzhao.org/lgl/ database are also included in the DisGeNET database indicate that these genes could be strong AML-associated genes.

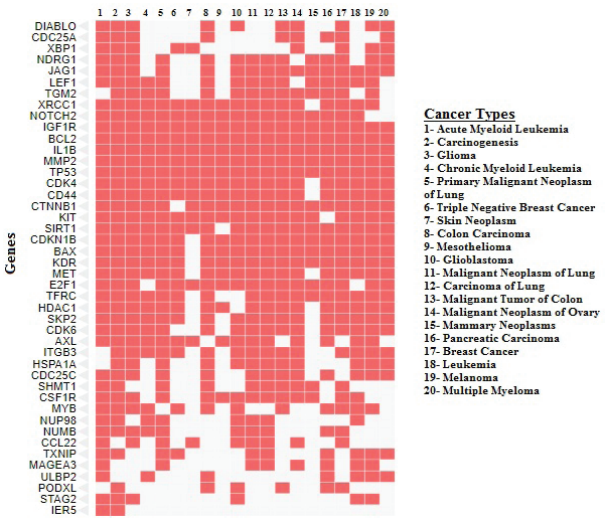

Figure 2: Forty-four genes are associated with other cancers, including AML. It was constructed via DisGeNET database (http://www.disgenet.org).

PPI of selected AML-related genes

The interaction of 44 genes suggesting there is a complex relationship between the selected genes except for the *SHMT1* gene is demonstrated in figure 3. Because this figure reveals that many of the selected genes may interact with the *TP53* gene, which is known as the genome's guardian, unraveling the interaction between the selected genes and miR-34a-5p may provide an essential clue in understanding the complicated molecular process of AML.

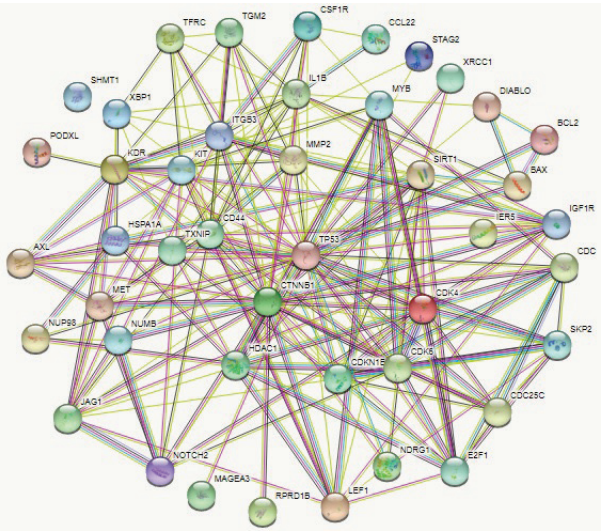

Figure 3: Using miR-34a-5p related 44 AML genes as seed, a total of 207 PPI enrichment was constructed by the STRING database (p<1.0e-16).(https://string-db.org/.)

Pathway analysis of selected AML genes

According to the results of the pathway analysis, 44 potential target genes of miR-34a-5p appear to be associated with many biological pathways. The analysis results show that one of the pathways most associated with these genes is the IGF1 pathway as seen in figure 4. In addition *NOTCH2, IGF1R, SKP2,* and *CDC25A* genes were found to be in the IGF1 pathway as shown in figure 5. This suggests that developing a potential therapy strategy based on miR-34a-5p targeting these IGF1 pathway genes may be effective in the AML cancer process.

Investigation of the relationship between selected genes and miR-34a-5p in AML in the literature

According to our research of the literature using PubMed, we noticed that all of the *NOTCH2, IGF1R, SKP2,* and *CDC25A* genes have critical roles in cancers including AML. However, it was observed that no study had yet been conducted showing that these genes are targeted by miR-34a-5p in AML cells.

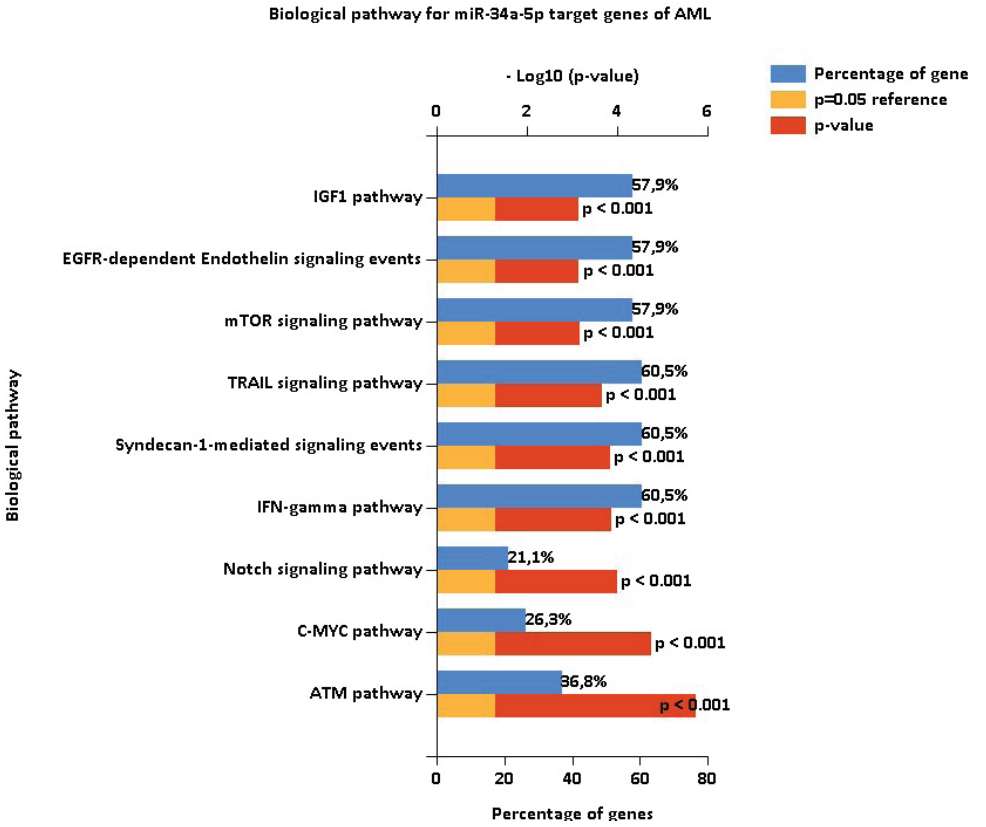

Figure 4: The defined 44 genes' pathway analysis results in AML. It was constructed using Funrich tool (http://www.funrich.org).

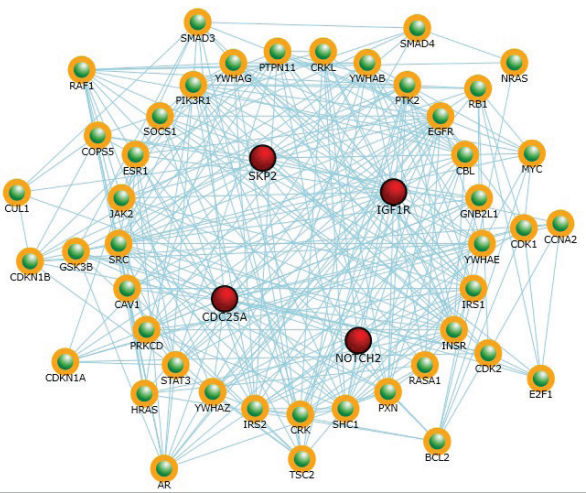

Figure 5: The *SKP2, IGF1R, CDC25A* and *NOTCH2* genes are related to the IGF1 Pathway. It was constructed via Funrich tool (http://www.funrich.org).

Identification of priority genes in elucidating AML/miR-34a-5p/target gene association

According to the TCGA data, it was detected that the *NOTCH2* gene expression level was noticeably increased in AML than in other cancer types as demonstrated in figure 6. The results of the overall survival (OS) analysis revealed that *IGF1R, SKP2*, and *CDC25A* genes had no statistically significant effect, but that the *NOTCH2* gene showed poor overall survival for AML patients as shown in figure 7. These findings suggest that *NOTCH2*, one of the 44 identified genes, is particularly significant in AML and that further research into the miR-34a-5p/*NOTCH2* axis might be beneficial.

In vitro study results

The effects of mimic transfection of miR-34a-5p on the AML cell proliferation

HL60 and NB4 cells transfected with mimic miR-34a-5p were found to have significantly higher expression of miR-34a-5p compared to the nt control group (figure 8). This indicates that mimic miR-34a-5p transfection into cells has been successfully achieved. It was determined that miR-34a-5p significantly reduced proliferation in mimic miR-34a-5p transfected HL60 and NB4 cells compared to the control group (figure 9). It was demonstrated that the values show a statistically significant decrease at both 48h measuring (HL60 p=0.00011; NB4 p=0.0031) and 96h measuring (HL60 p=0.00013; NB4 p=0.00018).

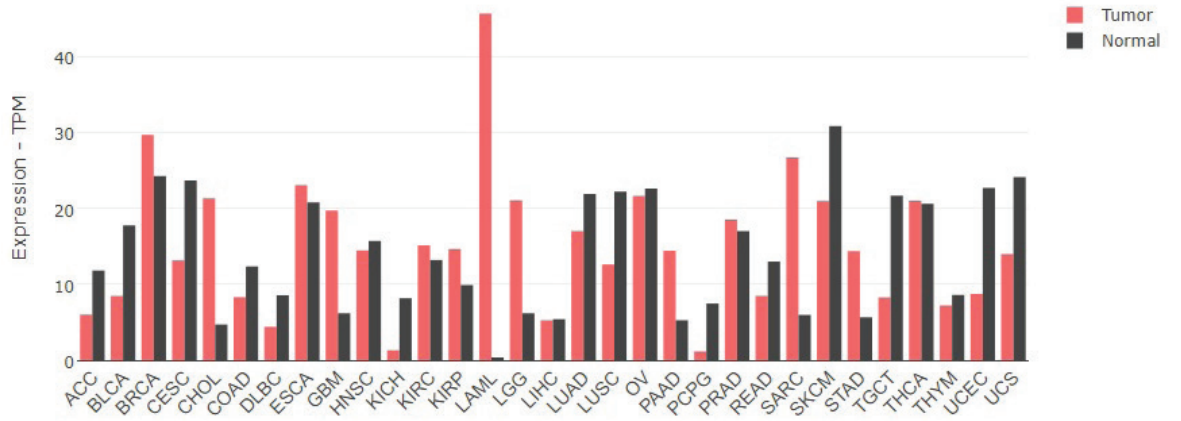

Figure 6: The expression levels of the *NOTCH2* gene in various cancer types. *NOTCH2* expression is overexpressed in many cancers, however, the increase in *NOTCH2* expression in AML is striking. LAML: Acute myeloid leukemia. TPM: Transcripts Per Million. It was constructed via GEPIA2 tool (http://gepia2.cancer-pku.cn/.).

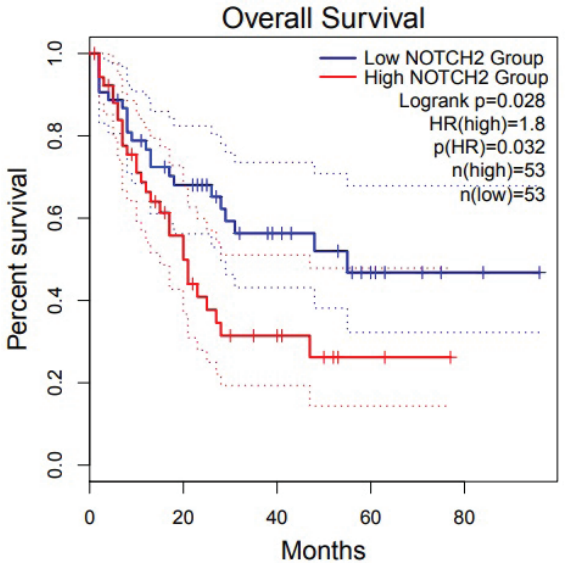

Figure 7: The impact of the *NOTCH2* gene on overall AML survival. It was created via GEPIA2 tool (http://gepia2.cancer-pku.cn/.).

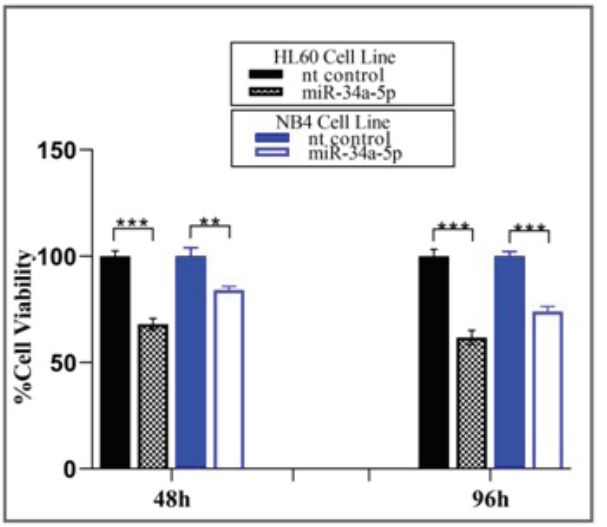

Figure 9: The effect of miR-34a-5p on cell proliferation in HL60 and NB4 cells (nt control: Non-targeting control. **p-value<0.01, ***p-value<0.001).

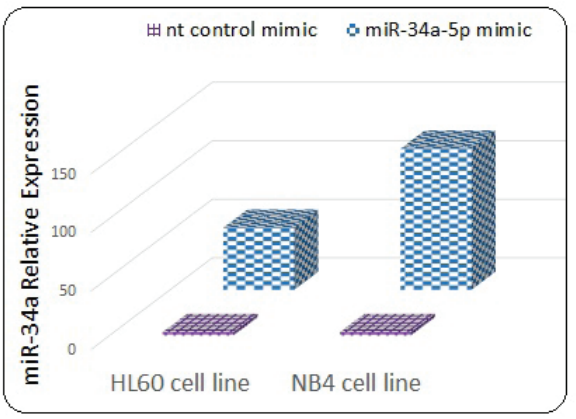

Figure 8: The transfection efficiency of miR-34a-5p in MM cells

qRT-PCR results of the selected genes

NOTCH2, IGF1R, SKP2 and *CDC25A* expression levels were detected to be statistically decreased in miR-34a-5p transfected HL60 cells (respectively p=0.003; p=0.02; p=0.01; p=0.0009) and NB4 cells (respectively p=0.02; p=0.02; p=0.01; p=0.0007) compared to the control groups (figure 10 and 11).

DISCUSSION

Over the last decade, studies have shown that miRNAs play crucial roles in nearly all biological events associated with AML, including cellular proliferation, migration, and metastasis. These findings support the idea that miRNAs could be used as biomarkers in AML. Although much has been learned about the roles of miRNAs in the initiation

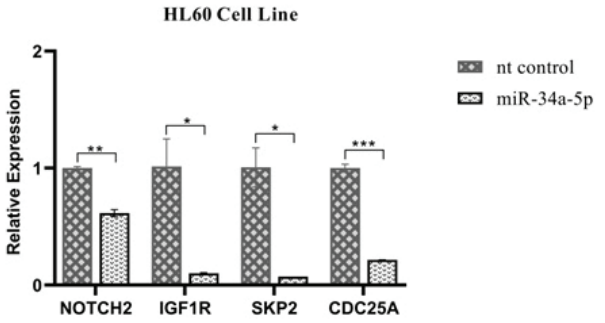

Figure 10: The expression value of selected genes in HL60 cells. *β*-actin gene expression was used for normalization (nt control: Non-targeting control, *p-value<0.05, **p-value<0.01, ***p-value<0.001).

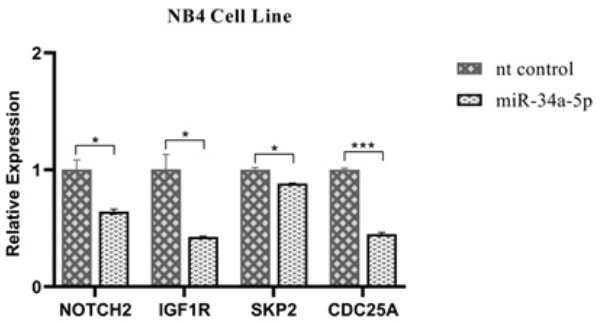

Figure 11: The expression value of selected genes in NB4 cells (nt control: Non-targeting control, *p-value<0.05, **p-value<0.01, ***p-value<0.001).

and progression of AML, many unanswered questions about the relationship between AML and miRNAs remain (16). MiR-34a-5p is a tumor suppressor, and miRNA (TsmiR) plays a vital role in oncogenesis and is essential for tumor progression inhibition (17, 18). Many studies in recent years have revealed that miR-34a-5p has a low expression level in various cancers due to the loss of its tumor suppressor effect (19-21). MiR-34a-5p is critical in the regulation of hematopoiesis. It has been reported, for instance, that miR-34a-5p can reduce mature B cells by targeting *FOXP1*, a B cell oncogene (22). MiR-34a-5p was shown to be significantly downregulated in patients with AML and cytogenetically normal-AML, and low expression of miR-34a-5p was linked with patients with intermediate/low-risk AML (23). MiR-34a-5p could influence the phenotype of leukemia cells in a variety of ways (22, 24, 25). Furthermore, miR-34a-5p is known to play a critical role in myeloid differentiation. Alteration of miR-34a-5p expression has been shown to reprogram granulocytic differentiation of blast cells with *CEBPA* gene mutations in AML (26). In the study by Liu X et al., miR-34a-5p expression was demonstrated to be downregulated in bone marrow mononuclear cells of AML patients and it was associated with tumor burden (27).

Our bioinformatics research and in vitro study results showed that *NOTCH2, IGF1R, SKP2,* and *CDC25A* genes may be closely related to miR-34a-5p in AML. These genes have been implicated as oncogenes in a variety of cancers (28-31). For example, *IGF1R* overexpression has been shown to facilitate the progression of lung metastasis (31). Zhang et al. showed that overexpression of Skp2 in breast cancer induces breast cancer cell proliferation (32). Silencing *CDC25A* has been demonstrated to suppress the proliferation of liver cancer cells by downregulating *IL-6* (33).

It has been reported that these genes are also closely related to AML. NOTCH signaling pathway having four receptors including *NOTCH2* is important in AML cell survival, which is regulated by bone marrow stromal cells. This might be the rational evidence for novel AML eradication approaches that may become useful for diagnosis. It has been shown that chemosensitivity can be partially restored by blocking the *NOTCH2* gene in AML (34). IGF1R, another important gene in our study that may be associated with miR-34a-5p, is a tyrosine kinase transmembrane protein receptor. This gene activates signaling pathways by binding to insulin-like growth factor ligands and controls cell proliferation, differentiation and apoptosis in AML (35). The another crucial gene, *SKP2*, has been demonstrated to downregulate *C/EBPa* (CCAAT/enhancer-binding protein) expression via ubiquitin-dependent proteasome degradation, resulting in differentiation block in AML (36). Due to the increased proportion of *FLT3* mutation (25-30 percent of total of AML) and its association with poor prognosis, many *FLT3* inhibitors have been designed and tried in various clinical studies, either as a single drug or in a combination with chemotherapy. *CDC25A* is an early-stage target in *FLT3*-ITD oncogenic signaling and is a key player in proliferation and differentiation of arrest events in AML cells (37). Therefore, the relationship between miR-34a-5p and *NOTCH2, IGF1R, SKP2, CDC25A* may be of particular importance for AML.

It has been reported that these four genes are overexpressed in AML as in other cancers and are involved in the disease process (38-41). Furthermore, miR-34a-5p has been shown to directly target these genes in a variety of cancers and other diseases (42-45). A recent study, for instance, revealed that miR-34a-5p inhibits cervical tumor progression and migration by suppressing *CDC25A* (43).

The identification of genes controlled by miRNAs is a key difficulty in miRNA studies. Our study findings indicate that if proper bioinformatics tools and in vitro applications are utilized and combined, the AML/miRNA/Target genes link may be identified more accurately. The current study has filled an important gap in the literature by determining the possible target

genes of miR-34a-5p, using various bioinformatics tools, and then performing validation studies at the mRNA level in HL60 and NB4 cells. According to the pathway analysis of the present study, the majority of the 44 potential target genes of miR-34a-5p are related to the IGF1 pathway (Figure 4.) *NOTCH2, IGF1R, SKP2,* and *CDC25A* genes, which are among the 44 genes in our study and whose expression levels decrease after miR-34a-5p transfection into HL60 and NB4 cells, are also related to the IGF1 pathway in the cell Figure 5). IGF1 signaling pathway is known to play a vital role in many important processes, including development, homeostasis, and aging. Many diseases, particularly cancers, are caused by various mutations in the genes involved in this pathway. AML has also been linked to irregularities in the IGF1 pathway. For instance, in a recent study, it was revealed that IGF1 autocrine plays a critical role in the constitutive *PI3K/AKT* activation of primary AML cells, and it was suggested that *IGF1R* could be targeted as a potential new treatment option (39). This demonstrates how important the IGF1 signaling pathway is in the development of therapeutic strategies. MiR-34a-5p has been identified in the literature as one of the miRNAs involved in the IGF1 pathway (46). Our intriguing and significant findings imply that miR-34a-5p may contribute to the cancer process in AML, particularly via the IGF1 pathway. Therefore, we suggest that the relationship between miR-34a-5p and the IGF1 pathway genes (particularly the *NOTCH2, IGF1R, SKP2,* and *CDC25A*) in AML should be studied further.

In the present study, the overall survival analysis of the *IGF1R, SKP2,* and *CDC25A* genes in AML was not found to be statistically significant, while it was demonstrated that the *NOTCH2* gene was an indicator for AML patients who had poor overall survival (Figure 7). The NOTCH pathway is a critical signaling mechanism that allows nearby cells to communicate and perform their developmental roles in a pathological environment. *NOTCH2* is one of the key players in this pathway. The *NOTCH2* gene is overexpressed in many cancers, including AML. It has been demonstrated that *NOTCH2* expression may be regulated in the cancer process by different miRNAs. For instance, Wang et al. showed that miR-181b/*NOTCH2* might overcome chemoresistance in NSCLC by modulating cancer stem cell-like characteristics (47). Jiang et al. revealed that miR-34c-3p reduced cell invasion and epithelial-mesenchymal transition in nasopharyngeal cancer by targeting *NOTCH2* (48). Our TCGA data analysis results show that *NOTCH2,* which is overexpressed in many malignancies, is much higher expressed in AML than in other cancer types (Figure 6). Literature data and our study results have revealed the fact that the miR-34a/*NOTCH2* relationship may be essential in AML.

The study's findings shed light on the relationship between miR-34a-5p and its target genes in AML. However, for more precise results, methods such as western blot and luciferase reporter assay should be used. Based on our study results, we suggest that miR-34a-5p and selected AML-related genes should be studied in more detail. Thus, the contribution of miR-34a-5p and target genes to cellular processes such as apoptosis, migration, and metastasis in AML will be determined.

Acknowledgements: The NB4 cell line was obtained from the Genetics Department of Aziz Sancar Institute of Experimental Medicine. We would like to thank Prof. Sema Sırma Ekmekci.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- M.K.; Data Acquisition- M.K., İ.S.; Data Analysis/Interpretation- M.K., İ.S.; Drafting Manuscript- M.K.; Critical Revision of Manuscript- M.K., İ.S.; Final Approval and Accountability- M.K., İ.S.

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

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

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RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1200910
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

EXPLORING MITOMIRS IN BREAST CANCER: AN *IN-VITRO* STUDY OF THEIR EMERGING ROLES

MEME KANSERİNDE MİTOMİR'LERİN TANIMLANMASI: *İN VİTRO* ÇALIŞMA İLE ROLLERİNİN GÖSTERİLMESİ

Pervin Elvan TOKGÜN[1], Ayşe Gaye TOMATIR[2]

[1]Pamukkale University, Faculty of Medicine, Department of Medical Genetics, Denizli, Turkiye
[2]Pamukkale University, Faculty of Medicine, Department of Medical Biology, Denizli, Turkiye

ORCID IDs of the authors: P.E.T. 0000-0001-9025-4140; A.G.T. 0000-0001-9251-9632

Cite this article as: Tokgun PE, Tomatir AG. Exploring mitomirs in breast cancer: an in-vitro study of their emerging roles. J Ist Faculty Med 2023;86(1):69-77. doi: 10.26650/IUITFD.1200910

ABSTRACT

Objective: Breast cancer is associated with a 5% genetic predisposition in women. Mitochondria play a role in important cellular events such as metabolism, cell death, and inflammation. Recent studies have highlighted precursor micro-RNA (pre-miRNA) to be located in mitochondria as well as mature miRNA. This study aims to reveal the occurrence of mitochondrial miRNA (mitomiR) in breast cancer cells.

Materials and Methods: The study has prepared the mitochondrial fractions using the magnetic-activated cell sorting (MACS) method and performed small RNA (sRNA) sequencing.

Results: The study has identified known and novel mitomiR sequences aligned to the mitochondrial genome.

Conclusion: Identifying new mitomiRs can provide significant contributions and illuminate the molecular mechanism underlying mitomiR biogenesis.

Keywords: mitomiRs, MACS, breast cancer, small RNA sequencing

ÖZET

Amaç: Kadınlarda meme kanseri %5 oranında genetik yatkınlıkla ilişkilidir. Mitokondri, metabolizma, hücre ölümü ve inflamasyon gibi önemli hücresel olaylarda rol oynar. Son çalışmalar, olgun miRNA'ların yanı sıra pre-miRNA'ların mitokondride bulunduğunu göstermişlerdir. Bu çalışmada meme kanseri hücrelerinde mitomiR'lerin varlığını ortaya koymayı amaçladık.

Gereç ve Yöntem: MACS yöntemi kullanılarak mitokondriyal fraksiyonlar hazırlandı ve küçük RNA dizilimi yapıldı.

Bulgular: Mitokondriyal genoma hizalanmış bilinen ve yeni mitomiR dizilerini belirledik.

Sonuç: Yeni mitomiR'lerin tanımlanması literatüre önemli katkılar sağlayabilir ve mitomiR biyogenezi moleküler mekanizmalar ile aydınlatılabilir.

Anahtar Kelimeler: mitomiR, MACS, meme kanseri, küçük RNA dizileme

INTRODUCTION

Breast cancer ranks first among cancer-related deaths in women and is associated with 5% genetic predisposition among women, with an autosomal dominant inheritance pattern having been observed (1). BRCA1 and BRCA2 gene mutations have also been associated with a very high risk of breast and ovarian cancer, with 65% -85% of women who carry these mutations bearing a lifelong risk for developing invasive breast cancer and a 15%-65% risk of contracting invasive ovarian cancer (1, 2).

Mitochondria are double-membrane organelles approximately 0.5–1 μm wide and 7 μm long. In addition to ATP production, these highly dynamic organelles play an essential role in regulation many physiological processes including metabolism, apoptosis, disease, and aging. While mitochondrial function is a key to cell survival and death, mitochondrial metabolism deregulation is also critical in the pathogenesis of many cancers (3, 4) and is also central to oxidative phosphorylation, with 95% of cellular energy being provided by oxidative phosphory-

Corresponding author/İletişim kurulacak yazar: Pervin Elvan TOKGÜN – parslan@pau.edu.tr

Submitted/Başvuru: 02.12.2022 • **Revision Requested/Revizyon Talebi:** 12.12.2022 •
Last Revision Received/Son Revizyon: 27.12.2022 • **Accepted/Kabul:** 02.01.2023 • **Published Online/Online Yayın:** 26.01.2023

lation in mitochondria. This process includes the five different protein complexes, including complexes I-V. The electron transport chain (ETC) in the inner membrane is associated with coenzyme Q and cytochrome c electron transfer components. Beta-oxidation of fatty acids and Pyruvate oxidation pathways occur in the tricarboxylic acid cycle (TCA) of the mitochondrial matrix. A total of 91 polypeptides, including cytochrome c, are directly associated with oxidative phosphorylation (OXPHOS). Some of these are encoded as nuclear proteins and others as mitochondrial proteins (5).

Depending on the restricted coding capacity of mitochondrial DNA (mtDNA), nuclear genes are required for biological functions and structural components. In addition, nuclear-encoded genes regulate mtDNA replication, transcription, and translation. For this reason, the collaboration of nuclear genes and mtDNA is necessary for regulating OXPHOS capacity in response to several physiological and disease states (6, 7). Mammalian cells contain more than 1,000 mitochondria and approximately 10,000 copies of mtDNA. The mitochondrial genome is 16.6 kb in size, contains no introns, has a circular double-stranded structure, and contains a total of 37 genes, 2 rRNA, 22 tRNA, and 13 polypeptides coding for the ETC and non-coding RNAs. Although the mitochondria genome has only 13 protein-coding genes, it contains around 1,500 proteins. Replication and transcription of mtDNA begin in the mitochondrial D-loop, which is known as the small non-coding region. All other proteins associated with replication, transcription, and translation are encoded by nuclear genes and imported to mitochondria through special transport systems (4, 8-11).

Although mitochondrial RNA (mtRNA) are transcribed from both strands such as polycistronic precursor transcripts, the process leads to the release of non-coding RNA and coding RNA containing tRNA, rRNA, and mRNA (12). Similarly, RNA transport is very important for mitochondrial function, although the relevant mechanisms have yet to be elucidated.

Non-coding RNA (ncRNA) have many functions, from catalyzing biological reactions, cellular defense, and developmental processes to cellular response. In addition, ncRNA regulate transcriptional and post-transcriptional gene silencing and chromosome remodeling. Many non-coding RNA in mitochondria can also be encoded by mtDNA (13). However, many types of RNA are known to enter and exit mitochondria. The most common RNA in mitochondria (e.g., tRNAs, 5S rRNA, RNAse MRP and RNAse P) are nuclear-encoded and transferred to the mitochondria (14, 15). Mitochondria also contain many non-coding RNA, such as miRNA, snRNA, Piwi-interacting RNA (piRNA), signal recognition particle RNA (srpRNA), and small nucleolar RNA (snoRNA) (12, 16). Unlike nuclear-encoded miRNA, mitochondrial miRNA (mitomiR) biogenesis is not yet fully known, but several hypotheses are found in this regard. The miRNA encoded by the mitochondrial genome are assumed to act in three different ways: 1) Nuclear genome-encoded miRNA suppress mRNA translation in the cytosol by targeting nuclear-encoded mitochondrial proteins, thus affecting the transport of specific mitochondrial proteins to the mitochondria; 2) nuclear-encoded miRNA regulate the translation of mitochondria-encoded proteins; 3) mitochondrial genome-encoded miRNA regulate the translation of mitochondrial genome-encoded proteins (17). Some pre-miRNA are processed in the mitochondria and can synthesize the mature miRNA that are simultaneously activated on mitochondrial transcripts or sent to the cytosol in order to combine with genomic mRNA (18). Bandiera et al. (19) discovered the presence of miRNA with different expression profiles in the nucleus and mitochondria called mitomiR and showed mitomiR to have both nuclear- and mitochondrial-encoded targets (19). The miRNA detected in mitochondria may vary depending on the cell type (16, 20). MitomiR differ from other miRNA in terms of their thermodynamic properties and dimensions and are expressed in the loci of almost all nuclear-encoded genes related to mitochondrial function. Compared to the miRNA found in the cytosol, mitomiR appear to be unable to preferentially target nuclear-encoded mitochondrial genes.

By considering one of the causes of cancer formation to be defects in mitochondria, this study aims to identify mitomiR that have different expressions in the mitochondria of breast cancer cell lines and to determine their target genes.

MATERIALS AND METHODS

Cell culture

The MCF-10A cells have been cultured in the DMEM/F-12 (Sigma, Germany) medium containing 1% penicillin-streptomycin, L-glutamine, 5% heat-inactivated Horse Serum (GIBCO, USA), 10 µg/ml insulin (GIBCO, America), 20 ng/ml epidermal growth factor (EGF; Miltenyi Biotec, Germany), and 0.5 µg/ml hydrocortisone (Sigma, Germany); the MDA-MB-231 cells have been cultured in the RPMI1640 Medium (GIBCO, USA) containing 5% heat-inactivated fetal bovine serum (FBS; GIBCO, USA), 1% penicillin-streptomycin (GIBCO, USA), and 1% L-glutamine (GIBCO, USA); and the MCF-7 cells have been cultured in the DMEM Medium (SIGMA, USA) containing 5% heat-inactivated FBS (GIBCO, USA), 1% penicillin-streptomycin (GIBCO, USA), and 1% L-glutamine (GIBCO, USA) incubated at 37°C at 5% CO2 and 95% humidity.

Isolating mitochondria from cells using MACS technology

A mitochondria isolation kit (Miltenyi Biotec, Germany) has been used to obtain a high yield with purity and integrity of mitochondria isolated from cells. In short, the cells were disrupted with the help of a Dounce homogenizer, treated with 9 mL of the 1X lysate separation buffer and labeled with 50 µl anti-TOM22 coated beads, incubated for 1 hr at 40°C using a rotator. The labeled lysate was then transferred to LS Columns (Miltenyi Biotec, Ger-

many). The mitochondria were eluted after being washed five times with 3 mL of the 1X lysate separation buffer.

The RNase A (10 mg/ml, ABM, Canada) treatment was performed to eliminate genomic contamination in the mitochondrial fractions. The pellet was treated with TRIZOL (MRC, USA) immediately after processing to inactivate the RNase A and isolate the mtRNA.

Small RNA (sRNA) sequencing

The sRNA sequencing was performed using the Illumina platform. In short, the purity and quality of the mtRNA were checked using the NanoDrop (Thermo, America) and Agilent 2100 Bioanalyzer (Agilent Technologies, America) devices. Libraries were prepared from the 50ng mtRNA samples using the SMARTer smRNA for Illumina Kit (CloneTech, America). First, the poly(A) tail was added to the input RNA using Poly(A) Polymerase in order to facilitate oligo(dT)-primed cDNA synthesis. Next, adapter-linked miRNA fragments were transformed into cDNA fragments followed by the PCR purification. The sizes of the amplified cDNA fragments were checked using a Bioanalyzer DNA High Sensitivity Chip. The cDNA fragments were sequenced according to the read length using the sequence by synthesis method on the Illumina platform.

Bioinformatic analysis

After sequencing, the raw sequence reads were filtered, then the adapter sequences were clipped away from the raw sequence readings using the program Cutadapt version 4.1. The clipped reads were then clustered, and these clusters contain reads that match 100% of the sequence and read length. Clustered reads were then aligned with the reference genome (hg19) and precursor miRNA from the miRBase (v21; https://www.mirbase.org/) to identify the defined miRNA. The miRDeep2 algorithm (https://github.com/rajewsky-lab/mirdeep2/releases/latest) was used to predict the potential hairpin structures of the miRNA. To classify other RNA types, clustered reads were mapped to the reference genome in miRBase (v21) and the non-coding RNA database Rfam (v9.1; https://rfam.org/). The number of reads for each miRNA was transferred from the mapped miRNA, and the distribution of each miRNA has been reported.

The web-based DIANA bioinformatics analysis program (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index) was used to analyze the functions of the miRNA, and the Gene Ontology (GO; http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (https://www.kegg.jp/kegg/pathway.html) analyses were performed to determine the biological processes, molecular functions, and biochemical pathways pertaining to these miRNAs. The Search Tool for the Retrieval of Interacting Genes / Proteins (STRING) database (v10.5; https://string-db.org/) was used to analyze the proteins that interact with the miRNA and gene regions that show homology.

This study is supported by Pamukkale University Scientific Research Projects Department (Project No: 2014SBE011).

RESULTS

Mitochondrial RNA (mtRNA) isolation and mitochondrial gene enrichment analysis

The study grew $5x10^7$ cells on T125 flasks to prepare the purified mitochondrial fractions from MCF-7, MDA-MB-231 and MCF-10A cells. A mitochondria isolation kit using MACS technology enabled the isolation of mitochondria and cytosol fractions from the same cells. Thus, the study evaluated the genomic contamination in the mitochondrial fraction at the RNA level by comparing it to the cytosol fractions. The 16S rRNA gene was used as a calibrator for calculating the mitochondrial/nuclear RNA ratio (figures 1a, 1b).

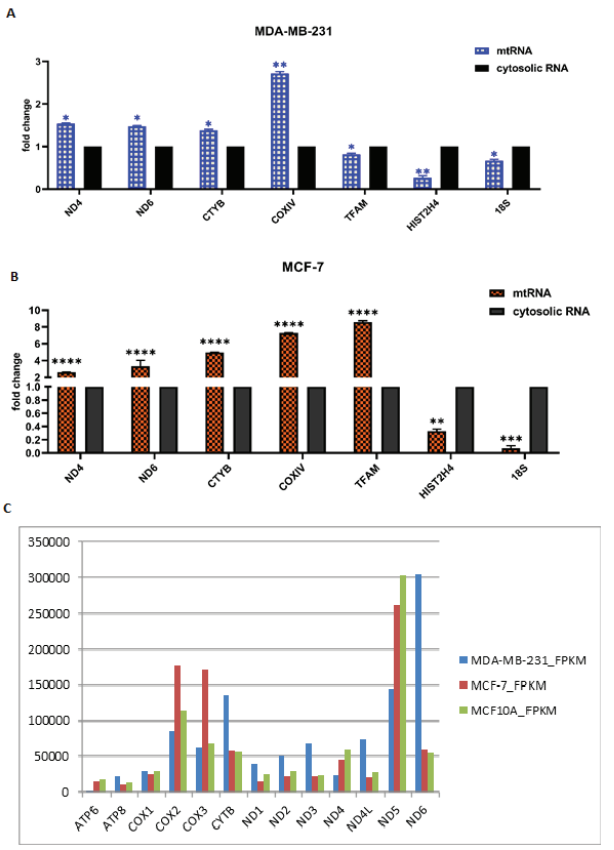

Figure 1: A, B) Mitochondrial genes were found to be significantly enriched in mitochondrial fractions compared to cytosolic fractions in both MCF-7 and MDA-MB-231 cells whereas HIST2H4, Nuclear gene histone cluster 2 H4 family, and 18S rRNA levels were vice versa. Two way ANOVA and Sidak's multiple comparisons test were used for statistical analysis. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) C) Fragments Per Kilobase of transcript per Million mapped reads (FPKM) of mitochondrial encoded genes as a result of small RNA sequencing of cells.

Enrichment of the mitochondrial genes in the mtRNA samples was also evaluated with the data obtained using the next-generation sequencing method (figure 1c). Thirteen mitochondria-encoded genes were evaluated and fragments per kilobase of exon per million mapped fragments (FPKM) were found to range between 320 and 303,493.

Mitochondria-associated sRNA library analysis

The study isolated the sRNA associated with mitochondria (18-30 nucleotides [nt]) and generated and sequenced libraries using the Illumina HT2500 platform. The peak seen around 175bp indicates the predicted size of the adapter-linked miRNA library, and libraries containing miRNA-derived sequences range in size from about 172-178 bp. The total base number, readings, GC percentage, Q20 (%) and Q30 (%) were calculated for each sample. Sequencing resulted in crude sequencing read lengths from the MDA-MB-231, MCF-7 and MCF-10A cells of 28,496,397, 32,712,495, and 35,790,420, respectively. The 3' adapter sequences of mature miRNAs with 24 bp length were removed using the program Cutadapt. By filtering sequences smaller than 15 nucleotides, the 24,781,073, 21,059,268, and 22,302,897 reads were then matched to the genome (hg19), respectively. The sRNA population including the miRNA, piRNA, and sRNA of respective lengths 21-22 nt, 30 nt, and 24 nt was categorized by performing a length analysis. The highest read rate in the samples was at 25 nt, which indicates the presence of miRNA, piRNA, and sRNA.

The clustered reads were mapped to the reference genome (hg19) and precursor miRNA separately in order to determine the defined miRNA. In addition, the Rfam database (v9.1) was used to identify the miRNA and other RNA species. The miRDeep2 score was chosen between±10. Figure 2 shows the small RNA class types in the samples.

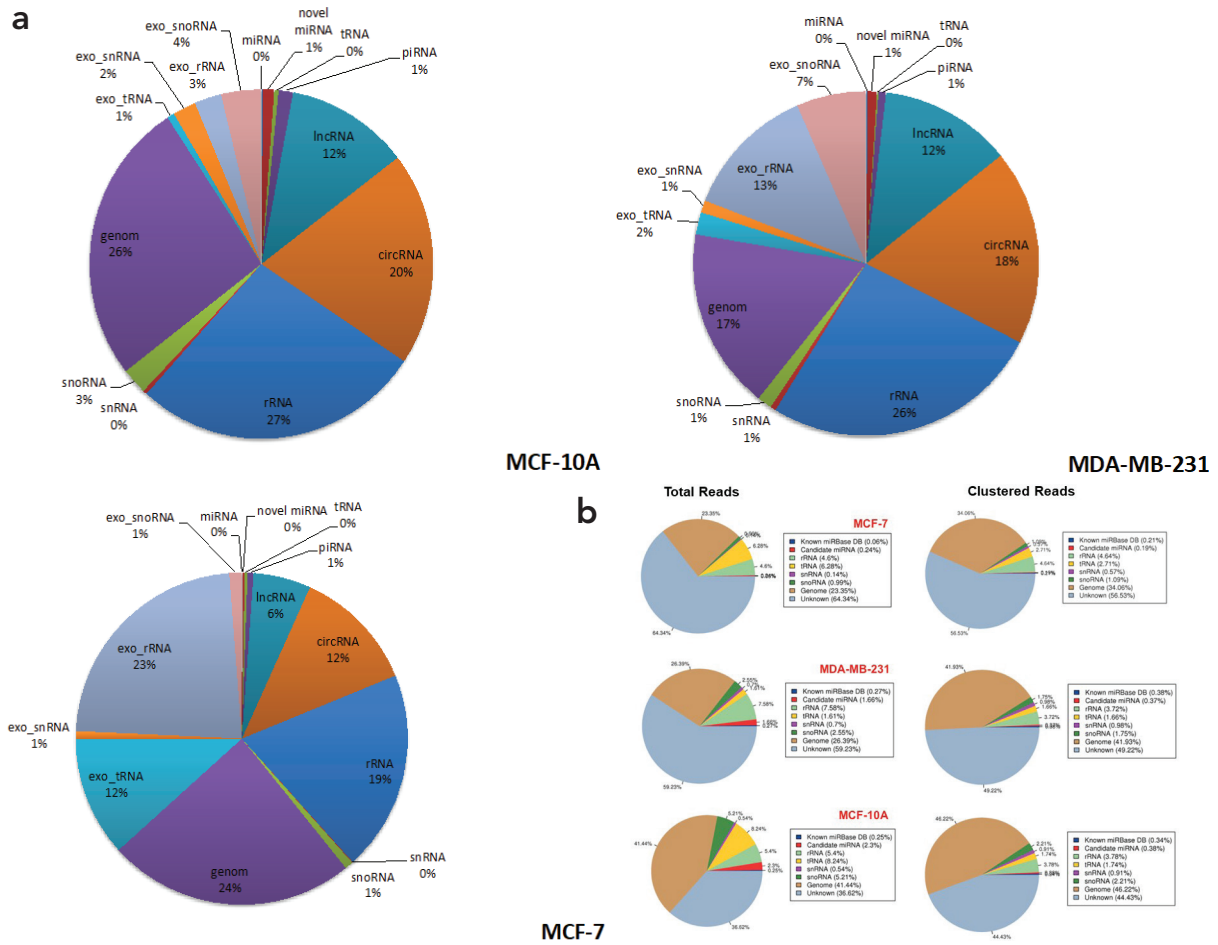

Figure 2: a) Pie charts corresponding to RNA contents in the cells. sRNA sequences are paired with RNAs from Genbank and Rfam databases in MCF-7, MDA-MB-231 and MCF-10A cells. b) Percentage of small RNA reads in the samples. smRNA class type (such as known miRBase, candidate miRNA, rRNA, tRNA, snRNA, snoRNA, Genome, Unknown) are shown for total reads (on the left) and clustered reads (on the right) respectively. Unique clustered reads are sequentially aligned to reference genome, miRBase v21 and run blast to non-coding RNA database, Rfam 9.1 to classify known miRNAs and other type of RNA such as tRNA, snRNA, snoRNA etc. in MCF-7, MDA-MB-231 and MCF-10A cells.

Identifying the mitochondria-associated miRNA

The numbers of miRNA frequencies in the mtRNA samples were determined to be between 1-12,384. According to the bioinformatic analysis results, 283 miRNAs in the MCF-10A cells, 234 miRNAs in the MCF-7 cells, and 220 miRNAs in the MDA-MB-231 cells were found to be compatible with the miRBase database (v21). The most frequently determined miRNA associated with mitochondria in the samples were hsa-miR-6087-5p, hsa-miR-3960-3p, hsa-miR-7641-5p, hsa-miR-3648-3p, hsa-miR-4488-5p, hsa- miR-4485-5p, hsa-miR-4449-3p, hsa-miR-4484, let-7 family members (let-7a, b, c, d, e, f, g, i), hsa-miR-1290 -3p, hsa-miR-423-5p, and hsa-miR-3687-3p. In addition, hsa-miR-1246-5p, hsa-miR-1275-5p, hsa-miR-663a-5p, miR-25-3p, miR-23a-3p, hsa- miR-423-5p, hsa-miR-320a-3p, hsa-miR-574-5p, and hsa-miR-7704-5p were also found to be associated with mitochondria (table 1).

In order to detect the presence of miRNA-targeting mitochondria-encoded genes among the miRNA, the precursor sequences were matched with the mitochondrial genome using the programs SerialCloner 2-6-1 (figure 3) and Mitowheel.

Table 1: Nuclear-encoded miRNAs detected in mitochondrial fractions of cells

miRBase ID	MCF-10A (number of reads)	MCF-7 (number of reads)	MDA-MB-231 (number of reads)
hsa-miR-6087-5p	11187	4175	1322
hsa-miR-3960-3p	5094	871	2438
hsa-miR-7641-5p	4851	232	408
hsa-miR-3648-3p	4620	1057	2405
hsa-miR-4449-3p	1709	234	154
let-7 family	1652	72	274
hsa-miR-1275-5p	86	7	25
hsa-miR-663a-5p	534	381	230
hsa-miR-423-5p	138	9	138
hsa-miR-320a-3p	138	62	76
hsa-miR-1296-3p	5	4	4
hsa-miR-574-5p	212	54	179
hsa-miR-221-3p	16	-	118
hsa-miR-3687-3p	636	202	256
hsa-miR-7704-5p	161	96	57
hsa-miR-664b-3p	102	37	56
hsa-miR-6724-5p	54	31	19
hsa-miR-193b-5p	48	9	5
hsa-miR-6126-5p	17	20	12

Figure 3: Matching defined miRNA (mitomiR) sequences with the mitochondrial genome using SerialCloner 2-6-1.

GO and KEGG pathway analysis of MitomiR and STRING protein-protein interaction analysis

Evaluations were made using Fisher's exact test analysis method in the DIANA database for the pathway analysis of the miRNA. The pathways in which the targets of the miRNA clusters are highly correlated were determined by considering the p-value. Figures 4 a and 4b summarize the gene-related pathways. This study performed

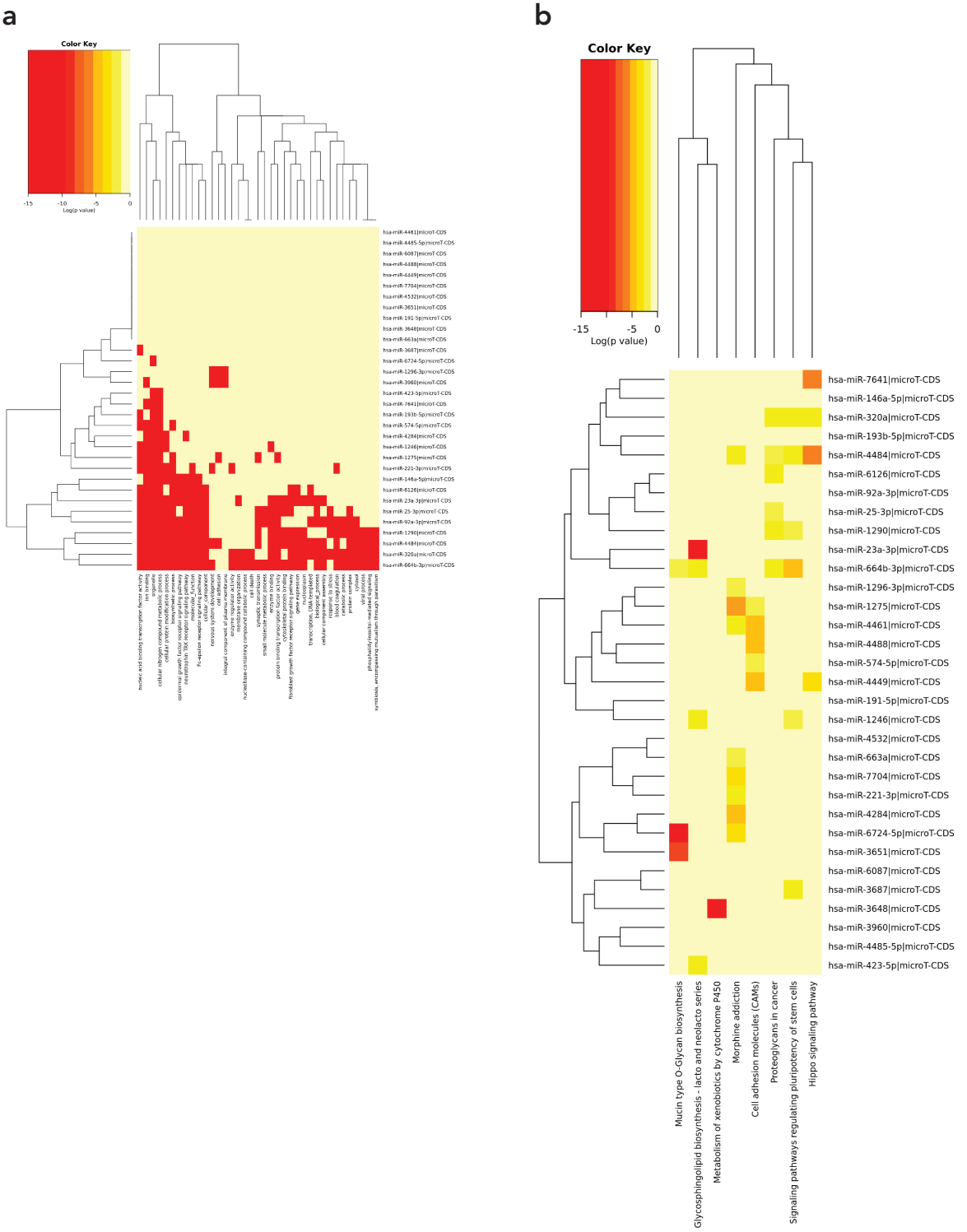

Figure 4: a) Heat-map analysis (DIANA database) of GO terms of the detected miRNAs b) Heat-map analysis (DIANA database) corresponding to KEGG pathways of the detected miRNAs

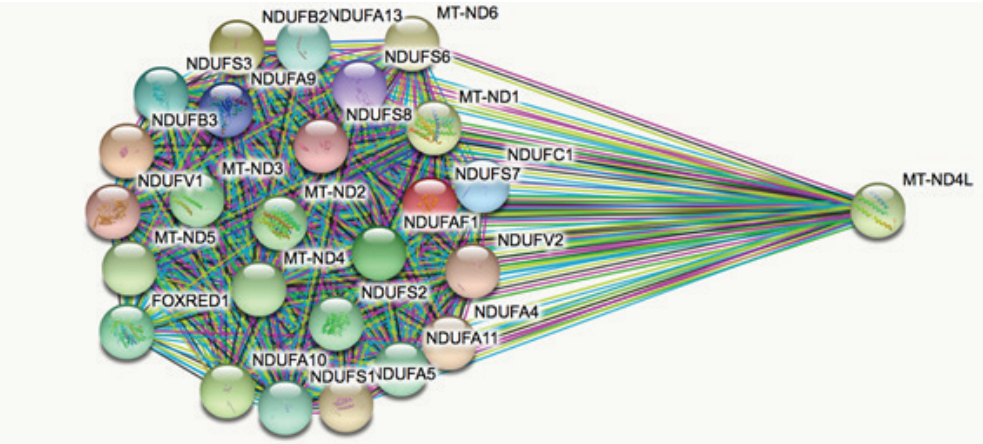

Figure 5: STRING analysis of hsa-miR-4461.

the protein-protein interaction analysis by detecting hsa-miR-4461 showing homology with the mitochondrial gene region MT-ND4L using the STRING v10.5 database (figure 5).

DISCUSSION

This study has determined the presence of miRNAs in mitochondrial fractions isolated from breast cancer cells and shown the identified miRNA targets' ability to regulate cellular pathways in which mitochondria play key roles. Because the MACS isolation method was shown to enable the isolation of mitochondria with a high purity and density equivalent to ultracentrifugation (21), the mitochondrial fraction was therefore first obtained from the cells using this technology. The most common problem in obtaining mitochondrial fractions is the presence of cytosolic contamination. The desired result removing the cytosolic contamination from the samples was achieved by using a method that enables the isolation of mitochondria using super magnetic beads conjugated with the outer membrane protein antibody anti-TOM22 and washing the isolated mitochondrial fractions with RNAse A.

Warburg hypothesized that increased aerobic glycolysis in tumor cells may be associated with impaired respiratory capacity in these cells. Although cancers are associated with reduced respiration in cells resulting in mitochondrial dysfunction, these defects do not cover OXPHOS as a whole. Some studies have shown the most aggressive breast cancer cells to have the most extensive OXPHOS defect, where malignant cells play a role in mitochondrial damage and result in OXPHOS deregulation (22). In the normal breast cell line (MCF-10A) and metastatic (MDA-MB-231) and non-metastatic (MCF-7) breast cancer cell lines used in the study, the expression of OXPHOS genes was primarily confirmed at the mRNA level.

The necessity for discovering the underlying molecular mechanisms for miRNA transport from the nucleus to the mitochondria has emerged alongside the discovery of the mitomiR. Many studies have shown the transport of nuclear-encoded RNA to the mitochondria to be able to occur through several transmission routes, mostly ATP-dependent. However, the molecular mechanisms of mitochondrial RNA transport can often show species-specific variability (23). Pre-miRNAs were previously shown to also be present in mitochondria as well as in mature miRNAs, and these findings increase the probability of mitochondrial miRNA biosynthesis (24). Some pre-RNA sequences are thought to be processed in mitochondria and to act on mitochondrial transcripts or form mature miRNAs that are transported to the cytosol to interact with genomically derived mRNAs. For this reason, the mitochondrial-processed miRNA are thought to contribute to the post-transcriptional regulation of gene expressions related to mitochondrial functions (4). This study identified sRNAs, especially miRNAs, in the MCF-10A, MCF-7, and MDA-MB-231 cell lines using sRNA sequencing and determined their relationship with mitochondria. Based on the idea that miRNA targets are able to regulate the critical cellular pathways in which mitochondria play a key role, bioinformatic analyses were performed as a result of the sRNA sequencing of the mtRNA samples, which enabled the identification of mature miRNAs in the samples. Library analyses revealed three mitochondria-encoded miRNAs (i.e., hsa-miR-4461, hsa-miR-4484, and hsa-miR-4485). Once the sequences of these three miRNAs were aligned with the mitochondrial genome, they showed respective homology with the ND4L, L-ORF, and 16S rRNA genes.

A dynamic relationship is known to exist between mitochondrial function and miRNA activity. The presence of the miRNA in mitochondria isolated from various tissues and cells, as well as the presence of the important proteins

AGO and Dicer (19, 21, 25) suggests that an active RNA interference (RNAi) mechanism may be present in mitochondria. According to other studies on miRNA, homology has been shown with mitochondrial genes in breast cancer cell lines, and the possible targets of hsa-miR-4485, which is localized on chromosome 11p15.4 and targets 16S rRNA, may involve many steps of tumor formation.

Sripada et al. demonstrated hsa-miR-4485's ability to inhibit glycolysis and reduce the clonogenic potential of breast cancer cells by using bioinformatics programs to determine the pathways that are associated with these targets to also be associated with tumor suppression, cancer cell migration, metabolic reprogramming, and cell cycle control (16). hsa-miR-4484 is localized on chromosome 10q26.2 and has been shown to regulate AATF by interacting with MRP3 K12/DLK, which is associated with apoptosis being triggered in cells. Hypoactivation of survival and proliferative pathways such as PI3K/AKT results in cancer onset that also affects mitochondria. PI3K also plays a role as a miR-4484-specific target (24). hsa-miR-4461 is localized on chromosome 5q31.1, and the nucleophosmin 1 (NPM1) gene, which can play a protective role against oxidative stress in hematopoietic stem cells, is one of its possible targets. Many cancer cells with high NPM1 expression are more resistant to ultraviolet or hypoxia-induced apoptosis (25). Anti-apoptotic functions were found to be associated with the ability of NPM1 to inhibit p53 localization in mitochondria (26).

In addition to the hsa-miR-4484, hsa-miR-4485, and hsa-miR-4461 mitomiRs that showing homology with the mitochondrial genome, the study also determined nuclear genome-encoded miRNA that are also associated with mitochondrial function (table 1).

As a result of the GO and KEGG pathway analyses made using the identified mitochondrial miRNA, the study also sheds light on which genes are targeted by targeting certain genes on specific pathways. Accordingly, GO terms such as transcription regulation (GO: 0006351), regulation of gene expression (GO: 0010467), stress response (GO: 0006950), cell death (GO: 0008219), biosynthetic process (GO: 0009058), and biological processes (GO: 0008150) were found to be quite meaningful. According to the KEGG analysis, the Hippo signaling pathway (hsa04390), proteoglycans (hsa0525), thyroid hormone signaling pathway (hsa04919), FoxO signaling pathway (hsa04068), TGF-β signaling pathway (hsa04350), mTOR signaling pathway (hsa04150), glycosphingolipids biosynthesis (hsaT30003), alanine-aspartate-glutamate metabolism (hsa00250), choline metabolism (hsa05231), PI3K-AKT signaling pathway (hsa04151), and insulin signaling pathway (hsa04910) are the possible targets.

In addition to the identified miRNA, other possible novel miRNA reads were also analyzed based on mature, star, and loop sequences using the RNAfold algorithm in the program miRDeep2, with 229 sequences from the MCF-10A cells, 112 sequences from the MCF-7 cells, and 139 sequences from the MDA-MB-231 cells being detected with the Randfold algorithm (table 2). Further analysis will be required in order to identify the possible functional roles of mitomiRs.

Table 2: Chromosomal locations of novel miRNA sequences

ID	Localization	Strand	Count	Sequence	Homology
MCF-10Am-0012	chr2:189355167..189355246	+	210	gggguuuggcagagaugu	locus MT-OHR
MCF-10Am-0013	chr2:191627126..191627186	+	24	gaggugaugauggag-gug	MTND5
MCF-10Am-0025	chr8:57649272..57649312	-	12	gagguugaagugagaggu	MTND5
MCF-10Am-0026	chr22:35822054..35822114	+	9	guagguggccugacuggc	MT-CO1
MCF7-m001	chr2:189355167..189355246	+	15	gggguuuggcagagaugu	locus MT-OHR
MCF7-m002	chr22:35822054..35822114	+	7	guagguggccugacuggc	MT-CO1
MDA-MB-231-0001	chrM:8293..8364	-	27	cacuguaaagaggu-guugg	ATP8
MDA-MB-231-0006	chr1:568841..568912	-	27	cacuguaaagaggu-guugg	MT-TK
MDA-MB-231-0014	chr2:189355166..189355247	+	28	gggguuuggca-gagaugug	locus MT-OHR
MDA-MB-231-0032	chr5:134260622..134260688	+	54	guugguuagguaguugag	MTND5
MDA-MB-231-0035	chr5:134263745..134263786	-	44	auggccuagacuacguac	MTND4L
MDA-MB-231-0036	chr22:35822054..35822114	+	56	guagguggccugacuggc	MT-CO1

CONCLUSIONS

Identifying new mitomiRs may provide significant contributions and possibly enlighten the molecular mechanism underlying mitomiR biogenesis.

Acknowledgements: Authors would like to thank Prof. Dr. Gülseren Bağcı for her laboratory support and valuable scientific comments, Assoc. Prof. Dr. Onur Tokgun for contributing with his valuable scientific support and Msc.Serap Kurt for laboratory support.

Peer Review: Externally peer-reviewed.

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

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

Financial Disclosure: This study is supported by Pamukkale University Scientific Research Projects Department (Project No: 2014SBE011).

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RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1164334
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

EVALUATION OF MITOCHONDRIAL DNA MUTATIONS IN SIX FAMILIES BY RESEQUENCING ARRAY

MİTOKONDRİYAL DNA MUTASYONLARININ TEKRAR DİZİLEME ARRAY YÖNTEMİ İLE ALTI AİLEDE DEĞERLENDİRİLMESİ

Guyem KOLBAŞI DEMİRCİOĞLU[1,2], Sezen GÜNTEKİN ERGÜN[1,3], Kıvılcım GÜCÜYENER[4], Ferda E. PERÇİN[1], Mehmet Ali ERGÜN[1]

[1]Gazi University, Faculty of Medicine, Department of Medical Genetics, Ankara, Turkiye
[2]Bornova Health Medical Center, Izmir, Turkiye
[3]Hacettepe University, Faculty of Medicine, Department of Medical Biology, Ankara, Turkiye
[4]Gazi University, Faculty of Medicine, Department of Child Neurology, Ankara, Turkiye

ORCID IDs of the authors: G.K.D. 0000-0002-4622-5016; S.G.E. 0000-0002-7133-5049; K.G. 0000-0002-3390-2794; F.E.P. 0000-0001-9317-8155; M.A.E. 0000-0001-9696-0433

Cite this article as: Kolbasi Demircioglu G, Guntekin Ergun S, Gucuyener K, Percin FE, Ergun MA. Evaluation of mitochondrial DNA mutations in six families by resequencing array. J Ist Faculty Med 2023;86(1):78-87. doi: 10.26650/IUITFD.1164334

ABSTRACT

Objective: Human mitochondrial DNA is a circular, double stranded molecule which is inherited through maternal lineage. Point mutations in tRNA, rRNA or protein coding genes and structural rearrangements such as partial deletions or duplications can cause mitochondrial disorders. The prevalence of mitochondrial diseases is estimated to be 1/5000 worldwide. For the analysis of mtDNA mutations, Sanger sequencing, Southern blot, long and quantitative PCR, Resequencing Array and next-generation sequencing methods can be used. In this study, we analysed whole mitochondrial genomes of six children (along with their mothers) who were admitted to Gazi University Hospital with symptoms suggestive of mitochondrial disease.

Materials and Methods: After the extraction of genomic DNA from six children and their mothers, mtDNA resequencing with the analysis of obtained data was performed. In order to determine whether one of the mutations found in Patient 4 was homoplasmic or heteroplasmic, PCR and RFLP techniques were also used.

Results: Among six patients included in this study group, none of the variants detected could be attributed to any mitochondrial diseases, except the pathogenic mutation detected in Patient 4. The m.3460 G>A mutation detected in Patient 4 was located in the *MT-ND1* gene that was known to be responsible for LHON. This mutation detected in Patient 4 was also detected both in his mother and sister with homoplasmic state. The lack of clinical findings in his mother and sister was thought to be due

ÖZET

Amaç: İnsan mitokondriyal DNA'sı, maternal kalıtılan dairesel, çift sarmallı bir moleküldür. tRNA, rRNA veya protein kodlayan genlerdeki nokta mutasyonları ve kısmi delesyonlar veya duplikasyonlar gibi yapısal yeniden düzenlemeler mitokondriyal bozukluklara neden olabilir. Mitokondriyal hastalıkların dünya genelinde prevalansının 1/5000 olduğu tahmin edilmektedir. MtDNA mutasyonlarının analizi ile ilgili olarak Sanger dizileme, Southern blot, kantitatif PCR, tekrar dizileme ve yeni nesil dizileme yöntemleri kullanılabilir.

Gereç ve Yöntem: Bu çalışmada, mitokondriyal hastalığı düşündüren semptomları olan ve Gazi Üniversitesi Hastanesi'ne başvuran 6 çocuk ve annesinden genomik DNA'nın elde edilmesinin ardından mtDNA yeniden dizileme yöntemi ile elde edilen verilerin analizi yapıldı. Dördüncü hastada bulunan mutasyonlardan birinin homoplazmik veya heteroplazmik olduğunu belirlemek için PCR-RFLP tekniği kullanıldı.

Bulgular: Çalışma grubumuza dahil edilen altı hastadan 4. hastada saptanan patojenik mutasyon dışında, saptanan değişikliklerin hiçbiri hastalarımızdaki mitokondriyal hastalık ile ilişkilendirilmemiştir. Dördüncü hastada saptanan m.3460 G>A mutasyonu: *MT-ND1* geninde lokalize olup LHON'dan sorumludur. Dördüncü hastada saptanan bu mutasyon, hastanın anne ve kız kardeşinde de homoplazmik olarak saptandı. Anne ve kız kardeşinde klinik bulgu olmamasının, kadınlarda hastalığın penetransının azalmasına ve nükleer genomdaki genlerin modifiye edilmesine bağlı olduğu düşünüldü.

Corresponding author/İletişim kurulacak yazar: Mehmet Ali ERGÜN – maliergun@gmail.com

Submitted/Başvuru: 19.08.2022 • **Revision Requested/Revizyon Talebi:** 14.09.2022 •
Last Revision Received/Son Revizyon: 10.10.2022 • **Accepted/Kabul:** 13.10.2022 • **Published Online/Online Yayın:** 26.01.2023

to decreased penetrance of the disease in females and modifying genes in nuclear genome.

Conclusion: Screening of mtDNA using resequencing method could provide fast, effective and more reliable results in the diagnosis of mitochondrial diseases. Also, currently, the NGS technology analysis of nuclear DNA along with mtDNA will provide more reliable results in diagnosis of mitochondrial diseases, thus allowing more accurate genotype-phenotype correlation.

Keywords: Mitochondrial DNA, mutation, Resequencing Array

Sonuç: mtDNA'nın yeniden dizileme yöntemiyle taranması mitokondriyal hastalıkların tanısında hızlı, etkili ve daha güvenilir sonuçlar sağlayabilir. Ayrıca, şu anda NGS teknolojisi ile nükleer DNA'nın mtDNA ile birlikte analiz edilmesi mitokondriyal hastalıkların tanısında daha güvenilir sonuçlar verecek ve böylece daha doğru genotip-fenotip korelasyonuna izin verecektir.

Anahtar Kelimeler: Mitokondriyal DNA, mutasyon, array

INTRODUCTION

Mitochondria are double-membraned organelles in eukaryotic cells that mainly function in oxidative phosphorylation and production of ATP by transportation of electrons through the electron transport chain. They also have a role in cellular signalling, apoptosis, beta oxidation and lipid and cholesterol synthesis (1). Each cell contains approximately 500-2,000 mitochondria; tissues with high energy demand, such as extrinsic eye muscles, heart muscle and neurons, contain a greater number of mitochondria compared to other tissues (2).

Mitochondrial proteins are encoded by mitochondrial and nuclear genomes. Human mitochondrial DNA (mtDNA) is a circular, double stranded molecule consisting of 16569 bp, which is inherited through maternal lineage. The mitochondrial genome encodes 22 transfer RNAs (tRNA), 2 ribosomal RNAs (rRNA), 13 proteins and subunits of respiratory chain complexes I, III, IV and V, while the remaining OXPHOS proteins, along with other proteins necessary for mitochondrial metabolism and maintenance, are encoded by the nuclear genome and transferred to the mitochondria through special import systems (3). MtDNA is highly polymorphic, so that variations can be observed even among people of the same ethnic group. Particular combinations of mtDNA variations observed in an individual form a haplogroup, which are thought to cause differences in terms of oxidative phosphorylation capacity and formation of reactive oxygen radicals (1, 4). The mtDNA has about a 10-17-fold high mutation rate compared to the nuclear genome, partly due to the absence of protective histones and the existence of endogenous reactive oxygen species. Some deleterious mtDNA mutations are homoplasmic, while others are found in the heteroplasmic state. The ratio of wild type to mutant mtDNA determines the phenotype. Clinical symptoms and tissue dysfunction occur after the mutant mtDNA rate reaches the threshold level, which varies among tissue types according to dependency on the OXPHOS metabolism (3). Point mutations in tRNA, rRNA or protein coding genes and structural rearrangements such as partial deletions or duplications can cause mitochondrial disorders. Mutations in the nuclear genome can also cause mitochondrial disorders by interfering with mtDNA replication, repairment or mitochondrial function (5). Although the exact prevalence of mitochondrial diseases cannot be ascertained, it is estimated to be about 1/5000 worldwide (6).

Regarding the analysis of mtDNA mutations, Sanger sequencing, Southern blot and long and quantitative PCR had been used. Resequencing Array has been reported to be faster and less expensive, allowing better resolution with respect to DNA sequencing (7). However, as these technologies are expensive and are limited in speed, throughput and sensitivity, next-generation sequencing (NGS) can also be used (8). Recently, long-read sequencing has been performed for the whole sequence of mtDNA molecules (9).

In this study, we analysed whole mitochondrial genomes of 6 children (and their mothers) who were admitted to Gazi University Hospital between 2010-2012 and had symptoms suggestive of mitochondrial disease, using a microarray-based resequencing method.

MATERIALS AND METHOD

Six children who were clinically diagnosed with mitochondrial diseases at the Gazi University Department of Pediatrics, Division of Child Neurology and their mothers were included in this study. Mitochondrial disease diagnostic criteria that were used in this study were Nijmegen Clinical Criteria for mitochondrial diseases (10, 11). Affymetrix Mitochip v2.0, a microarray-based resequencing method, was used for detection of variants. This study was accepted by the Local Ethics Committee of Gazi University Faculty of Medicine (Date: 29.09.2010, No:135). This study was supported by Gazi University Scientific Research Projects Coordination Unit (01/2011-54).

DNA isolation

After written consent was obtained from the patients and their mothers, 5 ml of venous blood was withdrawn from their antecubital veins. Genomic DNA was extracted using the high concentration salt precipitation method (12). The concentration of the DNA samples was measured using a spectrophotometer.

Human MtDNA resequencing

Genomic DNA of 50 ng/µl was used for one Long-Range PCR. After PCR reaction, the products were checked on a 1% agarose gel. The PCR products were purified and quantified using a spectrophotometer. Then, the fragmentation reaction was performed and checked on a 4% agarose gel. After labelling, the samples were loaded into the array and the hybridisation procedure was performed for 16 h in a hybridisation oven. Finally, the array was washed using the GeneChip® Fluidics Station 450 and scanned using the GeneChip® Scanner 3000. After scanning of the array, data analysis was performed with the GSEQ 4.1 program (Affymetrix®). Raw data (files with cell extension) was converted to an analysable file format (chp extension). Obtained sequences were then compared to revised Cambridge Reference Sequence (rCRS), which is the reference mtDNA sequence, and mtDNA variants were defined (13). All the variants detected in the patients were homoplasmic.

In this study, we did not analyse the nuclear genomes of the patients and their mothers.

Analysis of obtained data

The variants were named based on Mitomap online database (https://www.mitomap.org/). In order to interpret mtDNA variations, the American College of Medical Genetics (ACMG) and Association of Molecular Pathology (AMP) guidelines were used. The variants were classified into categories of Pathogenic, Likely Pathogenic, Uncertain Significance, Likely Benign and Benign (14-15). The incompatibility between some of the results of the patients and their mothers were due to the no-calls of the resequencing system.

PCR-RFLP analysis

In order to determine whether the m.3460G>A missense mutation found in Patient 4 was homoplasmic or heteroplasmic, the PCR and RFLP technique was used. After the isolation of DNA from Patient 4 with his mother and sister, forward and reverse primers with the BsaHI restriction enzyme were used. The primers were designed as follows: 5′-ATGGCCAACCTCCTACTCCT-3′ and 5′-GCGGTGATGTAGAGGGTGAT-3′.

RESULTS

In this study, we investigated mtDNA variations using the Mitochip resequencing method in patients that were clinically diagnosed with mitochondrial diseases. Since mitochondrial diseases are maternally inherited, mothers of the patients were also included in the study. Obtained raw data was converted into a suitable format using specific software, and all variants were classified based on Mitomap online database (www.mitomap.org) with ACMG/AMP guidelines.

Patient 1

A nine-month-old female patient was referred to the genetics department with hypotonia, muscle weakness and poor sucking. Her mother had a history of oligohydramniosis. A patent foramen ovale was detected by echocardiography. Cranial magnetic resonance (MRI) revealed that hemispheric grooves were prominent in anterior frontal and temporal lobes of the brain and myelinization was compatible with her age. Magnetic resonance (MRS) revealed minimal lipid and lactate peaks; persistence of this sign was considered compatible with neurometabolic diseases by child neurologists. The patient scored two points according to the mitochondrial disease scoring system. The missense variants detected in patient 1 and mother 1 are listed in table 1. No pathogenic mutations were detected either for the patient or her mother (table 1).

Patient 2

A four-month-old female patient was referred to our department with the prediagnosis of Leber Hereditary Optic Neuropathy (LHON), due to vision loss detected in routine examination. Routine metabolic tests were normal. Visual evoked potential was bilaterally not responsive. The patient scored two points according to the mitochondrial disease scoring system. The variants detected in patient 2 and mother 2 are listed in table 2. No pathogenic mutations were established in the patient or her mother (table 2).

Patient 3

This five years and three months old male patient's development was compatible with his age until the age of 4.5 years; at that age, he was referred to our department due to ataxia and difficulty in holding his head up and sitting up, all manifesting in a six months' period. Cranial MRI revealed increased density in cerebellum and basal ganglia, involvement in bilateral globus pallidus and cerebral crus, compatible with mitochondrial encephalomyelopathic diseases. MRS revealed hyperintense symmetrical signal alterations in bilateral globus pallidus in T2 and FLAIR sequences, elevated lactate peak and moderate cerebral atrophic changes compatible with mitochondrial metabolic disease. EEG revealed slow waves of 2 Hz in bilateral temporoparietal regions; there was no asymmetry between two hemispheres. Muscle biopsy specimen was stained irregularly with oxidase enzyme dyes (NADH, SDH, COX). Hearing was partially impaired in the left ear, and the right ear could not be evaluated due to epileptic jerks at the time of examination. Medical treatment was started with the prediagnosis of mitochondrial cytopathy. The patient scored six points according to the mitochondrial disease scoring system. The variants detected in patient 3 and mother 3 are listed in table 3. There were no pathogenic mutations in the patient or his mother (table 3).

Table 1: Variants detected in patient 1 and her mother

Variants	Patient 1	Mother 1	Locus	ClinGen pathogenicity
m.73A>G	+	+	D-LOOP	Benign
m.153A>G	+	+	D-LOOP	Benign
m.195T>C	+	-	D-LOOP	Benign
m.225G>A	+	-	D-LOOP	Benign
m.226T>C	+	-	D-LOOP	Likely benign
m.263A>G	+	-	D-LOOP	Benign
m.750A>G	+	-	MT-RNR1	Benign
m.1438A>G	+	-	MT-RNR1	Benign
m.1719G>A	+	-	MT-RNR2	Benign
m.3705G>A	+	-	MT-ND1	Benign
m.4769A>G	+	+	MT-ND2	Benign
m.6371C>T	+	+	MT-CO1	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8393C>T	+	+	MT-ATP8	Benign
m.8860A>G	+	+	MT-ATP6	Benign
m.11377G>A	+	-	MT-ND4	Benign
m.11719G>A	+	-	MT-ND4	Benign
m.12406G>A	+	-	MT-ND5	Benign
m.12705C>T	+	-	MT-ND5	Benign
m.13708G>A	+	-	MT-ND5	Benign
m.13966A>G	+	-	MT-ND5	Benign
m.14470T>C	+	-	MT-ND6	Benign
m.14766C>T	+	-	MT-CYB	Benign
m.15326A>G	+	-	MT-CYB	Benign
m.15927G>A	+	+	MT-TT	Likely benign
m.16189T>C	+	-	D-LOOP	Benign
m.16223C>T	+	+	D-LOOP	Benign
m.16278C>T	+	+	D-LOOP	Benign
m.16519T>C	+	+	D-LOOP	Benign

Patient 4

The patient was referred to our department at the age of 15 due to bilateral visual impairment. Visual impairment had started in the right eye, and the left eye was affected a month later. Ophthalmological examination revealed obscuration of bilateral optic disc margins. Medical history revealed that the patient's mother's grandmother also had bilateral visual loss at the age of 15. The patient scored 2 points according to the mitochondrial disease scoring system. The variants detected in patient 4 and mother 4 are listed in table 4.

The pathogenic m.3460G>A missense mutation was detected in patient 4 and his mother (table 4). After the detection of pathogenic mutation of m.3460G>A in patient 4 and his mother, the patient's sister was also analysed, and the same mutation was detected in his sister, as well. In order to determine the homoplasmic state of the mutation, PCR- RFLP were used. The primers mentioned in the Materials and method section were used to amplify a 215 bp PCR product. In order to genotype the amplified PCR products, BsaHI restriction enzyme was used at 37°C. The wild type allele (G) revealed two bands of 146 bp and 69 bp, whereas the mutant (A) allele revealed as 215 bp. This genotyping result confirmed that the patient, his sister and his mother had the G3460A mutation in homoplasmic state, with respect to two control subjects (figure 1).

Table 2: Variants detected in patient 2 and her mother

Variants	Patient 2	Mother 2	Locus	ClinGen pathogenicity
m.73A>G	+	+	D-LOOP	Benign
m.150C>T	+	+	D-LOOP	Benign
m.195T>C	+	+	D-LOOP	Benign
m.204T>C	+	+	D-LOOP	Benign
m.263A>G	+	+	D-LOOP	Benign
m.279T>C	+	+	D-LOOP	NR*
m.750A>G	+	+	MT-RNR1	Benign
m.961T>C	+	+	MT-RNR1	Likely benign
m.1119T>C	+	+	MT-RNR1	Likely benign
m.1438A>G	+	+	MT-RNR1	Benign
m.2706A>G	+	+	MT-RNR2	Benign
m.3497C>T	+	+	MT-RNR2	Benign
m.4769A>G	+	+	MT-ND2	Benign
m.5441A>G	+	+	MT-ND2	NR*
m.6221T>C	+	+	MT-CO1	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8860A>G	+	+	MT-ATP6	Benign
m.10398A>G	+	+	MT-ND3	Benign
m.11252A>G	+	+	MT-ND4	Benign
m.11719G>A	+	+	MT-ND4	Benign
m.13629A>G	+	+	MT-ND5	NR*
m.14766C>T	+	+	MT-CYB	Benign
m.15326A>G	+	+	MT-CYB	Benign
m.15346G>A	+	+	MT-CYB	Likely benign
m.15941T>C	+	+	MT-TT	Benign
m.16217T>C	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

Table 3: Variants detected in patient 3 and his mother

Variants	Patient 3	Mother 3	Locus	ClinGen pathogenicity
m.152T>C	+	+	D-LOOP	Benign
m.263A>G	+	+	D-LOOP	Benign
m.750A>G	+	+	MT-RNR1	Benign
m.1438A>G	+	+	MT-RNR1	Benign
m.2380C>T	+	+	MT-RNR2	NR*
m.2706A>G	+	+	MT-RNR2	Benign
m.4769A>G	+	+	MT-ND2	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.7094T>C	+	+	MT-CO1	NR*
m.7805G>A	+	+	MT-CO2	Likely benign
m.8860A>G	+	+	MT-ATP6	Benign
m.9797T>C	+	+	MT-CO3	NR*
m.15326A>G	+	+	MT-CYB	Benign
m.15670T>C	+	+	MT-CYB	Benign
m.16129G>A	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

Table 4: Variants detected in patient 4 and his mother

Variants	Patient 4	Mother 4	Locus	ClinGen pathogenicity
m.64C>T	+	-	D-LOOP	Benign
m.152T>C	+	+	D-LOOP	Benign
m.263A>G	+	+	D-LOOP	Benign
m.750A>G	+	+	MT-RNR1	Benign
m.827A>G	+	+	MT-RNR1	Benign
m.1438A>G	+	+	MT-RNR1	Benign
m.2442T>C	+	+	MT-RNR2	Likely benign
m.2706A>G	+	+	MT-RNR2	Benign
m.3460G>A	**+**	**+**	**MT-ND1**	**Pathogen**
m.3847T>C	+	+	MT-ND1	Likely benign
m.4769A>G	+	+	MT-ND2	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8674A>G	+	+	MT-ATP6	NR*
m.8860A>G	+	+	MT-ATP6	Benign
m.13188C>T	+	+	MT-ND5	NR*
m.13731A>G	+	+	MT-ND5	NR*
m.14766C>T	+	+	MT-CYB	Benign
m.15326A>G	+	+	MT-CYB	Benign
m.15930G>A	+	+	MT-TT	Benign
m.16126T>C	+	+	D-LOOP	Benign
m.16519T>C	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

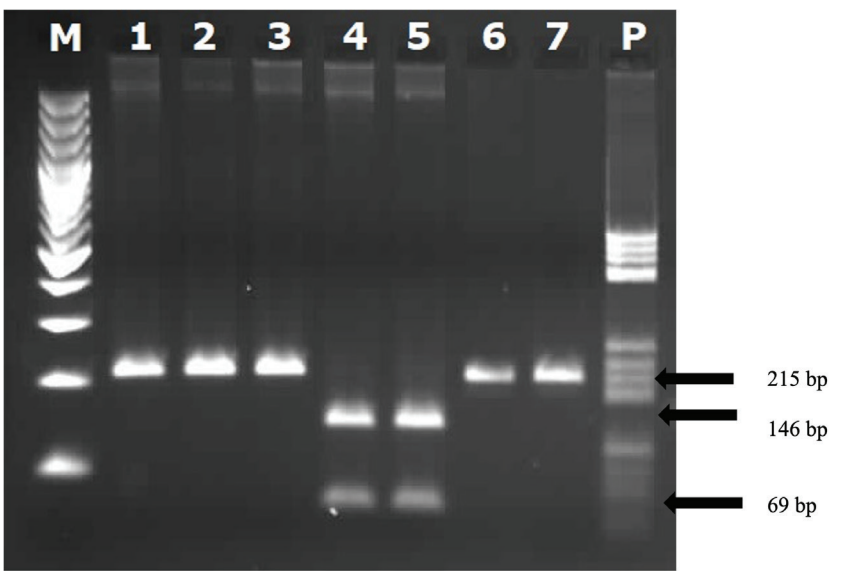

Figure 1: PCR-RFLP analysis of Patient 4, healthy sister and mother, after BsaHI restriction enzyme digestion. M: 100 bp marker; Lane 1: Patient, Lane 2: Sister, Lane 3: Mother, Lanes 4 and 5: control subjects, Lanes 6 and 7: uncut, Lane P: PBR3222 marker

Patient 5

A three-year-old female patient, who had no symptoms until the age of eight months, was referred to our department due to myoclonic jerks in extremities and seizures. The seizures were not responsive to antiepileptic treatment. Her developmental milestones were delayed; she could sit up at 10 months, walk at 17 months and say a few words at 2 years of age; she was still unable to form a sentence at the time of administration. Her liver edge was palpable 5 cm below the right costal margin. Metabolic screening tests revealed elevated urine lactic acid, 3-hydroxy butiric acid and acetoacetic acid. The patient scored 5 points in mitochondrial disease scoring system. The variants detected in patient 5 and mother 5 are listed in table 5. No pathogenic mutations were established in the patient or her mother (table 5).

Table 5: Variants detected in patient 5 and her mother

Variants	Patient 5	Mother 5	Locus	ClinGen pathogenicity
m.73A>G	+	+	D-LOOP	Benign
m.263A>G	+	+	D-LOOP	Benign
m.A512G	+	+	D-LOOP	NR*
m.709G>A	+	+	MT-RNR1	Benign
m.750A>G	+	+	MT-RNR1	Benign
m.1438A>G	+	+	MT-RNR1	Benign
m.1888G>A	+	+	MT-RNR2	Benign
m.2706A>G	+	+	MT-RNR2	Benign
m.4026A>G	+	+	MT-ND1	NR*
m.4216T>C	+	+	MT-ND1	Benign
m.4769A>G	+	+	MT-ND2	Benign
m.4917A>G	+	+	MT-ND2	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8697G>A	+	+	MT-ATP6	Benign
m.8860A>G	+	+	MT-ATP6	Benign
m.10463T>C	+	+	MT-TR	Benign
m.11251A>G	+	+	MT-ND4	Benign
m.11623C>T	+	+	MT-ND4	NR*
m.11719G>A	+	-	MT-ND4	Benign
m.12633C>A	+	-	MT-ND5	Benign
m.12634A>G	+	-	MT-ND5	Likely benign
m.13368G>A	+	+	MT-ND5	Benign
m.14034T>C	+	+	MT-ND5	NR*
m.14766C>T	+	+	MT-CYB	Benign
m.14905G>A	+	+	MT-CYB	Benign
m.15326A>G	+	+	MT-CYB	Benign
m.15452C>A	+	+	MT-CYB	Benign
m.15607A>G	+	+	MT-CYB	Benign
m.15928G>A	+	+	MT-TT	Benign
m.16126T>C	+	+	D-LOOP	Benign
m.16163A>G	+	+	D-LOOP	Benign
m.16186C>T	+	+	D-LOOP	Benign
m.16274G>A	+	+	D-LOOP	Benign
m.16294C>T	+	-	D-LOOP	Benign
m.16519T>C	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

Patient 6

This thirteen-year-old female patient was on antiepileptic treatment due to atonic seizures and the abnormal EEG. Medical history revealed she could walk at two years, after the operation she had due to bilateral hip dislocation, and she had ataxic gait. She started talking at the age of two but her speech was not fluent from the beginning; she had articulation defects and muttering. Deep tendon reflexes were hyperactive in the lower extremities and pathologic reflexes were present. CSF lactate level was elevated. Her condition was assessed as mild mental retardation according to psychometric evaluation. Cranial MRI revealed insignificant pathological signal increase in bilateral globus pallidus, predominant on the left side. MRS was normal. EMG revealed mixed type demyelinating neuropathy of sensorial and motor nerve fibres; needle EMG was consistent with myopathy. The patient scored five points according to the mitochondrial disease scoring system. The variants detected in patient 6 and mother 6 are listed in table 6. There were no pathogenic mutations in the patient or her mother (table 6).

Although mtDNA can be sequenced using the Sanger sequencing method, it can be inadequate to detect all the areas of mutation in mtDNA; since the percentage of heteroplasmic mutations, especially when analysed in mtDNA obtained from blood, can be under the threshold level of detection (18). Mitochondrial chip is a high throughput method, which helps to sequence all mitochondrial genomes and detect probable pathogenic variants with reasonable accuracy (19). The advantages of the Mitochip method over conventional sequencing are short analysis time and cost-effectivity. Furthermore, especially in heteroplasmic tissues, where normal and mutant mtDNA copies are both present, the microarray method can detect mutant alleles of even below 2% percentage, where detection with conventional sequencing requires a minimum allele percentage of approximately 30% (20). It is also used for haplotyping in population genetic studies (21). One of the disadvantages of microarray-based sequencing is that deletions or insertions cannot be detected with this method (19).

Table 6: Variants detected in patient 6 and his mother

Variants	Patient 6	Mother 6	Locus	ClinGen pathogenicity
m.263A>G	+	+	D-LOOP	Benign
m.480T>C	+	+	D-LOOP	NR*
m.750A>G	+	+	MT-RNR1	Benign
m.1438A>G	+	+	MT-RNR1	Benign
m.2706A>G	+	+	MT-RNR2	Benign
m.4655>A	+	+	MT-ND2	NR*
m.4769A>G	+	+	MT-ND2	Benign
m.7028C>T	+	+	MT-CO1	Benign
m.8860A>G	+	+	MT-ATP6	Benign
m.12192G>A	+	+	MT-TH	NR*
m.15115T>C	+	+	MT-CYB	Benign
m.15326A>G	+	+	MT-CYB	Benign
m.16311T>C	+	+	D-LOOP	Benign

*No records/ Not included in Mitomap's confirmed pathogenic mutations

DISCUSSION

The prevalence of mtDNA mutations among clinically affected patients is estimated to be approximately 1/5000 (6). Pathogenic mitochondrial DNA mutations result in mitochondrial DNA disorders, which are among the most common inherited human diseases (16). Both nuclear and mitochondrial genome mutations may cause mitochondrial diseases (17).

With the recent development, NGS is expected to become the method of choice for genetic analysis on mtDNA because it allows a rapid sequencing of the whole mtDNA with concurrent quantification of heteroplasmy levels for point mutations, down to low percentages (8).

Among six patients included in this study group, none of the variants detected in our study could be attributed to any mitochondrial diseases, except the pathogenic mutation detected in patient 4. The m.3460 G>A muta-

tion detected in patient 4 is located in the *MT-ND1* gene and it is one of the three primary mutations known to be responsible for LHON. Of all the LHON patients in the world, about 90% of the patients carry one of the mutations in nucleotides 11778, 3460 or 14484 (16). Incidence of these mutations are estimated to be 70, 15 and 10 percent for m.11778G>A, m.3460G>A and m.14484T>C, respectively. Heteroplasmy, secondary genomic or mitochondrial factors and environmental factors are suggested to affect the disease progression (22-23).

In patient 4, as the pathogenic m.3460G>A mutation was also detected in his sister and mother, the homoplasmic state of the mutation was confirmed by PCR- RFLP. Based on this finding, the lack of clinical findings in the mother and sister was thought to be due to decreased penetrance of the disease in females and modifying genes in nuclear genome. Also, the reason for this was thought to be the higher penetrance in males and possible existence of modifying loci in the nuclear genome (24). Since only 10% of female carriers exhibit LHON symptoms, whereas 50% of male carriers are affected, it was thought that other environmental or genetics factors that attribute to phenotypical expression must be present (25).

The small number of participants is one of the disadvantages of our study. In addition, the nuclear genome mutations that are associated with mitochondrial diseases could not be analysed in this study.

CONCLUSION

The MtDNA resequencing technique is a valuable diagnostic tool due to its reliability, short duration and the small quantity of required DNA. On the other hand, the heteroplasmy/homoplasmy state of detected mutations must be confirmed by a second method, such as PCR-RFLP, also used in this study. By increasing the number of studies like this one, a database for mitochondrial variants in the Turkish population could be founded. Also, currently, the NGS technology analysis of nuclear DNA along with mtDNA will provide more reliable results in diagnosis of mitochondrial diseases, thus allowing more accurate genotype-phenotype correlation.

Ethics Committee Approval: This study was approved by Gazi University Faculty of Medicine Institutional Review Board (Date: 29.09.2010, No: 135).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- G.K.D., K.G., F.E.P, M.A.E.; Data Acquisition- G.K.D., S.G.E.; Data Analysis/Interpretation- K.G., F.E.P., M.A.E.; Drafting Manuscript- G.K.D., K.G., F.E.P, M.A.E.; Critical Revision of Manuscript- G.K.D., S.G.E.; Final Approval and Accountability- G.K.D., K.G., F.E.P, M.A.E.; Material or Technical Support- G.K.D., K.G., F.E.P, M.A.E.; Supervision- K.G., F.E.P., M.A.E.

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

Financial Disclosure: This study was supported by Gazi University Scientific Research Projects Unit (Project number: 01/2011-54).

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RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1136469
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

AN EVALUATION OF RETROSPECTIVE RESULTS DETECTED IN THE HOSPITAL INFECTION RESEARCH LABORATORY

HASTANE ENFEKSİYONU ARAŞTIRMA LABORATUVARINDA SAPTANAN RETROSPEKTİF SONUÇLARIN DEĞERLENDİRİLMESİ

Niyousha MATLOOBI AGHDAM[1], Gülseren AKTAŞ[1]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkiye

ORCID IDs of the authors: N.M.A. 0000-0002-0688-6688; G.A. 0000-0002-1611-5289

Cite this article as: Matloobi Aghdam N, Aktas G. An evaluation of retrospective results detected in the hospital infections laboratory. J Ist Faculty Med 2023;86(1):88-94. doi: 10.26650/IUITFD.1136469

ABSTRACT

Objective: Hospitals are a potential source of infection risk during healthcare delivery. Since vancomycin-resistant enterococci (VRE) and carbapenemase producing Gram-negative rods cause persistent colonization and multi-drug resistant bacterial infections, they are important in nosocomial infections. In this study, we aimed to retrospectively evaluate rectal swab samples of patients hospitalized in the clinics in terms of VRE and carbapenem-resistant (CR) Gram-negative rods as nosocomial infection agents between 1st January 2020 and 31st December 2020.

Materials and Methods: Standard clinical laboratory methods were used to isolate and identify CR Gram-negative rods and VRE from rectal swab samples of hospitalized patients sent to our Hospital Infection Research Laboratory.

Results: There was growth in 777 (28.9%) of 2688 samples examined. Of the bacteria that grew, 627 (80.7%) were defined as VRE, and 150 (19.3%) as Gram-negative rods resistant to carbapenem. Seventy-five of these were defined as CR *K. pneumoniae*: 7 as CR K. oxytoca: 26 as CR *Enterobacter* species: 2 as CR *E. coli*: and 40 as CR *Acinetobacter* species. Vancomycin-resistant enterococci were detected most frequently in the internal medicine ward (56.3% - 353/627) and pediatric intensive care-neonatal ward (37.6% - 236/627). In pediatric services, 146 of total 296 bacteria isolated were identified as VRE.

Conclusion: With the surveillance studies carried out in the control of hospital infections, each health institution determines the microorganisms that make up its own hospital flora, their resistance status and their distribution. It is thought that the data we obtain will contribute to the infection control processes of the hospitals.

Keywords: Nosocomial infection, vancomycin-resistant enterococci, carbapenem-resistant bacteria, infection control measures

ÖZET

Amaç: Hastaneler, sağlık hizmeti sunumu sırasında potansiyel enfeksiyon riski kaynağıdırlar. Vankomisine dirençli enterokoklar (VRE) ve karbapenemaz üreten Gram-negatif çomaklar, kalıcı kolonizasyona ve çok ilaca dirençli bakteriyel enfeksiyonlara neden olduğundan hastane enfeksiyonlarında önemlidirler. Bu çalışmada, 1 Ocak 2020 / 31 Aralık 2020 tarihleri arasında kliniklerde yatan hastaların rektal sürüntü örneklerinin hastane enfeksiyonu etkeni olan VRE ve karbapenem-dirençli (KD) Gram-negatif çomaklar açısından retrospektif olarak değerlendirmesi amaçlanmıştır.

Gereç ve Yöntem: Hastanede yatan hastaların Hastane enfeksiyon Araştırma Laboratuvarı'na gönderilen rektal sürüntü örneklerinden KD Gram-negatif çomaklar ve VRE'yi izole etmek ve tanımlamak için standart klinik laboratuvar yöntemleri kullanılmıştır.

Bulgular: İncelenen 2688 örneğin 777'sinde (%28,9) üreme olmuştur. Üreyen bakterilerin 627'si (%80,7) VRE ve 150'si (%19,3) KD Gram-negatif çomaklar olarak tanımlanmıştır. Bunlardan 75'i KD *K. pneumonia*; 7'si KD *K. oxytoca*; 26'sı KD *Enterobacter* türleri; 2'si KD *E. coli*; ve 40 suş KD *Acinetobacter* türü olarak belirlenmiştir. Vankomisine dirençli enterokoklar en sıklıkla dahiliye servisinde (%56,3 - 353/627) ve pediatrik yoğun bakım-yenidoğan servisinde (%37,6 - 236/627) tespit edilmiştir. Pediatri servislerinde toplam 296 bakterinin 146'sı VRE olarak tanımlanmıştır.

Sonuç: Hastane enfeksiyonlarının kontrolünde yapılan sürveyans çalışmaları ile her sağlık kuruluşu kendi hastane florasını oluşturan mikroorganizmaları, direnç durumlarını ve dağılımlarını belirlemektedir. Elde edilen verilerin hastanelerin enfeksiyon kontrol süreçlerinde katkı sağlayacağı düşünülmektedir.

Anahtar Kelimeler: Hastane enfeksiyonu, vankomisine dirençli enterokoklar, karbapeneme dirençli bakteriler, enfeksiyon kontrol önlemleri

Corresponding author/İletişim kurulacak yazar: Gülseren AKTAŞ – gulserena2001@yahoo.co.uk

Submitted/Başvuru: 04.07.2022 • **Revision Requested/Revizyon Talebi:** 21.07.2022 •
Last Revision Received/Son Revizyon: 25.07.2022 • **Accepted/Kabul:** 22.11.2022 • **Published Online/Online Yayın:** 23.01.2023

INTRODUCTION

Nosocomial infections (NI) are a significant health problem that is now on our agenda with the developments in medicine, moreover it is a problem that concerns the whole world (1). Surveillance studies were initiated in hospitals in developed countries in the 1960s. In the United States of America (USA) in 1987, a series of definitions were introduced by the Infection Control and Prevention Centers (CDC) which were to be applied in hospitals participating in the National Nosocomial Infection Surveillance (NNIS) to determine the presence of nosocomial infection (NI). The implementation of the detected infection identification has also started. These definitions were used later all over the world (2).

The emergence of this problem in our country occurred in the 1990s. In 2005, it became mandatory to establish infection control committees in inpatient institutions (3). Between 450,000 and 700,000 patients experience at least one NI during their hospital stay. Therefore, monitoring and surveillance of NI are considered a very important activity for prevention and control programs (4). The records of the microbiology laboratory are the main source of surveillance. Therefore, microbiological and immunological reports from the laboratory constitute the starting point of research on endemic and epidemic nosocomial infections.

Nosocomial infections, also called hospital-acquired infections, can occur 48 hours after hospitalization. These infections can sometimes occur after the patient has been discharged. A patient is exposed to various microorganisms during a hospital stay. The probability of these microorganisms causing infection depends in part on the microorganisms' resistance to antimicrobial agents, virulence factors, and characteristics such as bacterial inoculum (5).

Health services are an environment where both infected people and people with a high risk of infection come together. Patients with infections or pathogenic microorganisms admitted to the hospital become a potential source of infection for other patients and staff if this is left uncontrolled.

Enterococci are inherently resistant to a variety of antimicrobials and have been recognized as one of the important nosocomial pathogens, due both to their virulence properties and their ability to acquire multiple antibiotics resistance (MAR) (6). Vancomycin has been the most reliable antibiotic used in the treatment of enterococcal infections with MAR. The emergence of vancomycin resistance in enterococci in the late 1980s made the treatment of these infections is a serious problem. The hospital environment is ideal for the growth of resistant Gram-negative bacteria due to the selective pressure of antibiotics. The seriousness of NI caused by such bacteria increases, and their treatment becomes difficult (7). Carbapenems have long been recognized as the most active and potent agents against MAR Gram-negative pathogens (8). However, due to life-threatening infections caused by MAR microorganisms, the use of carbapenem antibiotics is restricted, and treatment of NI with existing antibiotics becomes difficult (9).

Hospital infections constitute an important health problem in our country as well as all over the world. Surveillance studies are essential in ensuring the control of nosocomial infections. Thus, microorganisms forming the hospital's own flora and resistance patterns are determined. Each hospital determines its specific infection rates in line with the infection factors and rates obtained. It should identify high-risk services, take infection control measures accordingly, and rearrange the training programs of healthcare personnel according to their needs. Knowing each center's own patient profile, microorganisms that make up the hospital flora, their resistance patterns, along with the distribution and frequency of nosocomial infections in each unit enable the development of correct prevention strategies (10-12).

In the study, it was aimed to evaluate the retrospectively rectal swab samples, which were examined in the Hospital Infections Research Laboratory between 1 January 2020 and 31 December 2020 in terms of vancomycin-resistant enterococci (VRE) and carbapenem-resistant (CR) Gram-negative rods, which are important nosocomial infection factors.

MATERIAL AND METHODS

Cultivation of specimens

Rectal swab samples taken from the patients were sent to our laboratory with transport medium (Stuart transport medium, Letsswab, Turkey). Bile Esculin Azide agar (BEA) (Bile Esculin Azide Agar, Biolab Zrt., Hungary) supplemented with 6 mg/L vancomycin (Vancomycin HCL, Multicell) as vancomycin resistance screening medium and MacConkey agar (BBL ™ MacConkey Agar, BD; Becton, Dickinson and Company, USA) media supplemented with 3 mg/L meropenem (Meropenem Trihydrate, Tokyo Chemical Industry Co., LTD. Japan) as the screening medium of carbapenem resistance were used (13-14). Samples from Pediatric Intensive Care-Neonatal units were planted on both BEA agar with vancomycin for VRE and MacConkey agar with meropenem for the screening of bacteria producing carbapenemase, while samples from other clinics were only planted on BEA media with vancomycin for the screening of VRE.

After planting, all media were incubated for 24 hours in a 35 °C incubator, and the results were evaluated according to CLSI criteria (15-16). The control of the antibi-

otic discs used in the experiments was performed with the *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 standard strains. This study was approved by the ethics committee of Istanbul University, Istanbul Faculty of Medicine (Date: 13.11.2020, No: 196733).

Enterococcus identification

A Gram-stained and catalase test was performed to determine if there was a growth that hydrolyzed esculin in the BEA agar medium. Colonies with Gram-positive cocci and catalase-negative results were evaluated as enterococci. The PYR test was used for enterococcal confirmation. A disc diffusion sensitivity test was performed on these growths with vancomycin and teicoplanin antibiotics (13).

Identification of Gram-negative rods

To identify Gram-negative rods grown on MacConkey agar media supplemented with meropenem, triple sugar iron agar (TSI) (Triple Sugar Iron agar, BD; Becton, Dickinson and Company, USA), Motility-Indole-Ornithine (MIO) (Biolab Zrt., Hungary), Clark-Lubs (CM0043, MRVP Medium, OXOID LTD., England) and DNase agar (DNase Agar, Biolab Zrt., Hungary), the bacteria were identified by investigating various biochemical and enzyme properties of the strains (17-18). *Stenotrophomonas maltophilia* bacteria were not reported because they are naturally resistant to carbapenems (14).

Determination of sensitivity to antibiotics

Antimicrobial susceptibilities were determined by the disk diffusion method, and the results were evaluated according to CLSI criteria (14-16, 19). For the susceptibility test, 5-10 colonies were taken from each petri dish and inoculated into Mueller-Hinton broth (Mueller Hinton Broth, BD; Becton, Dickinson and Company, USA) medium, and the bacterial suspension was brought to the standard turbidity of McFarland and its pure culture was prepared. The pure culture of each strain was then homogeneously spread on the surface of the Mueller-Hinton agar (Mueller-Hinton II Agar, Biolab Zrt., Hungary) medium. Vancomycin (30 µg) (Oxoid Ltd., UK) and teicoplanin (30 µg) (Oxoid Ltd., UK) for *Enterococcus* strains, imipenem (10 µg) (Oxoid Ltd., UK), meropenem (10 µg) (Oxoid Ltd., UK) and ertapenem (10 µg) (Oxoid Ltd., UK) for Gram-negative rods were used. Antibiogram plates were incubated for 24 hours in a 35 °C incubator. The control of the antibiotic discs used in the experiments was performed with the *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 standard strains.

RESULTS

In total, 2688 rectal swab samples from various services of the university hospital in a one-year period between the first of January 2020 and December 31, 2020 were evaluated. In total, 1068 were female, and 1620 were male. It was determined that 895 of these samples belonged to pediatric patients and 1793 to adult patients. Of the adult patients, 713 were female, and 1080 were male (table 1).

The distribution of VRE growths detected in all rectal swab samples according to the clinics and the distribution of the isolated CR Gram-negative bacteria in the genus and species levels are shown in table 2.

Gram-negative rods producing carbapenemase were investigated only in the 741 samples sent from all Pediatrics Intensive Care and Neonatal units (including Intensive Care, Level 1, Level 2, Level 3, and Pediatrics Surgery). Vancomycin-resistant enterococci and/or CR Gram-negative rods growth was detected in 777 of the total samples (29% - 777/2688).

Accordingly, out of a total of 741 (27.5% - 741/2688) samples sent from the Pediatrics Intensive Care-Neonatal wards, growths were detected in 236 (31.8% - 236/741) of these samples as both 86 (11.6% - 86/741) VRE and 150 (20% - 150/741) CR Gram-negative rods (table 1, 2). As well as Pediatrics Intensive Care-Neonatal services, data from other services, including Pediatrics Infection; Pediatrics-Pandemic Intensive Care; Pediatrics-Emergency; Pediatrics-Nutrition; Pediatrics Gastroenterology; Pediatrics-Hematology and Oncology; Pediatrics-Cardiology; Pediatrics-Nephrology; Pediatrics-Neurology; Pediatrics-Special and Pediatrics-Clean were collected under Other Pediatrics services and are shown in table 1 (1). In total, 154 samples from Other Pediatrics services were investigated for VRE, and growth was detected in 60 samples (39% - 60/154). A total of 139 samples from various surgical services grouped under general Surgery

Table 1: Distribution of all examined rectal swab samples by clinics and gender

Sending services	Number of samples	Female	Male
1. Other pediatrics	154	67	87
2. Pediatrics Intensive Care-Neonatal	741	288	453
3. Surgical services	139	54	85
4. Internal medicine	1290	519	771
5. Intensive Care-Emergency Trauma	345	130	215
6. Other services	19	10	9
Total	2688	1068	1620

Table 2. Distribution of the growths of total VRE according to the services and CR rods

Units	VRE (627) (%)	CR Gram-negative rods (150)				
		K. pneumoniae	*K. oxytoca*	*Enterobacter* spp.	*E. coli*	*Acinetobacter* spp.
1. Other pediatrics	60 (9.6)	--	--	--	--	--
2. Pediatrics Intensive Care Neonatal	86 (13.7)	75	7	26	2	40
3. Surgical services	34 (5.4)	--	--	--	--	--
4. Internal Medicine	353 (56.3)	--	--	--	--	--
5. Intensive Car Emergency Trauma	88 (14)	--	--	--	--	--
6. Other services	6 (1)	--	--	--	--	--
Total	627	75	7	26	2	40

VRE: Vancomycin-resistant enterococci; CR: Carbapenem-resistant.

Table 3. Distributions of CR Gram-negative rods growth together with VRE strains in Pediatrics Intensive Care-Neonatal wards

Units	Number of samples	VRE and CR Gram-negative rods				
		K. pneumoniae	*K. oxytoca*	*Enterobacter* spp.	*E. coli*	*Acinetobacter* spp.
2. Pediatrics Intensive Care - Neonatal	19	12	1	3	1	2

VRE: Vancomycin-resistant enterococci; CR: Carbapenem-resistant.

Services (table 1) such as Chest surgery and Brain surgery were studied, and the growth of VRE was detected in 34 (24.5% - 34/139) samples. Vancomycin-resistant enterococci were detected in 353 of a total of 1290 samples (27.4% - 353/1290) sent from various services of Internal Medicine (table 1). A total of 345 samples were sent from various other Intensive Care-Emergency Trauma services were studied and VRE growth was detected in 88 (25.5% - 88/345) samples (table 1). The total number of samples coming from Other Services including Skin and Venereal Diseases, Chest Diseases, Eye Diseases, Clinical Microbiology outpatient clinic, Gynecological Oncology, Ear-Nose-Throat A-service, Neurology Dr. Edip Aktin Stroke service, Urology service was 19 and the growth of VRE was detected in 6 (31.6% - 6/19) samples (table 1).

In total, 627 VRE strains (23.3% - 627/2688) were isolated from all services. The VRE strains were isolated mostly in Internal Medicine Service with 353 (56.3% - 353/627), then 88 (14% - 88/627) in Intensive Care-Emergency Trauma, 86 (13.7% - 86/627) in Pediatrics Intensive Care-Neonatal, then 60 (9.6% - 60/627) in Other Pediatrics services, 34 (5.4%, 34/627) in Surgical Services and 6 VRE strains (1%, 6/627) in Other Services (table 2). Additionally, 150 CD Gram-negative rods were isolated from the samples of Pediatrics Intensive Care-Neonatal Wards. The distributions of these strains were as follows: 75 CR *K. pneumonia*, 7 CR *K. oxytoca*, 26 CR *Enterobacter* species, 2 CR *E. coli* and 40 CR *Acinetobacter* species (table 2).

In 19 rectal swab samples sent from Pediatrics Intensive Care - Neonatal wards, both VRE and CR Gram-negative rod growth were isolated together (table 3). In 12 of these samples, CR *K. pneumoniae* with VRE; In 1 example, VRE and CR *K. oxytoca*; VRE and CR *Enterobacter* species in 3 samples; VRE and *E. coli* in 1 example; In 2 samples, VRE and *Acinetobacter* species were isolated together.

DISCUSSION

Hospitals are a potential source of infection transmission risk during healthcare delivery (20-21). Vancomycin-resistant enterococcal infections are among the leading causes of healthcare-associated infections (22-24). Surfaces and medical devices in the rooms of patients colonized and/or infected with vancomycin-resistant enterococci are fre-

quently contaminated by this microorganism and form an important VRE reservoir within the hospital (2, 22).

In a study conducted in Germany, it was reported that 263 VR-*Enterococcus faecium* colonization were detected among 16350 patients hospitalized in six university hospitals between 2014 and 2018. In addition, it was also reported that VR-*E. faecium* prevalence rates increased from 0.8% to 2.6% in a five-year period (25). Carbapenem-resistant *Enterobacteriaceae* (CRE) poses a serious public health threat worldwide. Carbapenem-resistant *Enterobacteriaceae* infections appear rapidly and cause serious difficulties in treatment (26). One of the most important nosocomial pathogens from the *Enterobacteriaceae* family in the world is *Klebsiella pneumonia* (27-28). In other studies, risk factors for colonization or infection with CR *K. pneumoniae* (CRKP) in long-term intensive care hospitals have been evaluated. It has been reported that solid organ or hematopoietic stem cell transplantation, mechanical ventilation and lack of patient stool control are important risk factors for infection or colonization with CRKP (29). In a CRE prevalence study, 150 stool swabs from 150 patients from intensive care units and hematopoietic stem cell transplant services were examined. Among them, 25 (16.6%) CRE strains were isolated. Of the 25 CRE strains isolated, 17 (65.3%) were *K. pneumoniae*, 6 (23%) *Escherichia coli*, 1 (3.8%) *Citrobacter freundii* and one (3.8%) were defined as *Enterobacter* species (30). In another study conducted at a university hospital in China, CRE was investigated in 704 stool samples. Samples were taken from the Central Intensive Care Unit (CICU), Traditional Chinese Medicine Service (TCMS), pediatric service, and 62 other services. A total of 60 (8.5%) CRE were isolated from 704 stool samples examined. Of these, 42 (5.9%) were identified as *K. pneumoniae*, 7 *E. coli*, 3 *C. freundii*, 3 *K. oxytoca* , 3 (0.4%) *Enterobacter cloacae* and 1 (0.1%) *Enterobacter aerogenes* (12). In a research study conducted in a university hospital in our country to determine nosocomial infections and their causative agents to determine local data, in total 112 nosocomial infection agents had been isolated from 97 of 3254 patients (3.5% - 112/3254) who were hospitalized, followed up and treated in various services between January 2009 and March 2010. The most frequently isolated microorganisms causing the infections were followed by *Acinetobacter baumannii* 23.2%, *Klebsiella* spp. 20.5%, *E. coli* 19.6% and *Pseudomonas* spp. 11.6%. In the same study, it was reported that the rate of resistance to carbapenem antibiotics was quite high. In addition, *E. faecium* was detected at a rate of 9.2% (1). In another research study from Turkiye, meropenem resistance was reported at a rate of 5.7% in 53 *K. pneumoniae* strains isolated from various specimens of patients diagnosed with nosocomial infections in various clinics between 2011 and 2013 (28). Additionally, it was reported that the *Acinetobacter* genus was increasingly identified as a nosocomial infection agent, especially in patients in intensive care units (31-32). Şahin AR et al. investigated the epidemiology of *A. baumannii* and antimicrobial resistance in various clinical samples were taken from patients who were hospitalized and diagnosed with nosocomial infections between 2012 and 2017 (10). The most common isolation of it was from the anesthesia and reanimation intensive care units with 58.9% (284), then 21.7% (105) from internal medicine intensive care units, 9.5% (46) in neurology, 5.3% (26) in general surgery, 3.9% (19) in neurosurgery and in coronary intensive care units with 0.2% (1). It was reported that imipenem, meropenem and ertapenem discs and carbapenem resistance was detected in over 97% of these strains.

In our study, the rate of hospital infection was found to be 28.9% (777/2688) in the retrospective evaluation made between the 1st of January 2020 and December 31st, 2020. The distribution rate of this reproduction (777) according to services was as follows: Internal Diseases 45.43% (353/777), Pediatrics Intensive Care - Neonatal Services 30.37% (236/777) (150 CR bacteria + 86 VRE), Intensive Care Emergency Trauma Services 11.32% (88/777), 7.72% (60/777) in Other Pediatrics Services, 4.37% (34/777) in all Surgery Services, and 0.77% (6/777) in Other Services. Microorganisms causing nosocomial infections in Pediatrics Intensive Care-Neonatal Services were 86 VRE strains isolated from 741 samples of this unit and 150 CR Gram-negative rods as 75 CR *K. pneumoniae*, 7 CR *K. oxytoca*, 26 CR *Enterobacter* spp., 2 CR *E.coli* and 40 CR *Acinetobacter* spp.

Hospital-acquired *K.pneumoniae* is the main source of carbapenemase producing *Enterobacteriaceae* (CPE) infection in Europe. As a result, the emergence and spread of antibiotic resistance to last-line antibiotics, the opportunities to treat successfully CPE-infected patients in countries with a high prevalence of CPE have become less and less. However, it does not seem possible to introduce new and effective antibiotics to healthcare in a short time. Aims should include the prevention of cross-contamination, reduction of healthcare-associated infections by strictly following infection control measures, and infection control training should be planned for this target (33).

CONCLUSION

Nosocomial infections constitute an important health problem in the whole world. Surveillance studies are essential in ensuring the control of these infections. Thus, microorganisms forming the hospital's own flora and resistance patterns have been determined. Nosocomial infection rates differ between regions, countries and even hospitals. In line with the infection factors and rates obtained, each hospital should determine its own specific infection rates, determine high-risk services, take infection control measures accordingly, and rearrange

the training programs of health personnel according to the needs. It is essential for surveillance studies to keep the spread of infections to a minimum in the nosocomial infections control studies reducing the transmission and the spread of multiple antimicrobial resistant microorganisms and thus preventing epidemics. The fact that each center knows its own patient profile, the microorganisms that make up the hospital flora, their resistance patterns, the distribution and frequency of nosocomial infections in each unit, enables the development of correct prevention strategies.

Ethics Committee Approval: This study was approved by Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 13.11.2020, No: 28).

Peer Review: Externally peer-reviewed.

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

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

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

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RESEARCH / ARAŞTIRMA
DOI: 10.26650/IUITFD.1197098
İst Tıp Fak Derg 2023 / J Ist Faculty Med 2023

EVALUATION OF THE TREATMENT EFFICACY OF TIGECYCLINE AND REISHI SHIITAKE MAITAKE MUSHROOM EXTRACT IN MICE WITH THE VISCERAL LEISHMANIASIS MODEL

VİSERAL LAYŞMANYAZ MODELİ OLUŞTURULAN FARELERDE TİGESİKLİN VE REİSHİ SHİİTAKE MAİTAKE MANTAR EKSTRESİNİN TEDAVİ ETKİNLİĞİNİN DEĞERLENDİRİLMESİ

Özden BORAL[1], Deniz Gözde ÇELİK-PAYÇU[2], Halim İŞSEVER[3]

[1]Istanbul University, Istanbul Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkiye
[2]Medical University of Plovdiv, Plovdiv, Bulgaria
[3]Istanbul University, Istanbul Faculty of Medicine, Department of Public Health, Istanbul, Turkiye

ORCID IDs of the authors: Ö.B. 0000-0001-7145-1418; D.G.Ç.P. 0000-0002-9887-0332; H.İ. 0000-0002-5435-706X

Cite this article as: Boral O, Celik-Paycu DG, Issever H. Evaluation of the treatment efficacy of tigecycline and reishi shiitake maitake mushroom extract in mice with the visceral leishmaniasis model. J Ist Faculty Med 2023;86(1):95-102. doi: 10.26650/IUITFD.1197098

ABSTRACT

Objective: Visceral leishmaniasis (VL) is an infection that can be fatal if left untreated. Treatment of VL is becoming increasingly difficult due to the development of resistance to some drugs used. We aimed to investigate the efficacy of tigecycline (Tig) and Reishi-Shiitake-Maitake (RSM) mushroom extract alone and in combination in BALB/c mice infected with the *Leishmania donovani* strain (ATCC 30030).

Materials and Methods: To compare the treatment efficacy, the mice that were treated with amphotericin B (AMB) were used as the control group. BALB/c mice (n=40) were intravenously inoculated in the lateral vein of the tail with 10^7 stationary-phase *L. donovani* promastigotes in 100 µL of PBS. BALB/c mice were divided into 5 groups of 8. Tig group received 3.7 mg/kg tigecycline intraperitoneally for 5 days, RSM group received 10 mg/kg RSM extract by oral gavage for 5 days while Tig+RSM group received the same doses of both drugs via the same routes. Also, the AMB group received 15mg/kg amphotericin B by oral gavage for 5 days. The spleen and liver of all mice that were sedated with ketamine were collected on the 12th day. Parasite load was determined by Leishman Donovan Unit (LDU) and quantitative RT-PCR.

Results: When all groups were statistically evaluated according to LDU and RT-PCR findings, the lowest value was obtained in the AMB group compared to the value in the control group, while the second lowest value was obtained in the Tig+RSM group. The data obtained in the Tig+ RSM group were significantly lower

ÖZET

Amaç: Viseral layşmanyaz (VL) tropikal ve subtropikal bölgelerde yayılım gösteren, tedavi edilmediğinde ölümcül olabilen bir enfeksiyondur. VL tedavisi kullanılan bazı ilaçlara direnç gelişimi nedeni ile giderek güçleşmektedir. Çalışmamızda *Leishmania donovani* (ATCC 30030) suşu ile viseral layşmanyaz modeli oluşturulan BALB/c farelerde, tigesiklin (Tig), Reishi-Shiitake-Maitake (RSM) mantar ekstresi ve her iki ilacın birlikte tedavi etkinliğinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Tedavi etkinliğinin karşılaştırılmasında amfoterisin B (AMB) ile tedavi edilen fare grubu kullanılmıştır. BALB/c farelerine (n=40), 100 µL PBS içinde 10^7 strasyoner fazlı *L.donovani* promastigotları kuyruğun lateral damarına intravenöz yolla verildi. BALB/c fareler sekizli 5 gruba ayrıldı. Tig grubuna 5 gün intraperitonal yoldan 3.7 mg/kg tigesiklin, RSM grubuna 5 gün oral gavaj ile 10 mg/kg RSM ekstresi, Tig+RSM grubuna aynı yol, gün ve dozlarda tigesiklin ve RSM ekstresi, AMB grubuna 5 gün OG ile 15 mg/kg amfoterisin B verilmiş, kontrol grubuna hiçbir işlem yapılmamıştır. On ikinci günde ketamin ile sedatize edilen farelerin dalak ve karaciğeri alınmıştır. Parazit yükü Leishman Donovan Unit (LDU) ve kantitatif RT-PCR ile belirlenmiştir.

Bulgular: Tüm gruplar, LDU ve RT-RCR bulgularına göre istatistiksel olarak değerlendirildiğinde, kontrol grubuna göre en düşük değer AMB grubunda elde edilirken ($p<0,001$), ikinci en düşük değer Tig+RSM grubunda elde edilmiştir ($p<0,001$). Tig+RSM grubunda elde edilen değerler AMB grubu hariç ol-

Corresponding author/İletişim kurulacak yazar: Özden BORAL – ozden.boral@istanbul.edu.tr

Submitted/Başvuru: 31.10.2022 • **Revision Requested/Revizyon Talebi:** 19.12.2022 •
Last Revision Received/Son Revizyon: 20.12.2022 • **Accepted/Kabul:** 20.12.2022 • **Published Online/Online Yayın:** 24.01.2023

than the data in other groups, except for the AMB group.

Conclusion: Our study suggested that the use of RSM extract together with tigecycline may be an alternative in the treatment of VL. Further studies using different doses and routes of administration are needed to evaluate the efficacy of this combination.

Keywords: Visceral leishmaniasis, tigecycline, reishi shiitake maitake, treatment

mak üzere diğer gruplardan anlamlı ölçüde düşük bulunmuştur.

Sonuç: Çalışma bulgularımız tigesiklin ile birlikte RSM ekstresinin kullanılmasının VL tedavisinde alternatif olabileceğini göstermekle birlikte, farklı doz ve veriliş yollarının denendiği yeni çalışmalarla desteklenmesi gerektiğini düşündürmüştür.

Anahtar Kelimeler: Viseral layşmanyaz, tigesiklin, reishi shiitake maitake, tedavi

INTRODUCTION

Visceral leishmaniasis (VL) is a vector-associated infection caused by the species included in the *L. donovani* complex. It is estimated that 20,000 to 40,000 people have died due to this infection, which spreads in subtropical and tropical regions. It is noted that an estimated 50.000 to 90.000 new cases of VL occur worldwide each year, and only 25% to 45% of the cases are reported to the World Health Organization. VL is a parasitic infection with a fatal potential of over 95% if left untreated. VL is observed sporadically in eastern Anatolia, the Aegean, central Anatolian and the Mediterranean regions in Turkiye. seventy-one cases in 1997, 24 cases in 2000 and 2003, 20 cases in 2006, 6 cases in 2008, 22 cases in 2014 and 37 cases in 2016 were reported as VL in Turkiye (1, 2).

Pentavalent antimony compounds are still the first treatment option in VL. Despite the main advantages being the low cost and 90-95% efficacy, it has adverse effects that are temporary but life threatening, such as intramuscular administration, longer treatment time, development of resistance, cardiac arrhythmia, pancreatitis, and pneumonia. Liposomal amphotericin B is the first treatment option in developed countries with rapid and up to 100% recovery rates. However, it is expensive and toxic, and patients usually need close monitoring and hospitalization during treatment. Miltefosine is the only oral drug and is used against VL and Cutaneous Leismaniasis. However, resistance to miltefosine has been observed. Compounds with synergistic and/or additive activity can reduce the duration of treatment and the need for doses, toxic side effects and cost can be reduced, and most importantly may prevent the development of drug resistance (1-4).

Tigecycline is the first representative of the glycylcycline class, and has a broad spectrum of antibacterial activity similar to tetracyclines. It inhibits protein synthesis by binding to the 30S ribosomal subunit on the m-RNA ribosome complex of the amino acid t-RNA. It has been determined that tigecycline is well distributed in bone, bone marrow, spleen and thyroid in experimental animal models. Interestingly, although the mechanism has not been fully understood, tigecycline has shown significant antimalarial activity against the in vitro *Plasmodium falciparum* and in-vivo *P. berghei* infection (5, 6).

Innate immune system cytokines play an important role in early protection against leishmaniasis. interleukin (IL)-12, produced by dendritic cells, triggers natural killer (NK) cell activation. IFN-γ produced by NK cells can limit the spread of the parasite until the T-cell response develops. interferon gamma (INF-γ) is very important for increasing the killing capacity of macrophages, which are the primary target of promastigotes. Tumor necrosis factor (TNF) plays a critical role in the elimination of parasites in the liver and spleen, as well as in tissue damage. TNF production controls the formation of granuloma and the reproduction of the parasite. However, the excess amount of TNF causes cell damage. The immune response in patients with VL is characterized by high levels of antibodies, the presence of Leishmania-specific T-cell proliferation, and a low level or no existence of IL-2, IFN-γ. Recovery from VL is achieved by the stimulation of the Th1 response, which is prepared by IL-12, dendritic cells and macrophages. IFN-γ produced by the T cells induces nitric oxide (NO) -mediated killing of parasites. In contrast, the progression and worsening of the clinical picture in VL are associated with the intensive production of IL-10, Transforming growth factor beta (TGF-β), and IL-4 which are Th2-type of cytokines (7, 8).

Reishi (*Ganoderma lucidum*) is a medicinal mushroom that is a lanostane type source of triterpenoid (9). The pharmacological potential of *G.lucidum* is based on an existing polysaccharide-peptide complex, β–glucans and lectine triterpenoids which have a strong immunomodulating action supporting and improving the immune system. Particularly, the polysaccharides of *G. lucidum* trigger the release of cytokines, the functions of T and B lymphocytes, dendritic cells, macrophages and NK. The in-vitro pharmacological studies showed that *G. lucidum* polysaccharides have strong effects on macrophage functions by increasing the release of IL-1α, IL-6, IL-10, and TNF. One study showed that *G. lucidum* polysaccharides increased the concentration of IL-2, TNF-α and IFN-γ, promoting the functional maturation of dendritic cells by strengthening the cytotoxic activity of T cells and NK cells (10-12). Shiitake (*Lentinus edodes*) is an edible mushroom. The oral intake of its polysaccharides was shown to have modulated the functions of immunity, and increased the fagocytosis and TNF-α levels in macro-

phage cell cultures (13). Maitake (*Grifolia frondosa*) immunomodulation is the most well-known efficacy of *G. frondosa* biocomponents, and has been confirmed by various studies. These immunomodulatory components have been shown to increase the efficacy of many other immune-related cells, such as macrophages, cytotoxic T-cells, and NK cells (14, 15).

The aim of the present study was to investigate the therapeutic efficacy of tigecycline (Tig), RSM mushroom extract, and combined treatment efficacy of both drugs in BALB/c mice that were created in a VL model with the *L. donovani* strain (ATCC-30030).

MATERIAL AND METHODS

The Novy–MacNeal–Nicolle (NNN) medium was used for the production of the ATCC-30300 *L. donovani* strain in the present study. The solid phase of the medium was sterilized in an autoclave for 15 minutes by adding 1.4 g agar and 0.6 g NaCl to 90 ml of distilled water. 10 mL of defibrinated rabbit blood was added into the medium that was heated to approximately 50-55°C and transferred to the medium tubes to obtain 4 mL in each tube and was allowed to cool by tilting. 0.2 mL of penicillin G and streptomycin sulfate were added to the solidifying medium and taken to 8°C, and 1 ml of 10% fetal calf serum (FCS) containing RPMI-1640 was added to the medium tubes before culturing to create a liquid phase (16). As recommended by the ATCC, the ATCC-30030 *L. donovani* strain was opened and planted on the checked medium.

Six-week-old BALB/c female mice were used in the study. The mice were obtained from the laboratory of the Istanbul University, Aziz Sancar Institute of Experimental Medicine (DETAE), Department of Laboratory Animal Science and were monitored throughout the experiment at 20-22°C, with 50-60% humidity, 12 hours light, 12 hours dark cycle environment, and each cage including four mice. Food and water were provided as ad-libidum. Approval was obtained from HADYEK (Animal Experiments Ethics Committee) for the study (Date:25.02.2016, No: 2016/17).

The mice were divided into five groups, each group involving eight mice (n:40). Group 1: Control group (C), Group 2: Tig, Group 3: RSM, Group 4: Tig+RSM, and Group 5: Amphotericin B (AMB) group.

All mice were infected with 10^7 stationary phase *L.donovani* promastigotes in 100 µL of phosphate buffered saline (PBS) intravenously through the lateral vein of the tail.

1. Control group (C): No procedure was performed after the *L. donovani* strain was given to the mice, and the daily health conditions were monitored in this group (figure 1).

2. Tig group: Starting from the Day 7 after giving the *L. donovani* strain, 0.5 mL intraperitoneal 3.7 mg/kg tigecycline (Tygacil) was administered at the same time for 5 days.

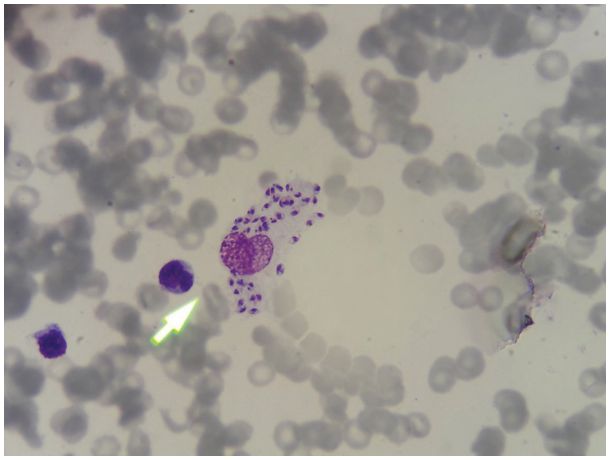

Figure 1: Amastigotes in spleen swab sample of positive control group mice (Giemsa stain x100)

3. RSM extract (Solgar) group: Starting from the Day 7 after giving the *L. donovani* strain, 0.2 mL oral gavage (OG) as to include 10 mg/kg was given at the same time for 5 days.

4. Tig+RSM group: Starting from the Day 7 after giving the *L. donovani* strain, intraperitoneal 3.7 mg/kg Tig was administered at 0.5 mL, and 10 mg/kg RSM 0.2 mL OG was administered at the same time for 5 days.

5. AMB (Ambisom-Gilead) group: Starting from the Day 7 after giving the *L. donovani* strain, 0.2 mL 15 mg/kg amphotericin B was administered with OG at the same time for five days.

At day 12, the spleen and liver were removed by opening the chest cavity after the mice in all groups were sedated with ketamine. The spleen and liver were weighed, and a swab was taken on the slide from the transverse cut surface of the right median lobe of the liver, stained with Giemsa, the number of amastigotes / 1.000 host cell nucleus was multiplied by the total weight of the liver (gr), and the Leishman Donovan Unit (LDU) and parasite load were determined by the quantitative PCR by DNA isolation from the spleen and liver samples (17). For DNA isolation, 25 mg of liver/spleen fragments were broken into pieces in a homogenizer and transferred to 1.5 ml tubes. The ZYMO RESEARCH Quik-DNA kit (USA) was used for the procedure, and the DNA isolation was performed in according to the manufacturer's recommendations. Samples were kept at -80°C. For qRT-PCR, the Techne qPCR *L. donovani* & *L. infantum* (UK) kit was used and studied in accordance with the test procedure.

Statistical evaluations were given as mean, standard deviation, median, minimum, and maximum values with

descriptive statistics of the results. The Shapiro-Wilk test was used to determine the suitability of the measurement results for the normal distribution. The differences in the measurements between the groups were performed by one-way analysis of variance (ANOVA), and multiple comparison tests were evaluated by Tukey-HSD. The relationships between the measurement levels were given by calculating the Pearson correlation coefficient. The statistical significance was accepted as $p<0.05$ and two-ways.

RESULTS

In our study, the number of amastigotes LDU and amastigote DNA copy numbers in mice in the RSM, Tig and Tig+RSM groups and in the control (C) and AMB groups were compared (table 1, figure 2). The mean LDU and copy/mL values were found to be statistically significantly higher in the control group compared with the values in all other groups ($p<0.05$). The parasite load was found significantly lower in Tig, RSM, and Tig+RSM groups when compared with the levels in the control (C) group

Table 1: Distribution of the amastigote count (LDU) and the number of DNA copies (copies/ml) in mice in the experimental groups

	Groups	N	Median	Std. deviation	Minimum	Maximum	Two way significance	
LDU	C	8	95.74	11.71	73.83	110.97	F=135.627	P<0.001
	Tig	8	71.98	10.91	53.50	84.50		
	RSM	8	54.81	4.95	48.45	61.10		
	AMB	8	3.64*	1.38	1.80	5.60		
	Tig + RSM	8	42.27§	8.18	32.64	59.80		
RT-PCR	C	8	61.50	4.93	52.00	67.00	F=208.3	P<0.001
	Tig	8	52.50	3.70	48.00	58.00		
	RSM	8	46.88	1.36	45.00	49.00		
	AMB	8	2.49*	0.99	1.20	3.80		
	Tig + RSM	8	29.55§	7.97	22.00	48.00		

*p<0.05 ; §:p<0.05; C: Control group, Tig : Tigecycline treatment group, RSM: Reishi Shiitake Maitake treatment group, AMB:Amfoterisin B treatment group, Tig+RSM:Tigecycline+Reishi+Shiitake Maitake treatment group

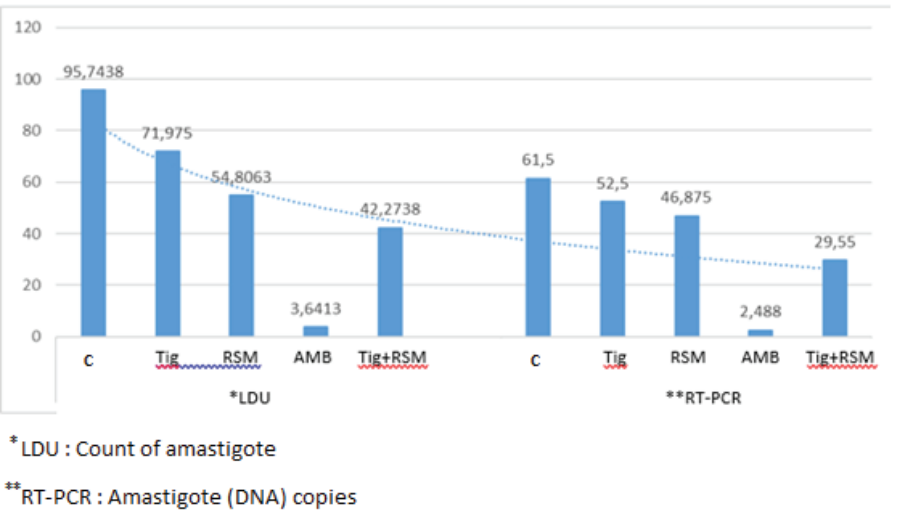

Figure 2: Graphical representation of the values obtained between the treatment groups according to the two measurement methods

C : Control,Tig : Tigecycline, RSM: Reishi-Shiitake-Maitake mushroom extract, AMB: Amphotericin B

($p<0.001$). The evaluation of the mean values showed that the lowest value was obtained in the AMB group, while the second lowest value was obtained in the Tig+RSM group. The values obtained in the Tig+RSM group were found to be significantly lower than in other groups, except for the AMB group.

DISCUSSION

Due to the fact that most anti-leishmania drugs are toxic, treatment requires hospitalization, their higher cost and the problem of increasing resistance make the treatment harder. Although in recent years improvements which reduce the treatment time and costs have been enabled with the combination treatment options, there is still a need for new drugs (4,17).

Tigecycline is the first representative of glycylcyclines. In a study, Tigecycline was shown to reach higher concentrations rapidly in human polymorphonuclear cells (5). Tigecycline was found to have antimalarial activity against the in-vitro *P. falciparum* and in-vivo *P. berghei* infections in the experimental mouse model. Researchers also found that the tigecycline antimalarial activity was strengthened by the combined association with chloroquine against the *P. falciparum* strain resistant to chloroquine. Malaria was completely eradicated using tigecycline, which was given 3.7 mg/kg IP for four days in combination with chloroquine in mice that were infected with *P. berghei* (6). Another study conducted in the same year reported that tigecycline had antimalarial activity against the culture-adapted, chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum* (18). In 2006, tetracyclines were suggested to be effective antimalarials and because they blocked the protein synthesis in prokaryotics, despite their unclear effect mechanism, it is suggested that they disrupted the mitochondria, and apicoplast including ribosomal subunits of prokaryotic origin in their genomes (19). In recent years, various antibiotics including tetracyclines and lincosamides have been shown to target apicoplast in *Plasmodiums* (20,21). The apicoplast is a plastid-like organelle from an endosymbiotic ancestor and is not found in humans. However, it is a good drug target because it is required for the survival of the parasite. Antibiotics that are effective against apicoplasts cause a typical "delayed death" effect on *Plasmodiums*, and the apicoplast function is lost in the parasite generations after exposure to the antibiotic (22,23).

The kinetoplast DNA (kDNA) involved in the single mitochondria of kinetoplastid parasites consists of mini- and maxi-circles adherent to a two-dimensional DNA network. The maxi-circles encode the mitochondrial proteins and the rRNA, while the mini-circles encode the guide RNA molecules that function in the regulation of mRNA transcripts (24). In light of this data, we may suggest that the rRNA in the kinetoplast DNA might be affected by the tigecycline used in our study.

In kinetoplastid parasites, all kinetoplasid-derived enzymes of trypanothion metabolism have been confirmed as drug targets by the genetic studies. Trypanothion reductase (TR) is one of the most well-protected gene regions in kinetoplastid parasites and is important for parasite survival. All attempts to obtain a mutant strain of trypanothione reductase on have failed in *L donovani*. Disruption of two TR alleles caused a significant decrease in the survival capacity of mutants with a low TR mRNA within the macrophage (25). Tigecycline used in the present study may disrupt the transcription by affecting the TR mRNA. In our study the number of amastigotes has significantly decreased in mice which were treated with only tigecycline compared to the numbers in the control group ($p<0.05$). One study investigating the in vitro synergistic efficacy of various antibiotics against L. donovani showed the synergistic efficacy of amodiaquine /quinine as 61.22%, pentamidine / quinine as 89.8%, pentamidine /amodiaquine 83.67% and gentamicin/amodiaquine as 100%, however, tetracycline and tigecycline were found to have no efficacy (26). No other studies which used the tigecycline against *L. donovani* were found in accessible resources. Our study is the first in vivo study which investigated the efficacy of tigecycline against *L. donovani*.

Reishi (*G.lucidum*) is a mushroom which has antifungal, antiviral effect in addition to its immunomodulatory and antiparasitic efficacy (11,12). *G. lucidum* extracts have shown antimalarial activity in mice infected with *P. berghei*. A group of mice infected with the *P. berghei* strain were given 100 and 250 mg/kg terpenoid extract of *G. lucidum*; 30 mg/kg chloroquine was given to the other group of mice with OG as standard therapy, and the results were compared with the control group of mice. In addition to antimalarial activity, *G.lucidum* has also been reported to provide improvement in liver damage associated with malaria and enabled significant decrease in AST, ALT, ALP and GGT levels (27). Similarly, RSM included 100 mg *G. lucidum* in our study. In another study, one ethyl acetate extract of *G. lucidum* was found to have antiplasmodial activity with 79% inhibition (at 4.9 µg/ml) (28). Seberi et al. suggested that the hydroalcoholic extract of *G.lucidum* at concentrations of 150 and 200 mg/ml enabled a significant decrease in the number of *L. major* living promastigotes and this effect was due to components such as tannins, flavanoids, terpenoids and polysaccharide (29). We also found in our study that it significantly reduces the number of amastigotes in vivo.

G.lucidum β-glucans have been shown to induce macrophages, neutrophils, monocytes, NK and dendritic cells. (30). The in vitro pharmacological studies with *G.lucidum* showed that *G.lucidum* polysaccharides induced macro-

phage phagocytosis by increasing the release of IL-1α, IL-6, IL-10, and TNF-α (11).

G. lucidum polysaccharides were found to have a positive effect on the Th1 response by increasing the IL-2 and IFN-γ levels. However, they have little or no effect on the production of IL-4, and IL-10 (31). Various experimental models and clinical studies showed that IFN-γ production and Th1 response in VL triggered a strong leishmanial mechanisms in phagocytes. In contrast, IL-4 and IL10 production and Th2 response inhibited the macrophage activation resulting with the intracellular replication of the parasite (32). With this data, *G. lucidum* included in RSM that was used in our study probably activates the Th1 response by increasing the IFN-γ level. However, not affecting the release of IL-4 and IL-10 was consistent with the results obtained from RSM and Tig+RSM group mice. IL-4, and IL-10 are the cytokines that contribute to pathogenesis in VL (32).

In many studies Shiitake (*L. edodes*), were reported to have immunomodulatory, antitumoral, antiviral, and antioxidant efficacy and these activities were mainly due to its pharmacological components such as proteins, peptides, polysaccharides, terpenoids, and sterols (12).

Individuals who consumed 5 gr or 10 gr of mushrooms or who did not consume mushrooms for four weeks were investigated in a comparison in this study; in the group consuming mushrooms, the T lymphocytes and NK cell proliferation, and the IL-4, IL-10, TNF-α, and IL-1α levels were found to have increased (33). Crespo et al. compared rabbits that were fed a conventional diet. The conventional diet consisted of 5% purified β-glucan in their in vivo study. They reported that in rabbits provided with β-glucan, the indicator of immune stimulation of IL-10 expression was downregulated with the expression induced by macrophage and IL-4, IFN-γ. Shiitake included high a amount of β-1-3 and β-1-6 D-glucan (lentinant), and revealed that the β-glucans induced the intracellular signaling, and interaction with different cellular receptors may activate the different profiles of the immune response (34). IL-10 is a cytokine that contributes to pathogenicity in human VL and experimental mouse models of VL, whereas IFN-γ has a protective effect (32). This data is consistent with the significant decrease in parasite load that occurred in the RSM mice compared to the control group in our study ($p<0.001$).

Goldman et al. found that 50 mg, 200 mg, and 400 mg/kg IV and IP administration of β-glucan four times separately to BALB/c mice 4 days after they were infected with the *L.major* strain prevented the development of lesions, and 400 mg/kg administration prevented even the first phase of lesion development. In another study, the IP administration of 0.45 mg glucan seven, five, three, and one day before infection on BALB/c mice which were infected with *L. major* promastigotes showed a significant decrease in the amastigote count on the spleen and liver compared with the levels in mice in the control group ($p<0.001$) (35,36). Ghosh et al. reported that β-glucan obtained from *Alcanigenes faecalis* through 10 mg IP administration 10, 15 and 20 days after infection in BALB/c mice of the VL model decreased the parasite load as 99% in the liver and spleen (37). They also observed in β-glucan-treated mice that the IL-12, IFN-γ, TNF-α and IL-1 β levels increased 4.6, 5.7, 8.2, and 5.1 times compared to the levels in the control group, respectively. In the interpretation of their study, they considered that the activation of Th1 cytokines could directly stimulate NO production.

Similarly, although the route of administration was oral gavage, the number of amastigotes decreased in the liver and spleen in mice receiving 10 mg/kg RSM for five days compared to the levels in the control group in our study ($p<0.001$).

Sandvik et al. administered 20 mg/kg *Saccaromyces cerevisiae* type β-1-3 and β-1-6 glucan for 14 days to mice who were created to have endotoxemia with *Escherichia coli*, and reported that OG administration was more effective compared to subcutaneous administration, and they found a significant decrease in the indicators of kidney and liver damage (38). They found that IL-10 expression, which is an indicator of immunostimulation, is downregulated in combination with macrophage, IL-4, IFN-γ-induced expression of β-glucan administered with oral gavage. Similarly, we administered RSM extract with OG in our study.

IL-10 contributes to pathogenesis in VL in humans and in experimental mouse models, while IFN-γ has a protective effect (32). Our study results are consistent with this data.

Maitake (*Grifolia frondosa*) is a type of edible mushroom. This mushroom is known as a natural immunomodulator as having no side effects related to increasing immunity. It contains high β-glucan and has β-1-6 branching points in addition to β-1-3 bonds in the main chain of β-glucan. In in-vitro studies β-glucan was shown to increase the production of TNF-α, IL-1 and IL-6 by activating the macrophages (15). In experimental animal models, IP administration was found to have significantly increased the production of TNF-α and the cytotoxicity of NK cells (14). Meng et al. found that the administration of polysaccharides obtained from *G. frondosa* with OG as 30, 60, 120 mg/kg for 14 days induced the fagocytosis, and increased the IL-1β , IL-2, IL-6 and IFN-γ levels in splenocytes in the immunosuppressed mice that were induced with IP cytoxane. The RSM extract given with OG includes 100 mg of maitake in our study (39). Sultana et al. showed in their study against the promastigote and amastigote forms of *L. donovani*, *L. tropica* and *L. major* strains that in macrophage cultures the semi-purified

extract of *G.frondosa* reduced amastigote replication in macrophages and the level of IL-10 and TGF-β from inflammatory cytokines more effectively than the reference drugs of amphotericin B, paromomycin, miltefosine and sodium antimony gluconate in VL treatment. In addition, the extract was also found to induced the apoptosis in promastigotes (40).

CONCLUSION

Our study results suggest that the combined use of RSM extract together with tigecycline may be used as an alternative approach in the treatment of VL, however, there is a need for further research that investigate the different doses and administration routes in order to show the treatment efficacy.

Ethics Committee Approval: This study was approved by Istanbul University Local Ethics Committee of Experimental Animals (Date: 05.02.2016, No: 2016/17).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- Ö.B., D.G.Ç.; Data Acquisition- Ö.B., D.G.Ç.; Data Analysis/Interpretation- H.İ., Ö.B.; Drafting Manuscript- Ö.B., D.G.Ç.; Critical Revision of Manuscript- Ö.B., D.G.Ç.; Final Approval and Accountability- Ö.B.

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

Financial Disclosure: This study was supported by the Scientific Research Projects Coordination Unit of Istanbul University (Project number: 20350)

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