BJMG

VOL 27
(1), 2024

ISSN 1311-0160

Balkan Journal of Medical Genetics

International Journal of Medical Genetics

VOL 27 (1), 2024

BJMG is an open access, peer-reviewed journal. All articles in the journal are immediately and permanently available online for all to read and use free of charge.
BJMG is published twice a year.
Indexed or abstracted in: EMBASE (the Excerpta Medica database), Elsevier Biobase (Current Awareness in Biological Sciences), Elsevier GeoAbstracts, PubMed Central, Scopus, Chemical Abstracts (CA), Science Citation Index Expanded, SCImago (SJR) Journal Citation Reports/Science Edition, De Gruyter, Sciendo, Academic Open Access Publisher.
BJMG is covered by Thomson Reuters services.

EDITORIAL OFFICE
Macedonian Academy of Sciences and Arts, Bul. Krste Misirkov 2, POB 428
1000 Skopje, Republic of North Macedonia.
Phone: + 389 2 3235 411, fax: + 389 2 3115 434, E-mail: bjmg@manu.edu.mk
Web page: www.bjmg.edu.mk

PUBLISHER
BJMG is published by Macedonian Academy of Sciences and Arts
Online edition

Front cover page: ??????????????????????????????????????

Back cover page: ??????????????????????????????????????

CONTENTS

Original Articles

Case Reports

BJMG 27 (1), 2024 • 5-14
DOI: 10.2478/bjmg-2024-0006
sciendo
ORIGINAL ARTICLE

ANXA5 AND *VEGFA* GENE VARIANTS IN WOMEN WITH EARLY PREGNANCY LOSSES FROM NORTH MACEDONIA

Terzikj M[1,#], Bozhinovski Gj[1,#], Branoski A[1], Dimkovska M[1], Kubelka-Sabit K[2,3], Plaseska-Karanfilska D[1,*]

[*]**Corresponding Author:** Corresponding author, Prof. Dijana Plaseska-Karanfilska, MD, PhD. Research Centre for Genetic Engineering and Biotechnology "Georgi D. Efremov", Macedonian Academy of Science and Arts, Skopje, North Macedonia, e-mail: dijana@manu.edu.mk

[#] equal contribution

ABSTRACT

Early pregnancy loss (EPL) is the most common pregnancy complication, found in approximately 15% of all clinically recognized pregnancy complications. Up to date, various maternal as well as fetal factors are reported as a cause of EPLs. However, in approximately 50% of EPL cases, the exact cause is not clearly identified and these cases are referred as idiopathic.

The aim of our study was to examine the association of four distinct variants in the *ANXA5* gene and two variants within the *VEGFA* gene in a cohort of women with EPLs from North Macedonia. This group was compared to a control group of women matched by ethnic background without pregnancy loss and at least one live birth. We also aimed to establish an effective and cost-efficient method for their detection based on multiplex single-base extension.

Among 190 women experiencing EPLs, and 190 samples from women without a history of pregnancy loss (control group), our results demonstrated a statistically significant prevalence of heterozygotes for the M2/*ANXA5* haplotype in women with EPLs, compared to the control group (p=0.0006). In the analyses comparing genotypic frequencies for the variants in the *VEGFA* gene, higher frequencies were generally observed among women experiencing EPLs, however without statistical significance.

Our study aligns with multiple studies showing that M2 and M1 *ANXA5* haplotypes are more prevalent in patients with pregnancy loss and presents an affordable genotyping technique for the specific *ANXA5* and *VEGFA* variants.

Key words: *ANXA5*, haplotypes, *VEGFA*, early pregnancy loss (EPL), multiplex single-base extension

INTRODUCTION

Early pregnancy loss (EPL), defined as a loss of the conceptus before the 12th week of gestation, is the most common pregnancy complication and is found in approximately 15% of all clinically recognized pregnancies [1]. About 5% of the couples trying for childbirth, experience recurrent pregnancy loss (RPL), a condition defined as two or more consecutive pregnancy losses, according to the European Society of Human Reproduction and Embryology (ESHRE) and American Society for Reproductive Medicine (ASRM) [2, 3], as well as three or more first trimester miscarriages, according to the Royal College of Obstetricians and Gynecologists, UK (RCOG) [4]. Up to now, various studies have reported that maternal as well as fetal factors may lead to RPL. Fetal chromosomal abnormalities are acknowledged as a significant contributor to pregnancy loss in approximately 50% of cases. This pattern is mirrored in our previous study concerning chromosomal abnormalities in early pregnancy losses (EPLs). In that study, chromosomal abnormalities were detected in 56.25% of uncontaminated products of conceptions (POCs), aligning with this observed trend [5]. Additionally, maternal endocrine dysregulations, autoimmune disorders, anatomical abnormalities, maternal thrombophilia, as well as genetic factors can contribute to RPL [6]. Concerning genetic factors, in a recent study that was designed and executed in our laboratory, we detected a high incidence

[1] Research Center for Genetic Engineering and Biotechnology "Georgi D. Efremov", Macedonian Academy of Sciences and Arts, Skopje, North Macedonia.

[2] Private Hospital "Acibadem-Sistina", Skopje, North Macedonia

[3] Faculty of medical sciences, University "Goce Delcev", Stip, North Macedonia

of Joubert Syndrome among a group of EPLs (2.03%), indicating that fetal monogenic diseases can be a common cause of EPLs [7]. Still, in a large portion of RPL cases, the exact cause is not clearly identified, and these cases are referred as idiopathic RPL [8].

Angiogenesis is a physiological process through which new blood vessels are formed. Decreased angiogenesis has been associated with several adverse pregnancy outcomes, such as infertility, preeclampsia, miscarriage, intrauterine fetal distress/growth restriction, and in severe cases, fetal demise. The Annexin A5 protein, encoded by the *ANXA5* gene, is found in abundance to hinder coagulation within the placenta and during the early angiogenesis. When calcium is present, Annexin A5 can attach to phosphatidylserine situated on the upper surface of syncytiotrophoblasts in the placenta to prevent clotting of maternal blood in the intervillous space. Additionally, the *ANXA5* gene plays a crucial role in promoting epithelial repair, upholding placental integrity [9]. Studies have indicated that the activity of *ANXA5* in placental tissue samples collected from patients with RPL was significantly diminished, accompanied by a suppressed *ANXA5* expression. These findings imply that a decrease in Annexin A5 protein expression elevates the risk of RPL, especially in early pregnancy [10]. Bogdanova et al. first reported that the combination of certain single nucleotide changes: rs28717001 (c.-210A>C), rs28651243 (c.-184T>C), rs112782763 (c.-229G>A) and rs113588187 (c.-135G>A) in the promoter region of *ANXA5* was associated with an increased risk of recurrent miscarriages. All four changes were defined as M2 haplotype, and the first two were designated as M1 haplotype [11]. As mentioned in a recent meta-analysis study [12], and a review article [13], dealing with the effect of the *ANXA5* haplotypes on RPL, several case-control studies have shown a favorable correlation between the M2/*ANXA5* haplotype and RPL across diverse ethnic populations, including cohorts from Germany [11, 14, 15], Japan [16], the United Kingdom [17] Italy [18] China [19] Malaysia [20] and Greece [21]. However, contrasting results were observed in studies involving the Chinese and Danish/Estonian ethnic groups. These groups did not reveal any association between the M2/*ANXA5* haplotype and RPL [22, 23].

The human placenta is rich in angiogenic factors such as *VEGF*, standing out as the most potent stimulator of angiogenesis. *VEGF* plays a crucial role in endometrial readiness, implantation, and the development of placental and fetal blood vessels in early pregnancy, and in the vascular adaptation during pregnancy in the mother [24]. Several single nucleotide changes (SNPs) have been identified in the *VEGFA* gene, affecting VEGF-A activity and expression. The most significant finding in most studies is that presence of the c.-1154G/A (rs1570360) variant is associated with recurrent early pregnancy losses in contrast to normal control groups. The earliest study demonstrating a significant difference goes back to 2005 in Greece [25]. The study involved 52 women with 3 or more recurrent pregnancy losses and 82 controls with live births and no history of pregnancy loss. The analysis of allele frequency for the polymorphic variant c.-1154G/A (rs1570360) yielded a p-value of 0.016, indicating an increased allelic frequency of the mutant allele A in patients with early pregnancy losses. The c.*237C>T variant (rs3025039) increases *VEGFA* expression as well and acts in a similar direction [26, 27]. Similarly, the effect of the variants in the *ANXA5* gene have been shown in some studies to contribute negatively to the rs1570360 and rs3025039 variants in susceptibility to recurrent miscarriages in different geographic groups [27, 28, 29]. Yet others have not confirmed this association [30, 31], therefore the need for further research to see if these relations exist. Hence, these allelic variants are unquestionably noteworthy in investigating the predisposition to RPL development.

In establishing an effective and cost-efficient method for variant detection based on multiplex single-base extension, we aimed to examine the association of the c.-210A>C, c.-184T>C, c.-229G>A, c.-135G>A variants in the *ANXA5* gene and c.-1154G>A, c.*237C>T variants in the *VEGFA* gene in a cohort of women with early pregnancy losses, compared to a control group of women without pregnancy loss and at least one live birth. Moreover, the determination of the status of these variants would provide a direction for future therapies in women with unexplained pregnancy losses, since the large amount of data suggest that the M2/*ANXA5* haplotype may contribute to the occurrence of this condition.

MATERIALS AND METHODS

Materials

For this study, a total of 380 DNA samples were observed using a multiplex single-base extension reaction assay. The samples were taken from 190 women experiencing EPLs, 104 from Macedonian and 86 from Albanian ethnic backgrounds and the same number of samples were obtained from women without a history of pregnancy loss as well as at least one healthy live birth as control group.

Following a methodology outlined by Noveski et al., the examined women with EPLs were additionally selected because no fetal chromosomal abnormalities were detected in their POCs [32]. Of the total number of women with pregnancy losses, 81 had single pregnancy loss (sporadic), and 109 had two or more pregnancy losses

(recurrent). Also, from the total number of women with early pregnancy losses, 151 did not have a previous live birth, and 39 had.

All participants gave informed consent for participation in the study. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Macedonian Academy of Sciences and Arts (09-1047/6 from 04.05.2016). This work was financially supported by the project "Molecular basis of spontaneous abortion" (project number: 08-707) funded by the Macedonian Academy of Sciences and Arts.

Methods

Blood samples were collected from each participant and utilized for DNA isolation from leukocytes using the conventional method involving phenol/chloroform extraction and ethanol precipitation, as described by Efremov et al. (1999). All individuals were analyzed for the presence of 4 different variants in the *ANXA5* gene: rs28717001 (c.-210A>C), rs28651243 (c.-184T>C), rs112782763 (c.-229G>A), rs113588187 (c.-135G>A) and 2 variants in the *VEGFA* gene: c.-1154G>A (rs1570360) and c.*237C>T (rs3025039). Taking inspiration from the methodology previously employed in our laboratory [33, 34, 35], we designed a multiplex single-base extension method for simultaneous detection of the 6 variants utilizing the Multiplex SNaPshot kit (Multiplex SNaPshot; Applied Biosystems, Warrington, WA) for the reaction, followed by capillary electrophoresis on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The PCR primers were designed to function in a multiplex mix and to produce PCR fragments of 427–550 bp. Briefly, the PCR amplification reaction contained 2 μL of 100 ng genomic DNA, 1.5 μL of a multiplex mix where each specific forward and reverse oligonucleotide primers are with a 10 pmol concentration (Table 1), 2 μL 2.5mM of each deoxyribonucleotide triphosphate (dNTP), 1.5 μL 25mM magnesium chloride, 5 μL GC rich enhancer and 0.2 μL of 1U Taq polymerase (Hot Fire polymerase; Solis Biodyne, Tartu, Estonia) in a total volume of 25 μL.

The PCR conditions were as follows: 10 min. initial denaturation at 95°C, followed by 33 cycles of 1 min at 95°C,1 min at 61°C for the primer annealing and 1 min at 72°C. The final elongation was set at 72°C for 10 min. Afterwards, purification was carried out using 1μL of ExoSAP-IT® (USB, Cleveland, OH) per 2.5 μL of PCR product. The process included an incubation step at 37°C for 20 minutes and subsequent inactivation of the enzyme at 86°C for 20 minutes.

The refined PCR products served as the basis for identifying the six mutations. In the subsequent single-base extension reaction, the detection primers (SNaPshot primers) were aligned adjacent to the single-nucleotide polymorphism position. Various lengths of poly (dC) tails were appended to the single-base extension primers (refer to Table 2). The SNaPshot reaction included a 1.5μL primer mix comprising all 6 single-base extension primers at 3.5μL of purified PCR products, and 1.5μL of the SNaPshot Multiplex kit (Applied Biosystems), adding up to a final volume of 6.5 μL. The cycling profile comprised of 25 cycles of 95°C for 10 s, 55°C for 10 s, and 60°C for 30 s. After the reaction, the 5'-phosphophoryl groups of unincorporated dideoxynucleotide triphosphates were eliminated by adding 1.0U of shrimp alkaline phosphatase (SAP; USB), followed by an incubation step at 37°C for 40 min and at 86°C for 20 min to deactivate the enzyme. Capillary electrophoresis was conducted using an ABI PRISM 3130 Genetic Analyzer, and the results were analyzed using Gene Mapper, Version 4.0 (Applied

Table 1. Nucleotide sequences of PCR primers

Primer ID	Sequence (5'-3')	Length (bp)
ANXA5-F	CAGCTACCGGGACAGCTC	18
ANXA5-R	CTCCAAAACCCCGAGCCC	18
VEGFA-F_IGU	TTCCTAGCAAAGAGGGAACG	20
VEGFA-R_IGU	GCTGACCGGTCCACCTAAC	19
VEGFA-F1_3'utr	ACACCATCACCATCGACAGA	20
VEGFA-R1_3'utr	GTCAGGATCTGAGTGGGAACA	21
rs112782763_ SShot	CCCCCCGCGGCCGGCCTGCGGTTG	24
rs28717001_ SShot	CCCCCCCTGCCCGGCTTGGCCCG	23
rs28651243_ SShot	CCCCCCCCCCCCCCCCCGGAAACGCCAGCGGCCCC	34
rs113588187_ SShot	CCCCCCCGCCGAGATGCAGACGCTGAAGGATC	32
rs1570360_Sshot	CCCCCCCCCCCCCCCCCGCCCGAGCCGCGTGTGGA	34
rs3025039_Sshot	CCCCCCCCCCCCCCCTGGCGAATCCAATTCCAAGAGGGACC	40

Table 2. Single base extension primers data

Variant	Nucleotide change	PCR fragment size (bp)	SNaPshot primer orientation	SNaPshot result (N/M)	SNaPshot fragment size-N (bp)	SNaPshot fragment size-M (bp)
rs112782763 (c.-229G>A)	G/A	551	Forward	G/A	27.1	30.3
rs28717001 (c.-210A>C)	A/C		Reverse	T/G	21.9	28.1
rs28651243 (c.-184T>C)	T/C		Reverse	A/G	34.1	32
rs113588187 (c.-135G>A)	G/A		Reverse	C/T	36.1	37.1
rs1570360 (c.1154G>A)	G/A	450	Forward	G/A	37.5	38.5
rs3025039 (c.*237C>T)	C/T	427	Reverse	G/A	41.5	42.5

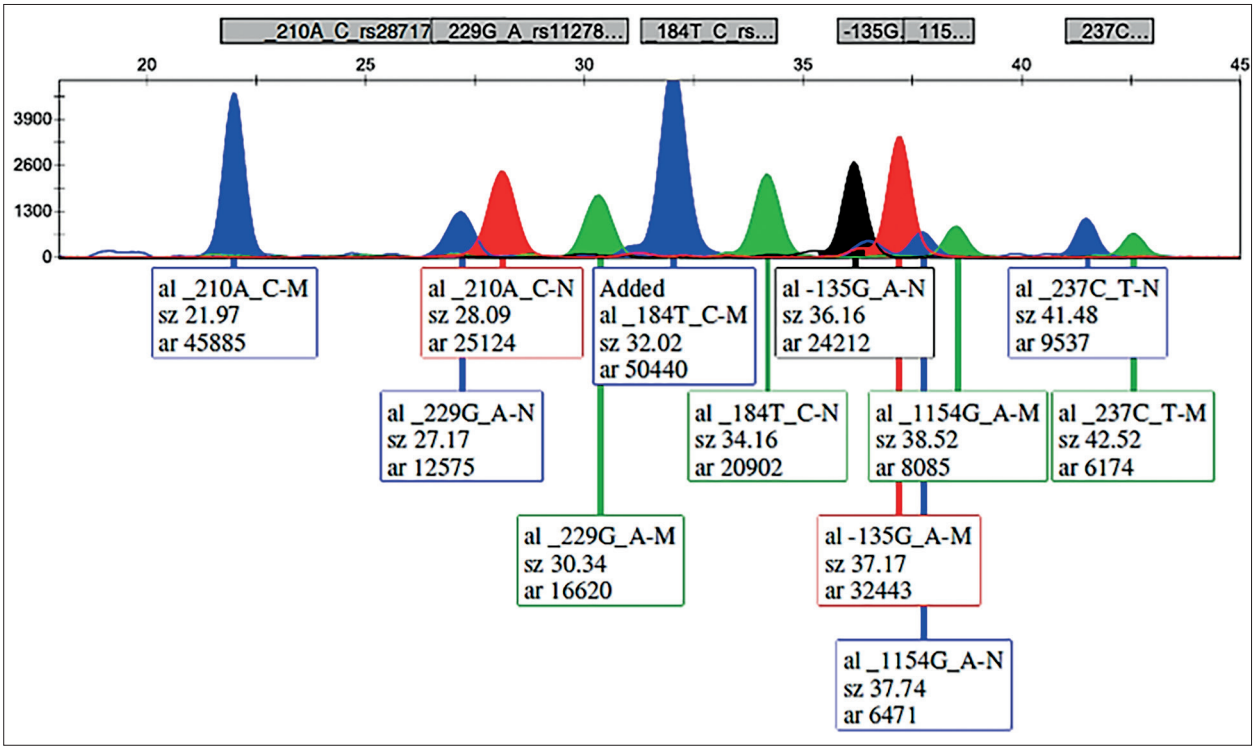

Figure 1. Electrophoretogram of an individual heterozygous for all ANXA5 and VEGFA examined variants.

Biosystems, Foster City, CA). Specifics of the multiplex PCR and SNaPshot reaction products can be found in Table 2. Due to the impact of the dye, size, and nucleotide composition on the mobility shift of DNA fragments, the reported sizes may deviate by a few bases from the actual sizes, especially with shorter fragments where the relative contribution of the dye is more pronounced. Figure 1 displays a representative electrophoretogram from a patient heterozygous for all six variants.

The Chi-square test as well as Fisher's exact test, were employed to assess the significance of the results in both the examined and control groups was determined based on the p-value. A p-value <0.05 was considered statistically significant.

RESULTS

ANXA5 variants

Haplotype-based classification

Given that the four alterations collectively constitute a haplotype, it is possible to categorize them based on the

haplotype's representation, the extent to which the M1 haplotype (comprising two changes) and the M2 haplotype (encompassing all four changes) are present. Moreover, various subcategories can be delineated to observe different combinations (haplotypes), including N/N (normal genotype), N/M1 (heterozygous for haplotype M1), M1/M1 (homozygous for haplotype M1), N/M2 (heterozygous for haplotype M2), M2/M2 (homozygous for haplotype M2), and M1/M2 (double heterozygous). All our examined samples belonged to one of the above-mentioned categories, i.e., a non-haplotype affiliation was not present in either of our cohorts.

We observed that the suggested pathological haplotypes (M1, M2 and their combinations) were more abundant among our group of women with EPLs, evident by the fact that the normal genotype was found to be significantly more frequent in the control group, with a p-value=0.0009. The M2 heterozygous haplotype was significantly more prevalent among the women with EPLs group, reaching a p-value of 0.0006. The heterozygous M1 and homozygous M2 haplotypes were both more frequent among the group of women with EPLs, however these differences did not reach statistical significance. Interestingly, no homozygous M1 and M1/M2 compound heterozygous haplotypes were detected among the patients group, while the control group included 1 and 5 representatives for each haplotype combination. These findings are presented in Table 3.

Comparison based on history of pregnancy loss and live births

Similarly, in these categorizations, our findings indicated a significantly higher frequency of the normal genotype observed in the control group, highlighting that the pathological M1, M2 haplotypes and their combinations were more prevalent among the women who had experienced EPL in our study (p-value=0.0001). Remarkably, statistically significant outcomes were noted for the presence of heterozygotes for the M2 haplotype in individuals who had experienced one early pregnancy loss, when compared to the controls, a pattern also observed in those with recurrent EPLs, however with borderline p-value of 0.057. Further, the occurrence of heterozygous M1 and homozygous M2 haplotypes were equally distributed in both categorizations and did not achieve statistical significance in comparison to the corresponding haplotypes observed in the controls (data presented in Supplementary Table 1).

Notably, among the women with EPLs and without a live birth, there was a statistically significant higher prevalence of heterozygotes for haplotype M2 compared to the control group. Also, the homozygotes for the M2 haplotype belonged to this subgroup of women with EPLs and without a live birth. The women with EPLs and a live birth showed higher presence of heterozygotes for the M1 haplotype, with a borderline statistical significance (p-value=0.05), as presented in Supplementary Table 1.

Comparison based on maternal age and on gestational week of the current pregnancy among the women with EPLs

When the maternal age (at the time of admission) was taken in consideration in our cohort, we observed statistically significant higher prevalence of the heterozygous M2 haplotype among the women with EPLs, ≤ 30 years of age (p-value=0.003) and ≥ 36 years of age (p-value=0.01), in comparison to the control group of the same age range. In contrast, the group of examined women between 31 and 35 years of age, presented a statistically significant difference in the heterozygous M1 haplotype amid the two cohorts (p-value=0.03), with this haplotype being more frequent among the women with EPLs. These data are presented in Supplementary Table 2.

A comparison based on gestational week of the current pregnancy was also performed and these findings are presented in Supplementary Table 2 as well. Unfortunately, data on this parameter was missing for more than a third (35.3%) of the women with EPLs. We divided the women in two major groups, the first having pregnancy loss between weeks 6-9, and the second group consisted of women with EPLs in weeks 10 and 11, based on the presumption that women carrying the M2 haplotype are

Table 3. The general results of the variants in the *ANXA5* gene in the group of women with EPLs and Control group

Haplotype	Women with EPLs (n=190)	%	p-value	Controls (n=190)	%
N/N	135	71.05	**0.0009**	161	84.73
N/M1	15	7.89	0.09	8	4.21
M1/M1	0	0.00	/	1	0.52
N/M2	36	18.95	**0.0006**	14	7.37
M2/M2	4	2.11	0.18	1	0.52
M1/M2	0	0.00	/	5	7.90
Total	190	100.00		190	100.00

Table 4. The genotypic frequency of the examined *VEGFA* variants in the group of women with EPLs and Control groups.

c.-1154G>A	Women with EPLs (n=190)	%	p-value	Controls (n=190)	%
GG	92	48.42	0.47	99	52.11
GA	76	40.00	0.53	70	36.84
AA	22	11.58	0.87	21	11.05
Total	190	100.00		190	100.00
c.*237C>T					
CC	137	72.10	0.64	141	74.21
CT	50	26.32	0.64	46	24.21
TT	3	1.58	1	3	1.58
Total	190	100.00		190	100.00

believed to face over twice the risk of fetal loss between 10 and 15 weeks of gestation compared to those who do not carry this haplotype. Again, the heterozygous M2 haplotype was significantly more present in both subgroups of women with EPLs, compared to the controls, reaching a p-value of 0.008 and 0.007 for both subgroups respectively.

VEGFA gene

The genotypic frequency of the two examined variants in the two specified groups are displayed in Table 4. The results indicate the absence of a statistically significant difference of the selected *VEGFA* variants between women with EPLs and the control group. The *VEGFA* c.-1154G/A (rs1570360) variant was identified in 76 patients (40%), in heterozygous state and in 22 (11.58%) patients in homozygous state. In the control group, 70 individuals were heterozygous (36.84%), while 21 (11.05%) were homozygous for this variant. The c.*237C/T (rs3025039) presented similar distributions between the women with EPLs and the controls, however, the homozygous mutant genotype was more rarely found in both groups, when compared to the c.-1154G/A (rs1570360) variant. Further, two women with EPL were found to be homozygous for both *VEGFA* variants (2/190, 1.05%), and 20 women with EPLs were heterozygous for the both *VEGFA* variants (20/190, 10.53%). Among the control group, those numbers were one (1/190, 0.53%), and 14 (14/190, 7.37%), respectively. Both differences were not statistically significant (p-value=0.8, and p-value=0.2, accordingly).

Comparison based on history of pregnancy loss and previous live birth

The results of the comparison based on history of pregnancy loss and previous live birth between the two groups are presented in Supplementary Table 3. Examining c.-1154G/A (rs1570360), among women with 2 or more early pregnancy losses, the mutant homozygous form A/A is represented by 14.68%, compared to 11.05% in the control group. However, this discrepancy did not reach statistical significance.

Likewise, in the case of the polymorphic variant c.*237C/T (rs3025039), no notable difference was observed in the subgroups of women with EPLs, compared to the controls. The distribution of the mutant form T/T was higher in the group of women with 2 or more EPLs, although the difference compared to the control group was not statistically significant.

Regarding previous live births, the patients were categorized into two groups: one comprised patients with early pregnancy loss and no prior live births, and the other included those with early pregnancy loss and at least one live birth. The group of women with EPLs and a live birth consisted of 39 cases, among which, 53.85% expressed heterozygosity for the c.-1154G/A (rs1570360) variant, compared to the 36.84% among the controls, and this difference was found to be statistically significant (p-value=0.04). Amid the subgroup of women with EPLs and a live birth, the genotypes containing the mutant allele of the c.-1154G/A (rs1570360) variant, were found to be more common, compared to the control group where the normal genotype was most frequent, reaching a borderline p-value of 0.05. The data on c.*237C/T (rs3025039), for this parameter did not show statistical significance. These data are depicted in Supplementary Table 3.

Comparison based on maternal age and gestational week of the current pregnancy among the women with EPLs

When maternal age was taken into account, in order to observe the distribution of the genotypes for the c.-1154G/A (rs1570360) and c.*237C/T (rs3025039) *VEGFA* variants, between the different age groups of the women with EPLs and controls, no statistically significant differences were obtained in any subcategory. These data are presented in Supplementary Table 4.

An analysis was conducted considering the gestational week of the current pregnancy, and the genotypes

of the *VEGFA* c.-1154G/A (rs1570360) and c.*237C/T (rs3025039) are displayed in Supplementary Table 4 as well. As previously noted, information regarding this parameter was unavailable for over a third (35.3%) of the women who experienced EPLs. The heterozygous c.-1154G/A (rs1570360) was much more common among the subgroup of women with EPLs in week 10-11, reaching p-value 0.03. In the same subgroup, the heterozygous c.*237C/T (rs3025039) was also more frequent, compared to the controls, with a borderline p-value of 0.05.

DISCUSSION

In this study, we have developed a SNaPshot genotyping technique, which identifies four distinct variants in the *ANXA5* gene and two variants in the *VEGFA* gene. The method efficiently and concurrently identifies all six selected variants and also stands out for its simplicity, accuracy, ease of execution, and cost-effectiveness. By employing this method, we have assessed the occurrence rates of the two haplotypes (M1 and M2) within the *ANXA5* gene and the two variants (c.-1154G/A (rs1570360) and c.*237C/T (rs3025039)) within the *VEGFA* gene among selected group of women of Macedonian and Albanian ethnic origin experiencing early pregnancy loss. Our analysis involved comparing these rates to controls matched by ethnicity and age.

Given the inconsistencies in the literature regarding the impact of the specified variants, as outlined in the introduction, the primary objective of our study was to investigate whether the six selected variants are linked to an elevated risk of early pregnancy loss, particularly within our two major ethnical groups (Macedonian and Albanian). It is important to emphasize that in our examined population fetal aneuploidy as a reason for EPLs was excluded.

Considering that the majority of studies have focused on individuals with subsequent pregnancy losses, and given the predominant nulliparous status among most patients, it became crucial to examine the distribution of haplotypes within various subgroups of our patients. Bogdanova et al. revealed four consecutive single nucleotide changes in the *ANXA5* promoter, which are transmitted as a haplotype called M2 that reduces promoter activity, thereby leading to reduced production of Annexin A5 mRNA as well as M1 haplotype covering the first two nucleotide substitutions. Certain analyzes showed that haplotypes M1 and M2 reduce *ANXA5* promoter activity by 40% and 60%, respectively, thereby significantly affecting *ANXA5* expression. Women with the M2 haplotype are thought to have more than a 2-fold higher risk of fetal loss between 10 and 15 weeks of gestation than non-carriers [11]. As highlighted in a recent meta-analysis [12] and a review [13] examining the impact of the *ANXA5* haplotypes on recurrent pregnancy loss (RPL), multiple case-control studies have demonstrated a positive association between the M2/*ANXA5* haplotype and RPL across a range of ethnic backgrounds [11, 14-21].

In line with numerous studies, supporting the concept that M2 and M1 *ANXA5* haplotypes are more common in patients experiencing pregnancy loss [10-21], our study presented higher frequency of the M2 haplotype among the women with EPLs compared to controls (p-value=0.0006).

Furthermore, when we did several sub categorizations, we observed evident statistical significance of the M2 haplotype among: women with recurrent as well as sporadic early pregnancy loss (p-value=0.057 and p-value<0.00001 respectively), women with EPLs and no live birth (p-value=0.0003), women ≤30 and ≥36 years of age (p-value=0.003 and p-value<0.001 respectively), and also in both subgroups of pregnancy loss between 6-9 GW and 10-11GW (p-value=0.008 and p-value<0.007 accordingly). The M1 haplotype was found to be more prevalent in the subgroup of women with EPLs and a live birth (p-value=0.05) and in the subgroup of women with ages between 31 and 35 years with a p-value of 0.01.

Furthermore, there are studies indicating that low-molecular-weight heparin might have a positive effect on miscarriage rate and recurrent implantation failure in treated M2/*ANXA5* haplotype carriers [36, 37], so, a research in this direction could be a further step.

VEGFA has gathered significant attention due to its pivotal role in angiogenesis, particularly notable for its implications in embryo development. Compelling evidence underscores its critical involvement in fetal and placental angiogenesis, suggesting that vascular formation irregularities or dysfunction contribute to RPL. Additionally, first-trimester trophoblast *VEGFA* expression was found to be weaker in placental samples from RPL cases compared to gestational age-matched normal placenta [38, 39]. The most significant finding in most studies is that presence of the c.-1154G/A (rs1570360) variant is associated with recurrent early pregnancy losses in contrast to normal control groups [25-29], while others have not confirmed the association [30, 31], so the need for further research of these relations exists. According to a meta study by Xu et al., [27] the c.-1154G/A (rs1570360) and c.*237C/T (rs3025039) variant demonstrated statistical significance concerning RPL risk across different geographical populations, and discrepancies such as in our present study may be explained by the small sample size and substantial errors from estimation.

In our examined cohort, we observed a general higher prevalence of the heterozygous and mutant homozygous genotypes for the both *VEGFA* variants, compared to the

controls, however without statistical significance. Nevertheless, we noticed borderline statistically significant difference (p-value=0.05) in heterozygotes for the c.-1154G/A (rs1570360) variant between the women with EPLs and a live birth, compared to the controls. Also, when we analyzed the results from the division based on gestational week of the last pregnancy, we noticed statistically higher frequency of the heterozygous genotypes for both *VEGFA* variants with a p-value<0.00001 for c.-1154G/A (rs1570360) and p-value=0.05 for c.*237C/T (rs3025039).

CONCLUSION

Our current study introduces a cost-effective genotyping method for selected *ANXA5* and *VEGFA* variants. Additionally, our study is in line with numerous studies, supporting the concept that M2 and M1 *ANXA5* haplotypes are more common in patients experiencing pregnancy loss. Moreover, since there are data on some experimental therapies for the individuals carrying primarily the M2/*ANXA5* haplotype, a research in that direction and clinical studies for therapy justification could be a further step.

ACKNOWLEDGMENT

The study was supported by grant No. 08-707/2023 by the Macedonian Academy of Sciences and Arts to DPK.

CONFLICT OF INTEREST
Not declared.

REFERRENCES:

1. Macklon NS, Geraedts JP, Fauser BC. Conception to ongoing pregnancy: the 'black box'of early pregnancy loss. Human reproduction update. 2002 Jul 1;8(4):333-43
2. Eshre Guideline Group on RPL, Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, Nelen W, Peramo B, Quenby S, Vermeulen N. ESHRE guideline: recurrent pregnancy loss. Human reproduction open. 2018;2018(2):hoy004.
3. Practice Committee of the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertility and sterility. 2020 Mar 1;113(3):533-5.
4. Regan L, Rai R, Saravelos S, Li TC, Royal College of Obstetricians and Gynaecologists. Recurrent Miscarriage Green-top Guideline No. 17. BJOG: An International Journal of Obstetrics &Gynaecology. 2023 Nov;130(12):e9-39.
5. Bozhinovski G, Terzikj M, Kubelka-Sabit K, Jasar D, Lazarevski S, Livrinova V, Plaseska-Karanfilska D. Chromosomal Abnormalities in Early Pregnancy Losses: A Study of 900 Samples. Balkan J Med Genet. 2024 Mar 12;26(2):11-20.
6. Turesheva A, Aimagambetova G, Ukybassova T, Marat A, Kanabekova P, Kaldygulova L, Amanzholkyzy A, Ryzhkova S, Nogay A, Khamidullina Z, Ilmaliyeva A. Recurrent Pregnancy Loss Etiology, Risk Factors, Diagnosis, and Management. Fresh Look into a Full Box. Journal of Clinical Medicine. 2023 Jun 15;12(12):4074.
7. Bozhinovski G, Terzikj M, Kubelka-Sabit K, Plaseska-Karanfilska D. High Incidence of CPLANE1-Related Joubert Syndrome in the Products of Conceptions from Early Pregnancy Losses. Balkan Med J. 2024 Mar 1;41(2):97-104.
8. Deng T, Liao X, Zhu S. Recent advances in treatment of recurrent spontaneous abortion. Obstetrical & gynecological survey. 2022 Jun;77(6):355.;
9. Gerke V, Moss SE. Annexins: from structure to function. Physiological reviews. 2002 Apr 1;82(2):331-71.
10. Cai Z, Zheng X, Chen Y, Chen F, Chen L, Deng X. Genetic analysis of *ANXA5* haplotype and its effect on recurrent pregnancy loss. Molecular medicine reports. 2022 Feb 1;25(2):1-7.)
11. Bogdanova N, Horst J, Chlystun M, Croucher PJ, Nebel A, Bohring A, Todorova A, Schreiber S, Gerke V, Krawczak M, Markoff A. A common haplotype of the annexin A5 (*ANXA5*) gene promoter is associated with recurrent pregnancy loss. Human molecular genetics. 2007 Mar 1;16(5):573-8.10.
12. Ang KC, Bogdanova N, Markoff A, Ch'ng ES, Tang TH. Association between M2/*ANXA5* haplotype and repeated pregnancy loss: a meta-analysis. Fertility and sterility. 2019 May 1;111(5):971-81.
13. Peng L, Yang W, Deng X, Bao S. Research progress on *ANXA5* in recurrent pregnancy loss. Journal of Reproductive Immunology. 2022 Jul 28:103679.
14. Tüttelmann F, Ivanov P, Dietzel C, Sofroniou A, Tsvyatkovska TM, Komsa-Penkova RS, Markoff A, Wieacker P, Bogdanova N. Further insights into the role of the annexin A5 M2 haplotype as recurrent pregnancy loss factor, assessing timing of miscarriage and partner risk. Fertility and sterility. 2013 Nov 1;100(5):1321-5.

15. Rogenhofer N, Engels L, Bogdanova N, Tüttelmann F, Markoff A, Thaler C. Paternal and maternal carriage of the annexin A5 M2 haplotype are equal risk factors for recurrent pregnancy loss: a pilot study. Fertility and sterility. 2012 Aug 1;98(2):383-8.
16. Miyamura H, Nishizawa H, Ota S, Suzuki M, Inagaki A, Egusa H, Nishiyama S, Kato T, Pryor-Koishi K, Nakanishi I, Fujita T. Polymorphisms in the annexin A5 gene promoter in Japanese women with recurrent pregnancy loss. Molecular human reproduction. 2011 Jul 1;17(7):447-52.
17. Demetriou C, Abu-Amero S, White S, Peskett E, Markoff A, Stanier P, Moore GE, Regan L. Investigation of the Annexin A5 M2 haplotype in 500 white European couples who have experienced recurrent spontaneous abortion. Reproductive biomedicine online. 2015 Nov 1;31(5):681-8.
18. Tiscia G, Colaizzo D, Chinni E, Pisanelli D, Sciannamè N, Favuzzi G, Margaglione M, Grandone E. Haplotype M2 in the annexin A5 (*ANXA5*) gene and the occurrence of obstetric complications. Thrombosis and haemostasis. 2009;102(08):309-13.
19. Lan Y, Wang J, Zhang Q, Yang X, Li L, Yin J, Li H, Song X, Chen Z, Liu Y. Genetic variations and haplotypes in the annexin A5 gene are associated with the risk of recurrent pregnancy loss. Journal of cellular physiology. 2019 Oct;234(10):18308-16.
20. Ang KC, Kathirgamanathan S, Ch'ng ES, Lee YY, Roslani AL, Naidu B, Kumar K, Abdullah R, Kadir SN, Yusoff NM, Abdullah WZ. Genetic analysis of the M2/*ANXA5* haplotype as recurrent pregnancy loss predisposition in the Malay population. Journal of assisted reproduction and genetics. 2017 Apr;34:517-24.
21. Dryllis G, Giannopoulos A, Zoi C, Pouliakis A, Logothetis E, Voulgarelis M, Zoi K, Kouskouni E, Dinou A, Stavropoulos-Giokas C, Kreatsas G. Correlation of single nucleotide polymorphisms in the promoter region of the *ANXA5* (annexin A5) gene with recurrent miscarriages in women of Greek origin. The Journal of Maternal-Fetal & Neonatal Medicine. 2020 May 2;33(9):1538-43.
22. Cao Y, Zhang Z, Xu J, Yuan W, Wang J, Huang X, Shen Y, Du J. The association of idiopathic recurrent pregnancy loss with polymorphisms in hemostasis-related genes. Gene. 2013 Nov 10;530(2):248-52.
23. Nagirnaja L, Nõmmemees D, Rull K, Christiansen OB, Nielsen HS, Laan M. Annexin A5 promoter haplotype M2 is not a risk factor for recurrent pregnancy loss in Northern Europe. PLoS One. 2015 Jul 2;10(7):e0131606.
24. Sinem AY, Fatma S, Servet OH, Ahmet U, Emine C, Ozturk O. Fetal Vegf genotype is more important for abortion risk than mother genotype. CanakkaleOnSekiz Mart University, Turkey (2014).
25. Papazoglou D, Galazios G, Papatheodorou K, Liberis V, Papanas N, Maltezos E, et al. Vascular endothelial growth factor gene polymorphisms and idiopathic recurrent pregnancy loss. FertilSteril (2005);83:959–6.
26. Sun Y, Chen M, Mao B, Cheng X, Zhang X, Xu C. Association between vascular endothelial growth factor polymorphism and recurrent pregnancy loss: A systematic review and meta-analysis. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2017 Apr 1;211:169-76.
27. Xu X, Du C, Li H, Du J, Yan X, Peng L, Li G, Chen ZJ. Association of VEGF genetic polymorphisms with recurrent spontaneous abortion risk: a systematic review and meta-analysis. PLoS One. 2015 Apr 20;10(4):e0123696.
28. Trifonova EA, Swarovskaya MG, Ganzha OA, Voronkova OV, Gabidulina TV, Stepanov VA. The interaction effect of angiogenesis and endothelial dysfunction-related gene variants increases the susceptibility of recurrent pregnancy loss. Journal of Assisted Reproduction and Genetics. 2019 Apr 15;36:717-26.
29. Su MT, Lin SH, Chen YC. Genetic association studies of angiogenesis-and vasoconstriction-related genes in women with recurrent pregnancy loss: a systematic review and meta-analysis. Human Reproduction Update. 2011 Nov 1;17(6):803-12.
30. Eller AG, Branch DW, Nelson L, Porter TF, Silver RM. Vascular endothelial growth factor-A gene polymorphisms in women with recurrent pregnancy loss. Journal of reproductive immunology. 2011 Jan 1;88(1):48-52.
31. Fortis MF, Fraga LR, Boquett JA, Kowalski TW, Dutra CG, Gonçalves RO, Vianna FS, Schüler-Faccini L, Sanseverino MT. Angiogenesis and oxidative stress-related gene variants in recurrent pregnancy loss. Reproduction, Fertility and Development. 2018 Mar 13;30(3):498-506.
32. Noveski P, Terzic M, Vujovic M, Kuzmanovska M, Sukarova Stefanovska E, Plaseska-Karanfilska D. Multilevel regression modeling for aneuploidy classification and physical separation of maternal cell contamination facilitates the QF-PCR based analysis of common fetal aneuploidies. Plos one. 2019 Aug 20;14(8):e0221227.
33. Madjunkova S, Volk M, Peterlin B, Plaseska-Karanfilska D. Detection of thrombophilic mutations related

to spontaneous abortions by a multiplex SNaPshot method. Genetic testing and molecular biomarkers. 2012 Apr 1;16(4):259-64.

34. Atanasovska B, Bozhinovski G, Plaseska-Karanfilska D, Chakalova L. Efficient detection of Mediterranean β-thalassemia mutations by multiplex single-nucleotide primer extension. PLoS One. 2012 Oct 26;7(10):e48167.
35. Plaseski T, Noveski P, Popeska Z, Efremov GD, Plaseska-Karanfilska D. Association study of single-nucleotide polymorphisms in FASLG, JMJDIA, LOC203413, TEX15, BRDT, OR2W3, INSR, and TAS2R38 genes with male infertility. Journal of andrology. 2012 Jul 8;33(4):675-83.
36. Rogenhofer N, Markoff A, Wagner A, Klein HG, Petroff D, Schleussner E, EThIG II Group, Thaler CJ. Lessons from the EThIGII trial: proper putative benefit assessment of low-molecular-weight heparin treatment in M2/*ANXA5* haplotype carriers. Clinical and Applied Thrombosis/Hemostasis. 2017 Jan;23(1):27-33.
37. Fishel S, Baker DJ, Greer IA. LMWH in IVF–Biomarkers and benefits. Thrombosis Research. 2017 Mar 1;151:S65-9.
38. Vuorela P, Carpen O, Tulppala M, Halmesmäki E. VEGF, its receptors and the tie receptors in recurrent miscarriage. Molecular human reproduction. 2000 Mar 1;6(3):276-82.
39. Plaisier M, Dennert I, Rost E, Koolwijk P, Van Hinsbergh VW, Helmerhorst FM. Decidual vascularization and the expression of angiogenic growth factors and proteases in first trimester spontaneous abortions. Human reproduction. 2009 Jan 1;24(1):185-97.

BJMG 27 (1), 2024 • 15-20
DOI: 10.2478/bjmg-2024-0003
sciendo
ORIGINAL ARTICLE

IGHV MUTATIONAL STATUS IN A COHORT OF BULGARIAN CLL PATIENTS: HIGH UNMUTATED CLL PREVALENCE IN NORTH-EAST BULGARIA

Yosifova A[1], Micheva I[4], Donchev M[3], Tincheva S[1,2], Ormandjiev S[1,2], Genova J[1], Pavlova Z[1,2], Todorova A[1,2]

[*]**Corresponding Author:** Angelina Yosifova, Genetic Medico-Diagnostic Laboratory "Genica", Sofia, Bulgaria. Email: andjim91@gmail.com; Phone: +359888979313

ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. One of the best established CLL prognostic markers is the somatic hypermutational status of the *IGHV* gene which is a part of the immunoglobulin heavy chain variable region. Technology for IGHV genotyping has been optimized and has been applied in routine diagnostics for the first time in Bulgaria. A total of 105 patients with CLL from different Bulgarian regions were tested. IGHV mutational status was determined by Sanger sequencing on total genomic DNA (gDNA) or RNA extracted from mononuclear cells. All sequencing profiles were analyzed with the IMGT/V-QUEST tool. Within the course of the analysis a high percentage of IGHV unmutated status was established in the Varna district on the Black Sea (Northeast Bulgaria). In addition, the IGHV genotyping performed on gDNA revealed a rare case with multiple rearrangements. The present data from IGHV genotyping will help in choosing the proper treatment for the benefit of Bulgarian CLL patients.

Keywords: CLL, IGHV mutational status, multiple rearrangements

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults and is a remarkably heterogeneous disease. Some of the patients present with an indolent form and never require treatment, while others manifest rapidly progressive disease, despite therapy. One of the best established CLL prognostic markers is the somatic hypermutational status of IGHV gene, which is a part of the immunoglobulin heavy chain variable region [1].

B cell malignancies arise from clonal expansion of a single mature B cell. The rearrangement of immunoglobulin (IG) heavy chain genes is unique for all malignant B cells of a patient and is used as a powerful prognostic marker: IGHV mutational status. The individual IGHV mutational status is examined in the context of its existing prognostic values and the effect it has on personalized CLL therapy [2]. The malignant B cells all have certain B cell receptor (BCRs) signaling, mainly expressing certain IgM and IgD isotypes. An important factor is the existing BCR stereotypy among CLL patients with a possible effect on the disease pathogenesis [3].

The assembly of the variable region of the immunoglobulin heavy chain in the process of formation and maturation of the B-cells represents a chromosomal recombination of V (variable), D (diversity), and J (junctional) segments. The specificity of the antibody is determined by three main complementarity-determining regions (CDRs) and a relatively constant sequence called the framework region (FR). Every V segment encodes the first three framework regions (FR1, FR2 and FR3) along with the CDR1 and CDR2 regions, as well as a part of the CDR3 region. Each D segment covers CDR3 completely. The J segment begins with its own recombination signal and encodes the complete FR4, as well as a part of CDR3 [4,5] (Figure 1).

[1] Genetic Medico-Diagnostic Laboratory "Genica", Sofia, Bulgaria
[2] Independent Medico-Diagnostic Laboratory "Genome Center Bulgaria", Sofia, Bulgaria
[3] National Specialized Hospital for Active Treatment of Hematological Diseases, Clinical Hematology Department 1, Sofia, Bulgaria
[4] University Multiprofessional Hospital for Active Treatment "Sveta Marina", Varna, Bulgaria

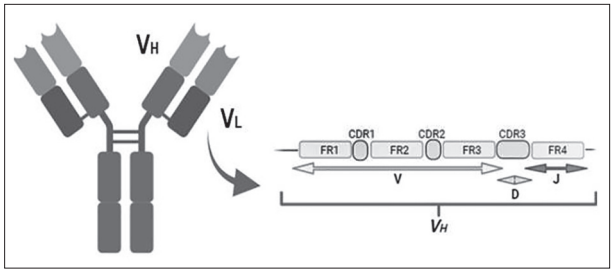

Figure 1. Structure of immunoglobulin heavy chain variable region.
VH – variable region of heavy chain; VL – variable region of light chain; V – variable segment; D – diversity segment; J – junctional segment; CDR – complementarity-determining region; FR – framework region

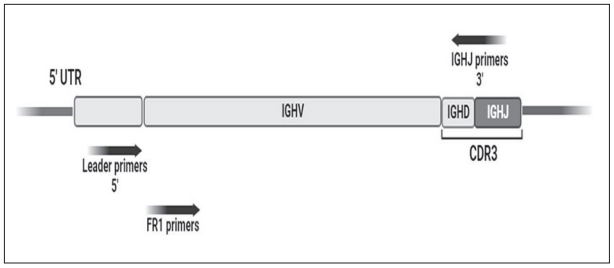

Figure 2. Locations of the primers for V-D-J gene rearrangements.
5' IGHV Leader primers are located upstream of the IGHV coding sequence; 5' IGHV FR1 (framework) primers are located within the rearranged IGHV gene; 3' IGHJ primers are located at the end of the rearranged IGHJ gene; UTR – untranslated region

This present study focusses on the implementation of the IGHV mutational status analysis in a cohort of Bulgarian CLL patients because of the high value of this marker in determining the disease outcome and selecting the appropriate treatment.

MATERIALS AND METHODS

The present prospective study includes a total of 105 newly diagnosed CLL patients (72 males and 33 females) at the ages of 38-80 years from different Bulgarian regions. To the best of our knowledge, all of them have previously tested negative by FISH for 17p13 deletion, and none of them have received treatment prior to IGHV testing. All patient samples were obtained after signing an informed consent form.

Samples collection and cells extraction: IGHV mutational status was determined by PCR/Sanger sequencing on genomic DNA (gDNA) or complementary DNA (cDNA) extracted from venous blood mononuclear cells collected in EDTA tubes. During the whole process of testing, ERIC Recommendations were strictly followed [6].

Mononuclear cells were obtained after density gradient separation by FiColl-Paque PLUS with some in-house modifications: after gradient centrifugation, the mononuclear cell pellet formed between the upper plasma layer and FiColl fraction was dissolved in Dulbecco's Phosphate-Buffered Saline (D-PBS 1x – calcium and magnesium free), instead of Roswell Park Memorial Institute (RPMI 1640 Medium 1x).

Total DNA/RNA was extracted from collected mononuclear cells. The preferred target for us is gDNA because there is no need for a reverse transcription step. High molecular weight gDNA was extracted by QIAGEN - QIAmp® DNA Blood kit following the manufacture's instructions. Total RNA (extracted by ZYMO Research - Quick-RNA Viral kit), followed by cDNA synthesis (SensiFAST cDNA Synthesis Kit) was used only in 9 problematic cases, in which gDNA amplification showed unproductive rearrangements or poor quality of the sequencing profile.

Following ERIC recommendations, the PCR amplification was performed with leader primers in a multiplex reaction. In rare cases (n=3 patients), the amplification of the clonotypic IG rearrangement was successful only by utilization of internal IGHV FR1 (framework) primers (Figure 2). In all 3 cases the amplification with the leader primers failed. Consensus primers targeting the IGHJ genes were used in reverse direction. These results were interpreted with caution, because FR1 primers do not amplify the entire V region [7].

The PCR amplification was performed with primers and protocols, according to ERIC recommendations and BIOMED2 [8,9].

The analysis of the rearranged IG sequences in FASTA format was performed with the IMGT/V-QUEST tool [10]. Cases with ≥98% identity were considered unmutated, while those with a homology less than 98% - mutant type, and cases were considered borderline when the homology is between 97-97.99% [11].

BCR stereotyped subsets were determined by ARResT/AssignSubsets online tool [12, 13].

Statistical analysis for correlation was performed by using Fisher's Exact Test.

RESULTS AND DISCUSSION

Following the 98% identity cut-off value, a total of 57 patients were genotyped as unmutated IGHV (U-CLL), 44 – as mutated (M-CLL), and 4 – as borderline (B-CLL) (Figure 3). Different BCR stereotyped subsets were found in 7 out of 105 cases (6.67%) (Table 1).

According to the published data, the expected ratio of unmutated and mutated cases at diagnosis are 40% vs. 60%, respectively [14,15]. Within the course of the analysis, a high prevalence of unmutated CLL patients was detected in the Varna district on the Black Sea (Northeast Bulgaria).

Table 1. Clinico-biological features and genetic characteristics of mutated and unmutated cases

Patients	Leukocytes count *(4.5 to 10x10⁹/L)**	General condition (Fit)	Treatment	17p deletion	Common IGHV genes	BCR stereotyped subsets (n)
44 mutated	15-180	Good condition	Newly diagnosed; Before first treatment	Negative	IGHV1-2 (4) IGHV3-7 (6) IGHV3-23 (4) IGHV4-34 (4) IGHV4-59 (4)	CLL#77 (1)
57 unmutated	17-328				IGHV1-69 (17) IGHV3-23(4) IGHV5-51 (5)	CLL#2 (1) CLL#5 (1) CLL#6 (2) CLL#8 (1) CLL#99 (1)

* White blood cell (WBC) count range
n – number of patients

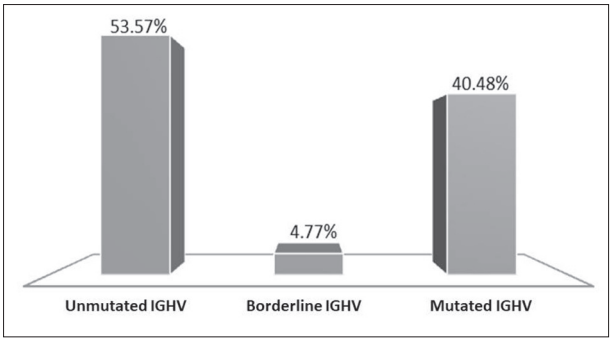

Figure 3. Distribution of Bulgarian CLL patients by their IGHV mutational status.
57 patients – unmutated IGHV; 44 patients – mutated IGHV;
4 patients – Borderline IGHV

From a total of 24 patients from the Varna region, 17 (75%) showed an unmutated status, hence more aggressive CLL, which we hypothesize might be related to the regional industrial activities. For the rest of the 81 patients originating from different regions in Bulgaria, the unmutated patients were 41 (51%). Fisher's Exact Test showed a statistically significant correlation between the region of origin of the patients and their IGHV mutational status (p=0.028, two-tailed Fisher's Exact Test), but this finding might be biased by the small number of patients tested (Table 2).

Furthermore, a difficult to categorize case with multiple rearrangements (triple productive rearrangements) was detected (Table 3). For diagnostic purposes, an analysis was performed on gDNA, and the obtained quality of the sequencing profiles was highly satisfactory, therefore RNA transcripts

Table 2. Fisher's Exact test for correlation between the region of origin of the patients and their IGHV mutational status.

	Origin of samples	
IGHV mutational status	**Varna (N)**	**Others (N)**
U-CLL*	17	41
M-CLL*	5	38
B-CLL*	2	2
two-tailed p value (α = 0.05)		0.028

*U-CLL – unmutated; M-CLL – mutated; B-CLL - borderline

were not tested. This case was interpreted and reported as unmutated, based on the published data showing a shift in favor of unmutated IGHV in a majority of the discordant cases [16]. In such cases, when discordant multiple productive rearrangements were detected, the resulting prognosis is inconclusive, and it is recommended to be considered and treated as a more aggressive unmutated status [16]. Following the current recommendations, cases with discordant multiple rearrangements, should be re-tested after six months [16].

In conclusion, the present data from IGHV genotyping could aid in estimating the disease's course and how to choose optimal initial treatment for Bulgarian CLL patients. Patients with unmutated IGHV CLL tend to relapse earlier due to the more aggressive course of the disease [17,18]. These patients have also demonstrated less benefit from treatment with chemoimmunotherapy and BCL2 inhibitors compared to patients with mutated IGHV, while Bruton Tyrosine Kinase (BTK) inhibitors have the same efficacy irrespective of the IGHV mutational status.

Table 3. Discordant mutational status in case with multiple IGHV rearrangements

IGHV gene	IGHV status	Identity %	CDR3 amino-acid length	Results
IGHV2-70	unmutated	100	19	Unmutated
IGHV3-30	mutated	96,53	16	
IGHV4-4	mutated	91,32	14	

ACKNOWLEDGEMENTS

The financial support of Johnson & Johnson Bulgaria for IGHV testing and blood sample transportation is gratefully acknowledged.

Our genetic team is extremely thankful to the European Research Initiative on CLL (ERIC) for their continuous support and provided training.

To all the physicians:

- Prof. Liana Gercheva MD and co-workers – Acibadem City Clinic "Tokuda" Hospital, Hematology Clinic, Sofia
- Assoc. prof. Evgenii Hadzhiev MD and coworkers - University and Multispecialty Hospital "Alexandrovska", Hematology clinic, Sofia
- Assoc. prof. Vanya Slavcheva MD and co-workers – University Hospital "Dr. Georgi Stranski", Hematology clinic, Pleven

Conflict of Interest: The authors report there are no competing interests to declare.

REFERENCES

1. Rajendra ND, Tarun W, Franco F et al. Ig V Gene Mutation Status and CD38 Expression As Novel Prognostic Indicators in Chronic Lymphocytic Leukemia, Blood, Volume 94, Issue 6, 1999, Pages 1840-1847.
2. Sutton, L. A., et al. Immunoglobulin genes in chronic lymphocytic leukemia: Key to understanding the disease and improving risk stratification. Haematologica. 102 (6), 968-971 (2017).
3. Ten Hacken E, Gounari M, Ghia P et al. The importance of B cell receptor isotypes and stereotypes in chronic lymphocytic leukemia. Leukemia. 2019 Feb;33(2):287-298. doi: 10.1038/s41375-018-0303-x. Epub 2018 Dec 16. PMID: 30555163; PMCID: PMC7182338.
4. Schroeder HW Jr, Cavacini L. Structure and function of immunoglobulins. J Allergy Clin Immunol. 2010 Feb;125(2 Suppl 2):S41-52. doi: 10.1016/j.jaci.2009.09.046. PMID: 20176268; PMCID: PMC3670108
5. Calis JJ, Rosenberg BR. Characterizing immune repertoires by high throughput sequencing: strategies and applications. Trends Immunol. 2014;35(12):581–590.
6. Agathangelidis A, Chatzidimitriou A, Chatzikonstantinou T et al. Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: the 2022 update of the recommendations by ERIC, the European Research Initiative on CLL. Leukemia 2022; 36, 1961–1968.
7. Huet, Sarah & Bouvard, Anne & Ferrant, Emmanuelle & Mosnier, Isabelle & Chabane, Kaddour & Salles, Gilles & Michallet, Anne-Sophie & Hayette, Sandrine & Sujobert, Pierre. (2020). Impact of using leader primers for IGHV mutational status assessment in chronic lymphocytic leukemia. Leukemia. 34. 10.1038/s41375-020-0716-1.
8. Agathangelidis A, Sutton LA, Hadzidimitriou A et al. Immunoglobulin Gene Sequence Analysis In Chronic Lymphocytic Leukemia: From Patient Material To Sequence Interpretation. 2018 J. Vis. Exp. (141)
9. Matthews C, Catherwood M, Morris TC et al. Routine analysis of IgVH mutational status in CLL patients using BIOMED-2 standardized primers and protocols. Leuk Lymphoma. 2004 Sep;45(9):1899-904.
10. Brochet X, Lefranc MP, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res. 2008 Jul 1;36(Web Server issue):W503-8.
11. Jain P, Nogueras Gonzalez GM, Kanagal-Shamanna R. The absolute percent deviation of IGHV mutation rather than a 98% cut-off predicts survival of chronic lymphocytic leukaemia patients treated with fludarabine, cyclophosphamide and rituximab. Br J Haematol. 2018; 180(1):33-40.
12. Darzentas N. ARResT/Interrogate Immunoprofiling Platform: Concepts, Workflows, and Insights. 2022 May 28. In: Langerak AW, editor. Immunogenetics: Methods and Protocols [Internet]. New York: Humana; 2022. Chapter 26. Available from: https://www.ncbi.nlm.nih.gov/books/NBK586953/ doi: 10.1007/978-1-0716-2115-8_26;
13. Bystry V, Agathangelidis A, Bikos V et al, also on behalf of ERIC, the European Research Initiative on CLL, ARResT/AssignSubsets: a novel application for robust subclassification of chronic lymphocytic leukemia based on B cell receptor IG stereotypy, Bioinformatics, Volume 31, Issue 23, December 2015, Pages 3844–3846,
14. Gaidano G, Rossi D. The mutational landscape of chronic lymphocytic leukemia and its impact on prognosis and treatment. Hematology Am Soc Hematol Educ Program. 2017 Dec 8;2017(1):329-337. doi: 10.1182/asheducation-2017.1.329. PMID: 29222275; PMCID: PMC6142556.

15. Hanson C. GHV and TP53 Sequencing: Clinical Utility in Chronic Lymphocytic Leukemia (CLL). Available from: https://news.mayocliniclabs.com/2019/08/12/ighv-and-tp53-sequencing-clinical-utility-in-chronic-lymphocytic-leukemia-cll
16. Plevova K, Francova HS, Burckova K et al. Multiple productive immunoglobulin heavy chain gene rearrangements in chronic lymphocytic leukemia are mostly derived from independent clones. Haematologica. 2014 Feb;99(2):329-38.
17. Rotbain EC, Frederiksen H, Hjalgrim H, et al. IGHV mutational status and outcome for patients with chronic lymphocytic leukemia upon treatment: a Danish nationwide population-based study. Haematologica. 2020 Jun;105(6):1621-1629
18. Gemenetzi K, Agathangelidis A, Zaragoza-Infante L et al. B Cell Receptor Immunogenetics in B Cell Lymphomas: Immunoglobulin Genes as Key to Ontogeny and Clinical Decision Making; Frontiers in Oncology 10, 2020;

BJMG 27 (1), 2024 • 21-30
DOI: 10.2478/bjmg-2024-0002
sciendo

ORIGINAL ARTICLE

COMPARISON OF FGF -8, FGF -10, FGF- RECEPTOR 2, ANDROGEN RECEPTOR, ESTROGEN RECEPTOR-A AND SS IN HEALTHY AND HYPOSPADIAC CHILDREN

Emaratpardaz N[1], Turkyilmaz Z[1], Karabulut R[1*], Dayanir D[2], Kaya C[1], Sert AAE[2], Arkan G[1], Ucaner FA[1], Kapisiz A[1], Eryilmaz S[1], Atan A[3], Sonmez K[1]

[*]**Corresponding Author:** Prof. Ramazan Karabulut, MD, Gazi University Faculty of Medicine, Department of Pediatric Surgery, Besevler, 06550, Ankara-Turkey. Tel: +90 312 2026210; Fax: +90 312 2230528. E-mail: karabulutr@yahoo.com, ramazank@gazi.edu.tr

ABSTRACT

In this study, we aimed to investigate the levels of Fibroblast Growth Factor-8 (FGF-8), FGF-10, FGF-Receptor-2 (FGFR-2), Androgen receptor (AR), Estrogen receptor alpha and beta (ER-α and ER-ß) in the foreskins of children with and without hypospadias.

Methods: Samples from the foreskins of 20 children with hypospadias and 20 skin samples from children without hypospadias between the ages of 14 months and 12 years were taken during circumcision or hypospadias correction surgery for immunohistochemical (IHC) examination of these markers. In IHC examination, it was shown that ER-α, ER-ß and AR receptors were more involved in the foreskin of children with hypospadias than in the foreskin of without hypospadias children, and FGF-8, FGF-10 and FGFR-2 were lower ($p<0.05$). ER and AR uptake were higher in hypospadias tissue samples and FGF-8, FGF-10, and FGFR-2 uptakes were lower compared to without hypospadias children's tissue samples, and these factors were supported by affecting each other in the development of hypospadias. The limited number of studies on this subject in the literature and the contradictory results of the findings indicate that more research should be done on this subject in the future.

Keywords: hypospadias, FGF receptors, androgen receptor, estrogen receptors, etiology

[1] Gazi University Faculty of Medicine, Departments of Pediatric Surgery, Ankara, Turkey

[2] Gazi University Faculty of Medicine, Departments of Histology and Embryology Ankara, Turkey

[3] Gazi University Faculty of Medicine, Departments of Urology, Ankara, Turkey

INTRODUCTION

Hypospadias is a genital anomaly characterized by the location of the external urethral meatus on the ventral side of the penis, in the scrotum, or perineum, affecting approximately one in every 200-300 boys. It is considered to be the most common congenital anomaly after undescended testis in boys [1,2]. There are many classifications for hypospadias. According to Duckett's classification, which is widely used by pediatric surgeons, hypospadias is classified as proximal and distal. In distal hypospadias, detected in approximately 70% of cases, the urethral meatus may be located in the glandular area, coronal area, and distal shaft of the penis. In proximal hypospadias, which is detected in approximately 30% of cases, the urethral meatus may be located in the middle penile region, proximal penile shaft, penoscrotal or perineal regions [3]. Investigations of genetic, endocrine and environmental factors in the etiology of hypospadias are still ongoing. Studies on the effects of environmental and endocrine factors have shown that factors such as small gestational age (SGA), placental insufficiency, maternal hypertension, preeclampsia, antiepileptic drugs use, multiple pregnancy, gestational diabetes, maternal obesity often play an effective role in the etiology of hypospadias [4]. Although many genetic syndromes include hypospadias, hypospadias due to genetic causes accounts for less than 10% of all cases. The risk of developing hypospadias in a brother of a patient with hypospadias is more than 10% [5]. Although it is known that the frequency of proximal hypospadias increases with some syndromes, there is usually sporadic occurrence for distal hypospadias. It has been shown that the expression of some genes and defects in androgen and estrogen production in the genital tubercle and urethral plate may also be effective in the etiology of hypospadias

(*SHH, ER1, ER2, GL1, GL2, GL3, ATF3, FGF-8, FGFR-2, VAMP7, WT1, BMP7, WNT5A), DGKK* etc.) [5,6]. It is known that Fibroblast Growth Factors (FGF) have a very important contribution to the fusion of the urethral folds and the development of the genital tubercle, and play a role in wound healing and tissue regeneration [7]. Additionally, androgen receptors (AR) are also found to play an important role in male sexual differentiation with the effect of gonadal androgens. Decreased expression of AR has also been reported to have consequences such as infertility, testicular atrophy, micropenis, hypospadias, and decreased sperm production. The decreased response to human chorionic gonadotropin (hCG) stimulation in children with hypospadias points to the importance of this receptor in the etiology of hypospadias [8]. Studies have found that there were significant differences in terms of estrogen receptors (ERs) in the foreskins of children with or without hypospadias, and these data showed that this difference may play a role in the etiology of hypospadias [9,10,11]. As well as the surgical treatment of hypospadias, investigation into ways of preventing the disease has gained importance due to its increasing prevalence [12]. Prevention of hypospadias or medical treatment research is important to prevent psychosexual problems of the patients due to possible chordee, fistula and poor cosmetic results that may occur in adulthood after surgical treatment applied in childhood.

In this study, levels of fibroblast growth factor 8 (FGF-8), FGF-10, FGF Receptor-2 (FGFR-2), AR, ER-α and ER-ß were compared in order to discover the etiology of hypospadias in foreskin tissues of children with and without hypospadias.

MATERIAL AND METHODS

Approval from the Gazi University Ethical Committee was obtained before the study began (07/03/2022-191). The study includes the children with hypospadias and the ones without who came to our clinic for circumcision. The children who underwent circumcision were the control group. After giving detailed information, written informed consent was obtained from their legal guardians (parents). Children who had previous hypospadias surgery, received hormone therapy, had accompanying urological anomalies such as undescended testis, had endocrine disorders and syndromes were excluded from the study. Between June 2022 and September 2022, 20 children with hypospadias and 20 children who applied just for circumcision were included. All children met the criteria. During circumcision and hypospadias correction surgery, the foreskin tissues about 1x1 cm from healthy and children with hypospadias were taken for histomorphological and immunohistochemical (IHC) studies. Levels of AR, ER-α and ER-ß, FGF-8, FGF-10, FGFR-2 were evaluated in these tissues.

Using Microsoft Office Excel Professional Plus 2016 (Microsoft Corporation, Redmond, WA, USA), statistical analysis was carried out. To compare the quantitative data between two groups that did not exhibit a normal distribution, the Mann-Whitney U test was employed.

Histomorphological Evaluation

Tissue samples were fixed in 10% neutral formaldehyde for 48 h. After washing the fixed tissues in running water for 24 hours, they were kept in 70% ethyl alcohol, 80% ethyl alcohol, 96% ethyl alcohol, Acetone I, Acetone II, Acetone III and Acetone IV, for 20 minutes each, respectively. Tissues extracted from acetone were kept in Xylene I and Xylene II for 30 minutes each. Before the tissues were embedded, they were kept in molten Paraffin I and Paraffin II for 1 hour in an oven at 60°C and embedded in paraffin blocks. Sections of 6μm thickness obtained from the blocks were kept in xylol for 2 times for 15 minutes. Samples extracted from xylol were kept in ethyl alcohol solutions at 100%, 96%, 90%, 80%, 70% and 50% concentrations, respectively, for 10 minutes, then rehydrated by soaking them in distilled water for 5 minutes, twice. Sections obtained from the blocks were then stained with Hematoxylin Eosin (H&E) for histological evaluation. The samples, which were dehydrated by passing through the alcohol series, were kept in xylol and covered with a lamella using entellan. All specimens were evaluated histopathologically by taking photographs with the Leica Q Vin 3 program with the help of Leica DM4000 (Germany) computer aided imaging system. These evaluations were examined separately by 2 instructors working in the Histology and Embryology Department.

Immunohistochemical evaluation

Tissue samples were processed similar to the histomorphological stage. Afterwards, the tissues were lined up on the immunohistochemistry bar in a humid environment and were scratched with PAP-Pen and washed 3 times with PBS (Phosphate Buffer Saline, pH: 7.4) for 3 minutes. Samples were treated with serum blocking solution for 10 minutes to prevent non-specific binding, AR Ab-1(Cat. No. MS-443-PO, Thermo Fisher Scientific, Cheshire, UK), ER-α (Cat. No. RM-9101-SO), Thermo Fisher Scientific, Cheshire, UK), ER-ß (Cat. No. orb448242, Biorbyt Ltd., Cambridge, UK), FGFR-2 (Cat. No. STJ91850, St Johns Laboratory Ltd., London, UK UK), FGF-8 (Cat. No. PA1216, Boster Biological Technology, Pleasanton, CA, USA), FGF-10 (Cat. No. E-AB-65862, Elabscience Biotechnology Inc., Houston, TX, USA) primary anti-

bodies were incubated at +4°C for 1 night. After incubation, a 3% hydrogen peroxide solution was applied to the samples washed with PBS for 15 minutes to inhibit endogenous peroxidase activity. After washing the samples with PBS, a secondary antibody with biotin was applied. Again, the samples were washed 3 times with PBS for 3 minutes, and chromogen containing diaminobenzidine (DAB) substrate was applied and left until a visible immune reaction occurred. Mayer's Hematoxylin was used as background dye for the samples washed with PBS. The samples, which were dehydrated by passing through the alcohol series, were kept in xylol and covered with a lamella using entellan. All samples were evaluated with the help of the Leica DM4000 (Germany) computer aided imaging system. Photographs were taken using the Leica Q Vin 3 program. Uptake of AR, ER-α and β in cell counts provided in 10 independent fields selected for each slide and were evaluated with the scoring system specified by Qiao et al. [9] (Table 1).

The evaluation of FGF-8, FGF-10 and FGFR-2 was done as follows, taking the study of Haid et al. as an example [7];

Epidermis assessment
- Pattern 1: Limited to basal involvement
- Pattern 2: Less than 50% of keratinocytes are involved
- Pattern 3: Involvement of more than 50% of keratinocytes

Table 1. Quantitative scoring system of stratified squamous epithelium by immunohistochemistry for AR, ER-α and ER-β.

Score	Grade
0	No involvement
+	Involvement in basal keratinocytes
++	Involvement limited to the lower 1/3 of the epidermis
+++	Involvement limited to the lower 2/3 of the epidermis
++++	Involvement in all epidermis layers

Dermis evaluation:
- Pattern A: No positive cells
- Pattern B: diffuse involvement in less than 50% of all visible dermal cells
- Pattern C: Clustered positive cells more than 50% of all visible dermal cells.

RESULTS

The study was carried out with a total of 40 children. The children's ages ranged from 14 months to 12 years with a mean age of 65.92±33.20 months. The mean age in the control group was 59.40±31.80 months (12-108 months) and the mean age in the hypospadias group was 72.45±34.09 (14-132 months). Of the patients with hypospadias, 2 had coronal, 11 had subcoronal and 7 had mid-penile hypospadias. In the H&E stained sections of the preputial tissues obtained from both groups, the epidermis was observed to be consistent with the features of the stratified squamous epithelium. There was no significant histomorphological differences between two groups ($p>0.05$).

IHC uptake of AR, ER- α, ER- β markers was evaluated with the quantitative scoring system of stratified squamous epithelium [9]. While the score was (+) in 18 patients in the AR control group, the score was (+++) in 15 patients with hypospadias and (++) in 5 patients. Similarly, ER-α and ER-β markers were detected in a larger area in the epidermis in the hypospadias group (Table 2), (Fig. 1, 2 and 3). When the AR and ER receptors were scored, it was shown that the foreskin of children with hypospadias contained statistically higher AR and ERs than the foreskin of children without hypospadias (Table 4) ($p<0.05$).

The levels of FGFR-2, FGF-8, FGF-10 in epidermis and dermis were quantitatively evaluated. Th involvement pattern was classified as 1, 2, 3 for epidermis and classified as A, B, C for dermis. When the epidermis area was examined, Pattern 3 involvement was observed

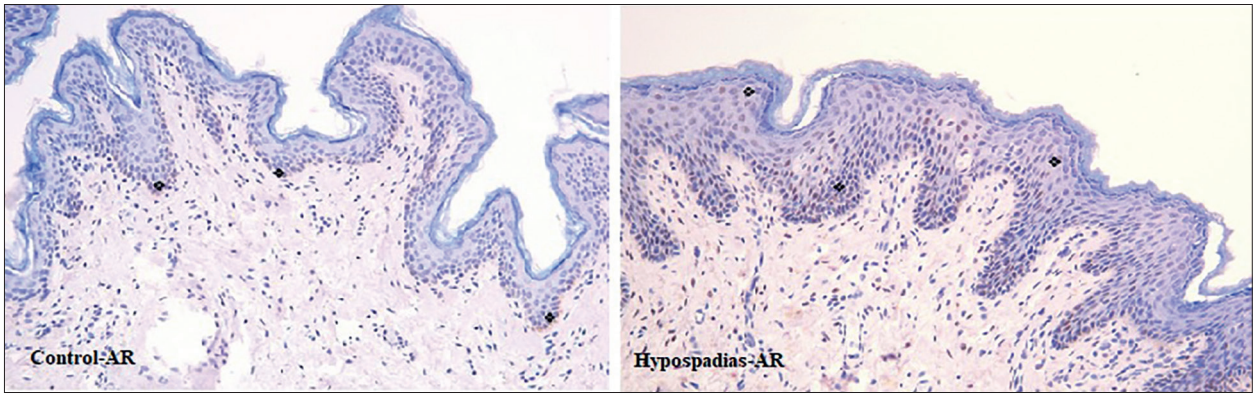

Figure 1. AR uptake was observed in basal keratinocytes in control group. AR involvement was limited to the lower 2/3 of the epidermis in the hypospadias group (IHC Staining, 200 X).

Table 2. Quantitative scoring results of stratified squamous epithelium for AR and ERs (n=20).

	AR		Estrogen Receptor- α		Estrogen Receptor- β	
Score	**Control**	**Hypospadias**	**Control**	**Hypospadias**	**Control**	**Hypospadias**
0	1	-	2	-	-	-
+	18	-	17	-	18	-
++	1	5	1	4	2	3
+++	-	15	-	16	-	17
++++	-	-	-	-	-	-

Table 3. Epidermis and dermis involvement of FGFR-2, FGF-8, FGF-10(n=20).

		FGFR-2		FGF-8		FGF-10	
	Pattern	**Control**	**Hypospadias**	**Control**	**Hypospadias**	**Control**	**Hypospadias**
Epidermis	1	-	-	-	16	-	17
	2	4	-	5	4	2	3
	3	16	5	15	-	18	-
Dermis	A	-	1	-	3	-	2
	B	3	19	14	17	3	18
	C	17	-	6	-	17	-

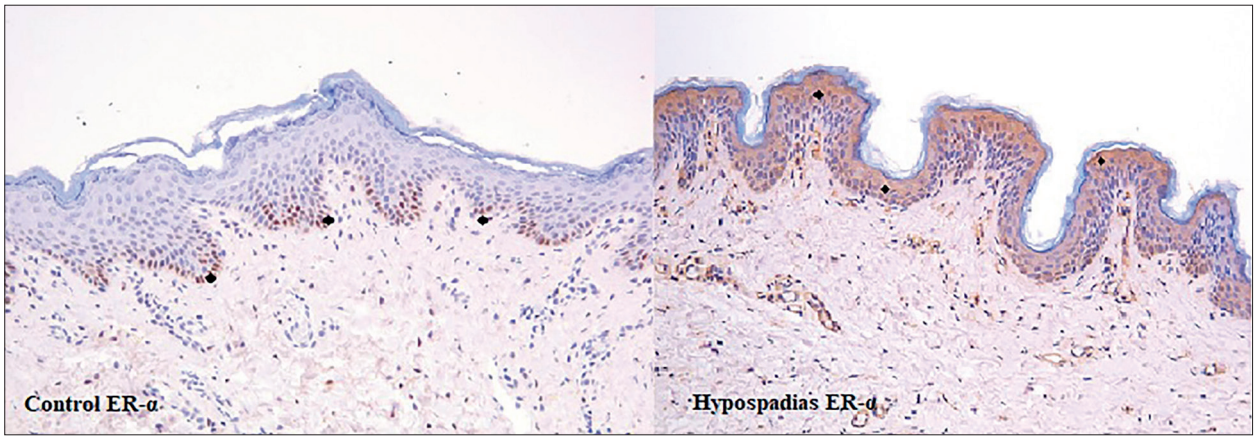

Figure 2. ER-α uptake was observed in basal keratinocytes in control group. ER-α involvement was limited to the lower 2/3 of the epidermis in the hypospadias group (IHC Staining, 200 X).

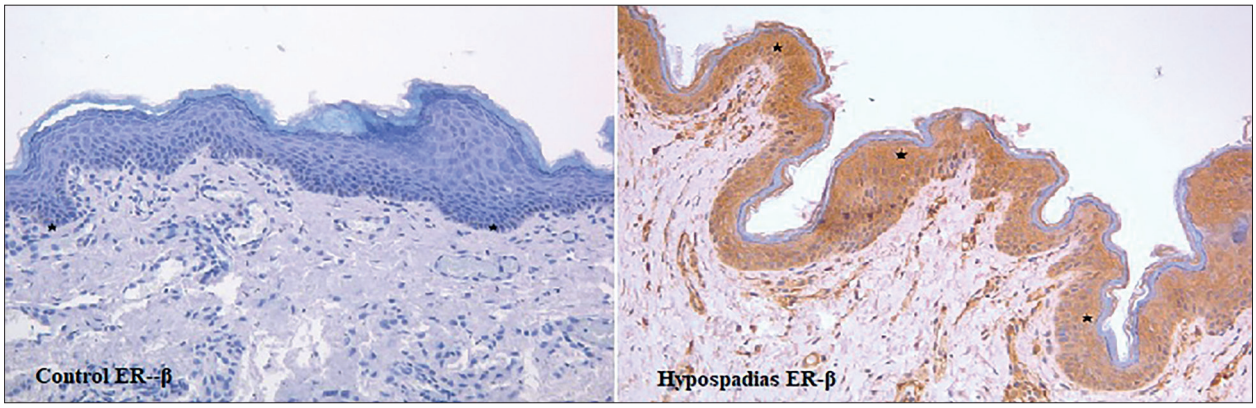

Figure 3. ER-β uptake was observed in basal keratinocytes in control group. ER-β involvement was limited to the lower 2/3 of the epidermis in the hypospadias group (IHC Staining, 200 X).

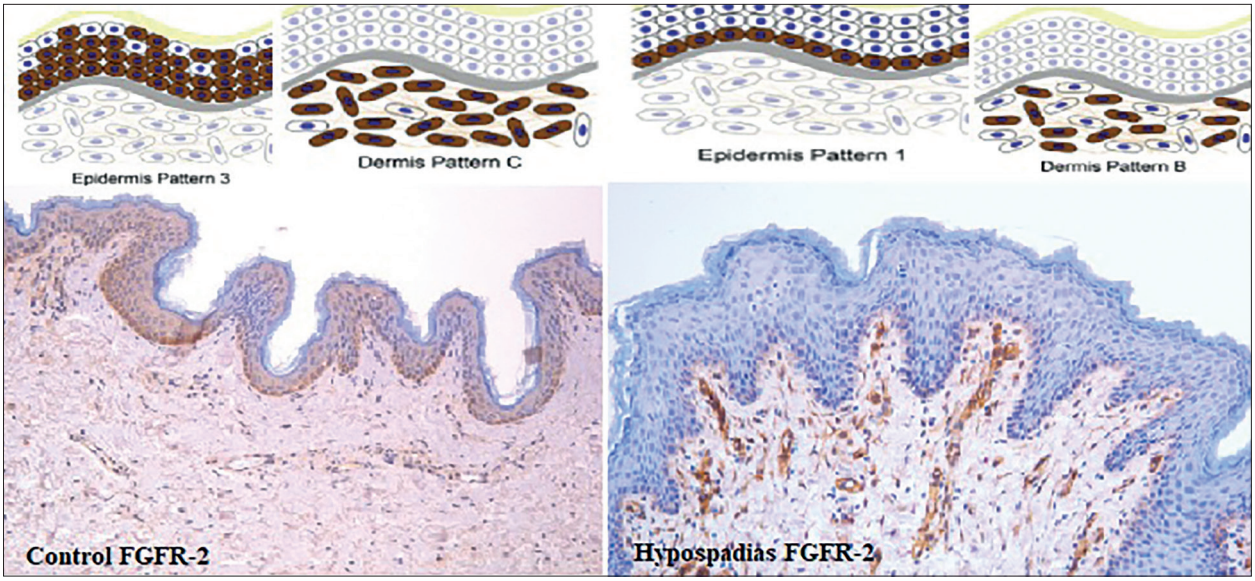

Figure 4. In the control group, FGFR-2 uptake was observed in more than 50% of Keratinocytes (Epidermis Pattern 3) and clustered positive cells in more than 50% of all visible dermal cells (Dermis Pattern C). Limited to basal involvement in hypospadias group (Epidermis Pattern 1) and involvement of less than 50% of dermal cells (Dermis Pattern B) (IHC Staining, 200 X).

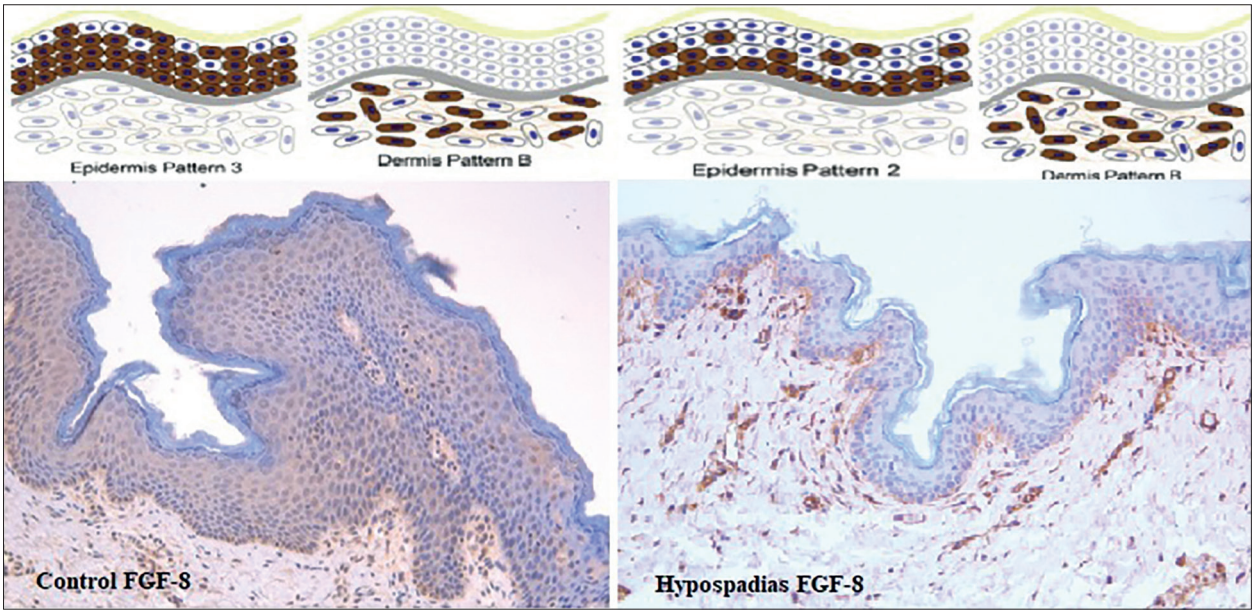

Figure 5. In the control Group, more than 50% of keratinocytes were involved (Epidermis Pattern 3) and less than 50% of dermal cells are involved (Dermis Pattern B) for FGF-8. In the hypospadias group, it was limited to basal involvement (Epidermis Pattern 1) and involvement of less than 50% of dermal cells (Dermis Pattern B) (IHC Staining, 200 X).

predominantly in control patients, while Pattern 1 involvement was observed in patients with hypospadias. When the dermis areas were examined, it was observed that 17 of the patients in the control group of FGFR-2 had Pattern C, 3 had Pattern B, 19 patients with hypospadias had Pattern B and 1 had Pattern A involvement (Fig. 4). If FGF-8, 6 Pattern C and 14 Pattern B uptake were detected in control patients, while 17 Pattern B and 3 Pattern A uptake were detected in hypospadias patients (Fig.5). It was Pattern C in 17 of the FGF-10 control patients, Pattern B in 3 of the patients with hypospadias, it was observed as Pattern B in 18 of the patients with hypospadias and as Pattern A in 2 of the patients with hypospadias (Fig.6), (Table 3).

Table 4. Mean ± SD values of the markers in the groups for IHC evaluation. *P<0.001, **P<0.05.

	Control	Hypospadias
Androgen Receptor	*2±0,32*	3,75±0,44*
Estrogen Receptor- α	*1,95±0,39*	3,80±0,41*
Estrogen Receptor -β	*2,10±0,30*	3,85±0,36*
FGFR-2 Epidermis	*3,80±0,41*	2±0,32*
FGFR-2 Dermis	*2,85±0,36*	1,95±0,22*
FGF-8 Epidermis	*3,75±0,44*	2,15±0,48*
FGF-8 Dermis	*2,30±0,47*	1,85±0,36**
FGF-10 Epidermis	*3,90±0,30*	2,15±0,36*
FGF-10 Dermis	*2,85±0,36*	1,90±0,30*

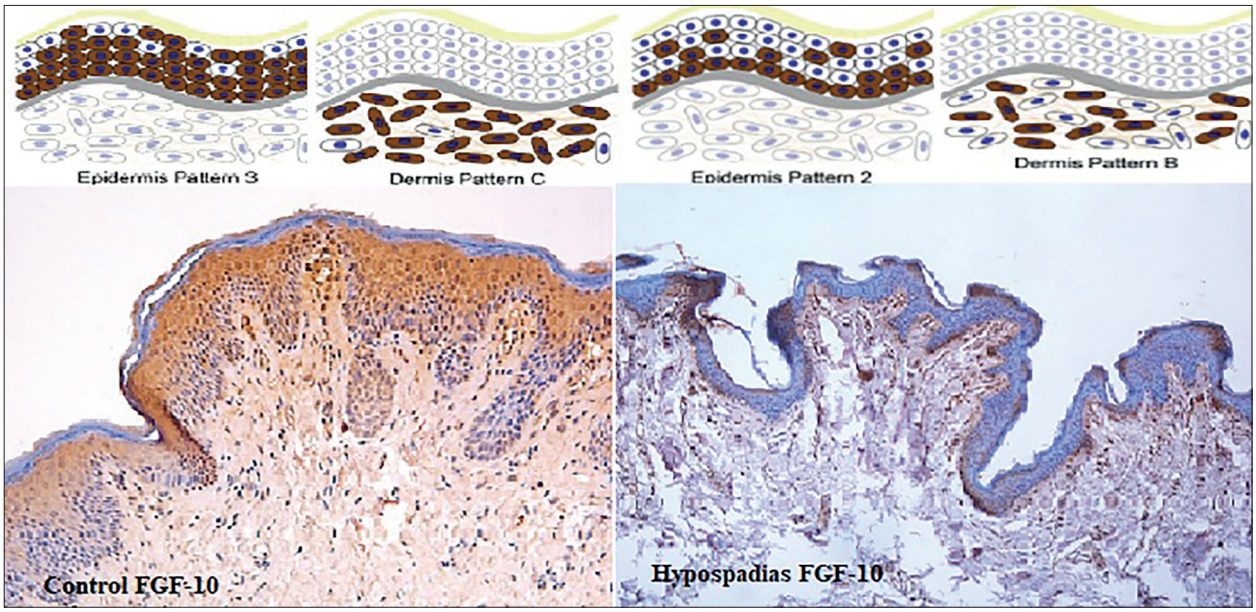

Figure 6. More than 50% involvement of keratinocytes (Epidermis Pattern 3) and clustered positive cells more than 50% of all visible dermal cells (Dermis Pattern C) for FGF-10 in the control group. In the hypospadias group, less than 50% of keratinocytes were involved (Epidermis Pattern 2) and involvement of less than 50% of dermal cells (Dermis Pattern B) (IHC Staining, 200 X).

There was a higher rate of uptake in both epidermis and dermis areas for FGFR-2, FGF-8 and FGF-10 in the control group. There was a statistically significant difference between two groups (p<0.05) (Table 4).

DISCUSSION

Hypospadias is considered the most common congenital anomaly after undescended testis in males. It is thought that there are multifactorial causes in the etiology of hypospadias, and there are many studies in the literature on molecular, genetic and environmental factors for etiology [1,2,13,14]. Hypospadias can arise as a result of embryological defects that affect the development of the urethra, which is connected to mesothelial-epithelial interaction, as well as the preputium and penile skin, which are of ectodermal origin [7]. It has been demonstrated that androgens enable the development and differentiation of the mouse and human penis, and that hypospadias results from a lack of androgens. Pregnant women who receive diethylstilbestrol (DES), a synthetic estrogen, have an increased risk of developing hypospadias [15]. Preoperative androgen stimulation has demonstrated to enhance penile size but not the risk of postoperative problems, and the degree of hypospadias does not alter the lengthening of the penis in response to androgen stimulation [16]. AR mutations have also been found to be associated with hypospadias [17]. Low serum testosterone levels, decrease in androgen-dependent pathways in the cell nucleus and increase in cytoplasmic estrogen-dependent pathways may cause hypospadias and undescended testis [14]. Intrauterine factors, such as abnormal androgen production by

the fetal testes, partial androgen insensitivity in the target tissues of the developing genitalia, and early atrophy of Leydig cells and premature cessation of androgen stimulation, have been implicated. In hypospadias, especially in its severe forms, abnormalities have been reported in the enzyme 5-alpha-reductase type 2, which catalyzes the conversion of testosterone to dihydrotestosterone [10]. For both the male and female sexes, it is well established that ERs and estrogen are essential for healthy genital development. The estrogen derivative diethylstilbestrol and the anti-estrogen letrozole (an aromatase inhibitor) have been administered to people and animals, and studies have shown that hypospadias develop as a result of an imbalance in estrogen levels [15].

Contrary to what Khanna et al.'s investigation found in the foreskin of children with and without hypospadias circumcision samples showed enhanced ER expression and decreased AR expression [10]. According to our research, children with hypospadias have more ER and AR receptors in their foreskins. Again, in the preliminary study of Pichler et al. on this subject, it was shown that AR mRNA expression and IHC AR protein level and AR protein staining in nuclear staining increased in the foreskin of children with hypospadias, and this increase was correlated with the degree of hypospadias. They stated that the elevation of AR mRNA and AR protein in preputium samples of boys with hypospadias is an indirect indicator of decreased AR DNA binding capacity. Therefore, they pointed to a signal defect indicating more deficient polypeptide encoding as a possible cause of hypospadias. They stated that more research on the notion should be conducted using structural analysis of AR to ascertain whether the degree of AR signaling malfunction and the severity of hypospadias are indicated by differences in the expression of AR mRNA and AR protein [8]. Although expression studies were not performed in our study, AR levels were found to be high in the tissue, and results supporting this theory were obtained. In the study of Celayir et al., in which the skin obtained from 33 children with hypospadias was examined, ER was found to be positive in 29 (87.8%) tissues and AR in 12 (36.4%) tissues. The progesterone receptor was found to be negative (0%) in all specimens [18].

According to Cunha et al., estrogens are essential for the development of the penile and clitoral regions in hyenas, as well as for determining the location of the urethral orifice, providing flexibility to the urethral meatus, and facilitating epithelial fusion events that are required for the correct formation of the distal urethra, urogenital sinus, and foreskin. Significant tissue expression of androgen and estrogen receptors is linked to the effects of prenatal androgens, anti-androgens, and decreased production of estrogen, indicating that estrogen, like androgen, plays an active role in prenatal penile development [19]. Celayir et al. described the predominant expression of ERs in the penile tissue of children with hypospadias as a postnatal reflection of impaired ER and AR interaction during the intrauterine development of external genitalia and interpreted that impaired AR and ER interaction may play a role in the development of hypospadias and external genital organs [18]. Similarly, Qiao et al. investigated the effects of estrogen in hypospadias and circumcision tissue. They found the mRNA expression of ER-α and β (dominant character in foreskin without hypospadias) significantly lower in hypospadiac skin compared to the control group [9]. In addition, ER-β was IHC stained weakly in preputial tissue, but prominently in skin without hypospadias. ER-α, on the other hand, was weakly stained in without hypospadias and mildly hypospadiac skin but could not be detected in severe hypospadias [9,10]. As a result, they stated that there is a tendency towards lower ER expression levels in severe hypospadias compared to mild hypospadias, and changes in ER levels play a role in the development of normal and abnormal foreskin [9]. In our study, both ER and AR were found to be increased in hypospadiac skin. These results are evidence that both estrogen and androgens play a role in the development of the genital system and the formation of hypospadias. The increased ER and AR receptor scoring in patients with hypospadias can be shown as the continuation of the above-mentioned and beginning of the intrauterine ER and AR balance, and perhaps the body's attempt to balance this imbalance.

The union of the urethral folds and the formation of the genital tubercle depend on FGFs, especially FGF-8, FGF-10, and FGFR-2. The development of severe hypospadias in mutant mice has also been demonstrated. Since members of the FGF family have a role in the interaction between mesenchyme and urothelium, the development of genital tissues, it has been demonstrated that abnormalities increase the risk of hypospadias [20]. Single-base mutations in FGF-8, FGF-10, and FGFR-2 are associated with hypospadias in humans, and it has been shown to increase the risk (3-4 times) of hypospadias compared to the control group [7]. However, studies on the concentration or receptor density of these factors in the foreskin or penile tissue are limited. In the pioneering study of Haid et al. investigating the density of FGF family components in the hypospadias foreskin, no difference was found in the expression levels of FGF-8, FGF-10, FGFR-2 mRNA in the foreskin of 32 hypospadias patients compared to the normal foreskin, but a significant difference was observed in the IHC staining distribution [7]. Compared to the control group, IHC staining of these markers were found to be higher (2/3) in a higher pattern, especially in those with hypospadias in the epidermis, while these markers were

more pronounced in those with proximal hypospadias than in those with distal hypospadias ($p<0.05$). While these HGFs were stained in a higher pattern in the dermis than in the control group (B/C), they showed a similar staining pattern among hypospadias types. Haid et al. emphasized that they showed that FGFs play an active role in the formation of hypospadias by associating their results with hypospadias caused by mutations of FGF-8, FGF-10 and FGFR-2 in mouse models. These findings offer compelling evidence in favor of the theory that mutations in FGF components associated with the initial phases of genital development could impact tissues produced from human ectodermal tissue. This implies that people with hypospadias may have significantly changed dartos fascia microanatomy as a result of FGFs acting on mesenchymal tissues. The induction of overlaying epithelial differentiation by mesenchymal FGF production is a well-established phenomenon. Cross-epithelial-mesenchymal boundaries have been shown to exhibit both directionally and reciprocally active FGF signaling. They did not make any specific comments about the development of hypospadias, saying that poor wound healing and the frequency of complications are mostly reflected in the changes in this FGF family in patients with severe hypospadias [7]. In our study, which is the second study on this subject, no mRNA expression study was performed, but lower scores were obtained in the hypospadias group in the IHC studies of FGF-8, FGF-10, and FGFR-2 in both epidermis and dermis.

Expression of FGF-8 and FGF-10, especially FGFR-2, is positively regulated by AR receptors based on androgen response element sequences in human hypospadias foreskin tissue. However, AR expression works independently of FGFs and without feedback [21, 22]. The changes in these FGF values in our study and Haid's study may be due to the disruption of the balance between FGFs and AR, which was described above [7]. Despite the low FGF in our study, the low AR levels may be due to the lack of feedback.

While endodermal FGFR-2 deletion causes mild hypospadias and defective urethral epithelium, ectodermal FGFR-2 deletion causes severe hypospadias and ventral prepuce loss [23]. FGF-8, FGF-10, and FGFR-2 are closely linked through Sonic Hedgehog regulation, particularly with the formation of the limbs and genital tubercle at an early androgen-independent stage of embryonic development. Mesenchymal FGF signaling affects the ectodermal and endodermal epithelium, causing overgrowth of the genital tubercle. Although it has been shown in the literature in mouse models that FGF-8, FGF-10 and FGFR-2 affect the development of tissues of ectodermal origin, such as penile skin and foreskin, this finding has not yet been confirmed in human tissue [23]. Whether it is the study of Haid et al. or our own study, although there are different results due to the limitations of the studies on this subject, penile tissue FGF-8, FGF-10 and FGFR-2 uptake and scores were different compared to the control group in these studies, and the FGF family also had genital development in humans, and evidence that it is associated with hypospadias [7].

The limitations of this study are the limited number of cases due budget limitations, the inability to measure blood estrogen and androgen levels, and the inability to express mRNA. As in other hypospadias studies, the results reflect a small group because they sample a single ethnic population or race. The strength of this study is that AR, ER, and FGF-8, FGF-10, and FGFR-2 are the only studies that looked at the same time.

CONCLUSIONS

IHC studies on the foreskin of children with hypospadias and without hypospadias revealed that ER and AR uptake were higher in hypospadias tissue samples, also that FGF-8, FGF-10, and FGFR-2 uptakes were lower compared to the children's tissue samples who did not have hypospadias. These factors were supported by affecting each other in the development of hypospadias. There are not many studies on this topic in the literature, and the conflicting results for some of the analyzed criteria suggest that further study on this topic needs to be done in the future.

Funding

This study was sponsored by the Scientific Research Foundation of Gazi University (9/2022-7828).

Authors' Contribution

NE, ZT, RK, AA and KS **performed to** study conception and design. NE, GA and FAU **performed data** acquisition. DD, AAMS, NE and RK **performed analysis** and data interpretation. ZT, RK and KS **read** and re-written the article. KS and AA **were** involved in revision of the article and mentorship throughout. All authors read and approved the final version of the manuscript

Ethical approval and consent to participate

The study (07/03/2022-191) began with approval from the Gazi University Ethical Committee. In conformity with the Helsinki Declaration, all procedures were carried out.

Competing interests

None declared.

REFERENCES

1. Bouty A, Ayers KL, Pask A, Heloury Y, Sinclair AH. The Genetic and Environmental Factors Underlying Hypospadias. Sex Dev. 2015;9(5):239-259.
2. Blaschko SD, Cunha GR, Baskin LS. Molecular mechanisms of external genitalia development. Differentiation. 2012;84(3):261-268.
3. van der Horst HJ, de Wall LL. Hypospadias, all there is to know. Eur J Pediatr. 2017;176(4):435-441.
4. George M, Schneuer FJ, Jamieson SE, Holland AJ. Genetic and environmental factors in the aetiology of hypospadias. Pediatr Surg Int. 2015;31(6):519-527.
5. Kalfa N, Gaspari L, Ollivier M, Philibert P, Bergougnoux A, Paris F, et al. Molecular genetics of hypospadias and cryptorchidism recent developments. Clin Genet. 2019;95(1):122-131.
6. Chen Z, Lei Y, Finnell RH, Ding Y, Su Z, Wang Y, et al. Whole-exome sequencing study of hypospadias. iScience. 2023;26(5):106663.
7. Haid B, Pechriggl E, Nägele F, Dudas J, Webersinke G, Rammer M, et al. FGF8, FGF10 and FGF receptor 2 in foreskin of children with hypospadias: an analysis of immunohistochemical expression patterns and gene transcription. J Pediatr Urol. 2020;16(1):41.e1-e10.
8. Pichler R, Djedovic G, Klocker H, Heidegger I, Strasak A, Loidl W, et al. Quantitative measurement of the androgen receptor in prepuces of boys with and without hypospadias. BJU Int. 2013;112(2):265-270.
9. Qiao L, Rodriguez E Jr, Weiss DA, Ferretti M, Risbridger G, Cunha GR, et al. Expression of estrogen receptor alpha and beta is decreased in hypospadias. J Urol. 2012;187(4):1427-1433.
10. Khanna S, Shankar Raman V, Badwal S, Vinu Balraam KV. Quantification of the Androgen and Estrogen Receptors in the Penile Tissues of Hypospadias in Comparison with Normal Children. Fetal Pediatr Pathol. 2023;42(2):175-186.
11. Kocaturk H, Özkaya F, Adanur S, Yapanoglu T, Aksoy Y. Expression of androgen receptor and estrogen receptor-beta in foreskin tissue of hypospadiac and healthy boys. Arch Esp Urol. 2020;73(2):119-125.
12. Nikolaev VV, Solontsov YN. [Epidemiology and theories of the origin of hypospadias]. Urologiia. 2018;(3):141-145.
13. Marrocco G, Grammatico P, Vallasciani S, Gulia C, Zangari A, Marrocco F, et al. Environmental, parental and gestational factors that influence the occurrence of hypospadias in male patients. J Pediatr Urol. 2015;11(1):12-19.
14. Zhou X, Zhang X, Zhou X, Abulimiti G, Wang Y, Zhang Q, et al. Identification of endocrine-disrupting chemicals targeting the genes and pathways of genital anomalies in males. Ecotoxicol Environ Saf. 2022; 247:114241.
15. Cripps SM, Mattiske DM, Black JR, Risbridger GP, Govers LC, Phillips TR, et al. A loss of estrogen signaling in the aromatase deficient mouse penis results in mild hypospadias. Differentiation. 2019;109: 42-52.
16. Do MT, Kim L, Im YJ, Hahn S, Park K. Effect of Preoperative Androgen Stimulation on Penile Size and Postoperative Complication Rate in Patients with Hypospadias: A Systematic Review and Meta-analysis. World J Mens Health. 2023; 41(3):558-574.
17. Nagaraja MR, Gubbala SP, Delphine Silvia CRW, Amanchy R. Molecular diagnostics of disorders of sexual development: an Indian survey and systems biology perspective. Syst Biol Reprod Med. 2019;65(2):105-120.
18. Celayir A, Moralioglu S, Cetiner H, Kir G, Celayir S. Expression of androgen, estrogen, and progesterone hormone receptors in the penile tissues of children with different types of hypospadias. North Clin Istanb. 2018;6(2):110-116.
19. Cunha GR, Risbridger G, Wang H, Place NJ, Grumbach M, Cunha TJ, et al. Development of the external genitalia: perspectives from the spotted hyena (Crocuta crocuta). Differentiation. 2014;87(1-2):4-22.
20. Joodi M, Amerizadeh F, Hassanian SM, Erfani M, Ghayour-Mobarhan M, Ferns GA, et al. The genetic factors contributing to hypospadias and their clinical utility in its diagnosis. J Cell Physiol. 2019; 234(5):5519-5523.
21. Lorenzi MV, Long JE, Miki T, Aaronson SA. Expression cloning, developmental expression and chromosomal localization of fibroblast growth factor-8. Oncogene. 1995;10(10):2051-2055.
22. Gnanapragasam VJ, Robson CN, Neal DE, Leung HY. Regulation of FGF8 expression by the androgen receptor in human prostate cancer. Oncogene. 2002;21(33):5069-5080.
23. Gredler ML, Seifert AW, Cohn MJ. Tissue-specific roles of FGFR-2 in development of the external genitalia. Development. 2015;142(12):2203-2212.

BJMG 27 (1), 2024 • 31-36
DOI: 10.2478/bjmg-2024-0004
sciendo

ORIGINAL ARTICLE

THE SPECTRUM AND FREQUENCY OF CYSTIC FIBROSIS MUTATIONS IN ALBANIAN PATIENTS

Kasmi I[1,*], Kasmi G[2], Basholli B[1], Sefa HS[3], Vevecka E[1]

[*]**Corresponding Author:** Ass. Prof. Irena Kasmi, Pediatric Clinic, Pediatric Department, University Hospital Center "Mother Teresa", Tirana, Albania. Address: Street Dibra 372, Tirana, Albania. Tel: + 355 67 2066175, E-mail: irenakasmi@hotmail.com

ABSTRACT

BACKGROUND

Cystic fibrosis (CF) is a genetic disease characterized by a wide spectrum of severity, resulting from the inheritance of a mutant allele of the gene for cystic fibrosis transmembrane conductance regulator (CFTR). The aim of the study was to present a CFTR mutation analysis among the Albanian population and to identify rare variants.

METHODS

We identified CFTR mutations in a representative cohort of CF patients comprising of Albanian patients and some Kosovo patients followed up by the Department of Pediatrics at the University Hospital Center "Mother Theresa" (UHCMT). Compiled clinical and genotypic data include 133 previously analyzed patients, of whom 116 have two identified mutations, 6 have only one known mutation, and 11 are unexamined.

RESULTS

The most frequent mutation is F508del (83.19%), followed by 621+1G>T (2.45%). Other mutations identified in decrease order are E822X, G85E, G542X, R1066C, R1070Q, R1158X, G1349D, N1303K, S466X, 1811+1G->C, E831X, CFTRdele2,3(21kb).

CONCLUSIONS

The data suggest that most of these patients can benefit from new modulatory therapies targeting CFTR mutations, translating to very hopeful prospects for these patients.

The Albanian population would benefit from Cystic Fibrosis neonatal screening, since outcomes can be improved through early diagnosis.

Keywords: CFTR, cohort, variants, modulatory, neonatal.

INTRODUCTION

Cystic fibrosis (CF) is a genetic multisystem disease resulting from the inheritance of a mutant allele of the gene for cystic fibrosis transmembrane conductance regulator (CFTR) from each parent. Cystic fibrosis (CF) is the most common life shortening condition in Caucasians [1]. About 162,428 people are estimated to be living with CF worldwide, of which 37,002 are estimated to be diagnosed in North America and more than 47,650 in Europe, while an estimated 57,076 CF patients are undiagnosed. [2]. Whilst prevalence is broadly similar in populations which had their origin from northern Europe, there are considerable variations through Europe from as high as 1 in 1,400 live births in Ireland, 1 in 4,200 in Italy and 1 in 25,000 in Finland [3]. Prevalence rates are much lower among non-Caucasian populations [4,5].

A major step forward was achieved by grouping CFTR mutations with a similar effect on CFTR protein synthesis or function in the same mutation class [3]. In view of drug development and drug distribution, it is therefore also useful to know the relative prevalence of these mutation classes [3].

[1] University Hospital Center" Mother Teresa" (UHCMT), Pediatric Department, Tirana

[2] University of Medicine, Tirana (UMT), Laboratory Department

[3] Laboratory Department, Catholic Hospital "Our Lady of Good Counsel", Tirana, Albania

In Albania since 1992, cystic fibrosis patients are followed up by the Department of Pediatrics, in UHCMT. The CF diagnosis is based predominantly on clinical criteria and two positive sweat test results for patients suspected of the disease. The diagnosis is confirmed by the identification of two CF causal mutations. Genetic assessment has been in practice since 2004, when the search of the genetic mutations in patients with cystic fibrosis began to be recorded.

Unfortunately, there was no national registry for Cystic Fibrosis in Albania until 2017. However, every pediatric patient diagnosed in the Department of Pediatrics in the UHCMT has been documented throughout the course of the disease. Practically, this is the only center which diagnoses and follows cystic fibrosis patients in our country. Since 2017 Albania is part of the European Cystic Fibrosis Society Patient Registry (ECFSPR) which collects anonymized demographic and clinical data from consenting people in Europe with CF [6].

The aim of this paper is to present a summary of the distribution of the CFTR mutations in 133 CF patients diagnosed in the Pediatrics Department in the UHCMT. Furthermore, we aim to compare our data with other countries in Balkan Peninsula and Europe.

MATERIALS AND METHODS

This retrospective study takes into consideration 133 pediatric patients diagnosed with Cystic Fibrosis followed in the Department of Pediatrics in the UHCMT, during the year (2019) of follow up and who have been included in the European Patient Registry of Cystic Fibrosis.

The examination of the CF mutations is conducted over a 25-year period from 1994-2019 for 116 patients at the Center of Molecular Diagnosis and Genetic Research in the University Hospital of Obstetrics and Gynecology "Queen Geraldine".

Whole blood was collected into EDTA anti-coagulant tubes. The genomic DNA was isolated from peripheral blood using Qiagen extraction kit (DNA Blood Isolation Kit, Qiagen, Valencia, California, USA). CFTR gene mutations were analyzed by PCR/OLA protocol (Abbott Applied Biosystems), testing for 33 mutations (Cystic Fibrosis V3 Genotyping Assay).

Undetermined samples of 15 cases were sent to the University Hospital Motol, in Prague, Czech Republic as part of the project: GENOTYPING IN UNDERTESTED / UNTESTED CYSTIC FIBROSIS POPULATIONS IN MIDDLE EAST, TRANSCAUCASIA, TURKEY & EASTERN NORTH AFRICA (2015-2019; Vertex grant scheme - CG-2015-104643).

Firstly, the most common mutations of the CFTR gene were examined using the commercial Elucigene CF-EU2 kit, where 50 mutations are simultaneously tested (CFTRdele2,3 / 21kb /, I507del, 2789 + 5G> A, E60X, F508del, Q890X, P67L, 1677delTA, 3120 + 1G > A, G85E, V520F, 3272-26A> G, 394delTT, 1717-1G, A, R1066C, 444delA, G542X, Y1092X (C> A), R117C, S549N, M1101K, R117H, S549R (T> G), D1152H , Y122X, G551D, R1158X, 621 + 1G, T, R553X, R1162X, 711 + 1G, T, R560T, 3659delC, L206W, 1811 + 1.6kbA, G, 3849 + 10kbC, T, 1078delT, 1898 + 1G> A S1251N, R334W, 2143delT, 3905insT, R347P, 2184delA, W1282X, R347H, 2347delG, N1303K, A455E, W846X, including IVS8-T variants (5/7/9) .

If just one mutation was detected or both causal mutations are not detected, but the patient shows clear clinical and laboratory signs, next generation sequencing of the whole coding region of CFTR (NGS CFTR Devyser) was provided. This ensures maximum capture of causal mutations within the diagnostic process we provide. All pathogenic variants were confirmed by Sanger sequencing or MLPA protocol https://www.mlpa.com/.

Also, the databases CFTR1 http://www.genet.sickkids.on.ca/, CFTR2 https://cftr2.org and CLIN Var https://www.ncbi.nlm.nih.gov/clinvar, were used for clinical interpretation.

RESULTS

Based on the number of pediatric patients diagnosed with CF every year from 1992 to 2017, and the birth rate during the same period [7], the incidence of CF in Albania is 1:4041. It is important to note that this incidence is not entirely correct since in the UHCMT, there are also CF patients from Kosovo, or Albanian children born in other countries.

Table 1. Incidence of Cystic fibrosis in Albania

Time Period	Birth Rate in Albania	CF cases diagnosed at UHCMT	Incidence
1992-2017	1144138	285	1:4014

We have presented in Table 2 an overview of the demographic as well as the clinical and laboratory characteristics of Albanian patients diagnosed at UHCMT who were included in the study, although data on some births are missing.

Allele Frequencies.

Among the studied group of patients, 116 of 133 patients have two CFTR alleles identified, and 6 patients have only one mutation determined. Although genetic examination was made possible, 11 patients remained unexamined due to their lack of interest in performing this analysis. As shown in Table 3, 14 CFTR mutations were identified from 122 patients, 116 of whom with two

Table 2. Overview data about patient with CF in Albania*

Year of follow up 2019	**Number (n=)**	**Percentage (%)**
No. of patients	133	100
Male	72	54.14
Female	61	45.86
Mean Age (years) at follow up. (Min-max)	10.5 0.5-28.0	
Mean Age (years) at diagnosis. (Min-max)	0.75 0.0-16.0	
No. of patients <18 years	115	86.47
No. of patients > 18 years	18	13.53
Homozygote for F508del	87	65.41
Compound heterozygous mutation F508del	23	17.29
Non- F508 mutation patients	6	4.51
One determined mutation	6	4.51
Unexamined	11	8.27
Mean BMI Z-score: ± SD. >18 years old <18years old	 -1.2 -0.7	
Mean FEV1 (% predicted)	89.7	
Chronic *S.aureus*	39	29.32
Chronic *P.aeruginosae*	33	24.8
CF liver disease	54	40.60
CF-related diabetes	6	4.51
Pancreatic sufficient cases	3	2.2

*Note data from ECFSPR 2019

Table 3. Allele frequencies in 122 Albanian patients and Classes according to their effect on the synthesis and/or function of the CFTR protein.

***CFTR* gene mutations NM_00492.3** **Legacy name, coding DNA, protein name**	**Mutation Type**	**Class of Mutation**	**All Alleles N= 244(%)**
F508del, c.1521_1523delCTT, p. Phe508del	deletion	II	203(83.19)
621+1G>T, c.489+1G>T	splicing	I	6 (2.45)
E822X, c.2464G>T, p. Glu822Ter	nonsense	I	5 (2.04)
G85E, c.254G>A, p. Gly85Glu	missense	II	5 (2.04)
G542X (c.1624G>T) p. Gly542Ter	nonsense	I	4 (1.63)
R1066C, c.3196C>T, p. Arg1066Cys	missense	II	3 (1.22)
**R1070Q, c.3209G>A, p. Arg1070Gln	missense	Unclassified	3 (1.22)
R1158X, c.3472C>T, p. Arg1158Ter	nonsense	I	2 (0.81)
G1349D, c.4046G>A p. Gly1349Asp	missense	III	2 (0.81)
N1303K, (c.3909C>G), p. Asn1303Lys	missense	II	2 (0.81)
**S466X, c.1397C>G, p. Ser466Ter	nonsense	I	2 (0.81)
1811+1G->C, c.1679+1G>C	splicing	V	1 (0.40)
E831X, c.2491G>T, p. Glu831X	nonsense	I	1 (0.40)
CFTRdele2-3(21kb), c.54-5940_273 + 10250 del, p. Ser18Argfs*16	deletion	I	1 (0.40)

The new mutations for the Albanian population detected by University Hospital Motol are bolded.
**2.0 complex alleles S466X-R1070Q
N: number of alleles; %: percentage rounded up to max. 2 digits after the full stop (thus may not add up exactly to 100%).
HGVS – Human Genome Variation Society nomenclature (www.hgvs.org/mutnomen/);
Legacy nomenclature according to the Cystic Fibrosis Mutation Database (www.genet.sickkids.on.ca/app)

CFTR mutations determined and 6 patients with only one mutation determined.

The most frequent mutation is F508del with 83.19% (203/244 alleles). F508del mutation is present in 87 homozygous patients, 23 patients with heterozygous mutations, and in all 6 patients who have only one known mutation. The second most frequent mutation is 621+1G>T with 2.45% (6/244 alleles), followed by G85E and E822X with 2.04% (5/244 alleles), G542X (1.63%), R1066C (1.22%), R1070Q (1.22%).

The mutations R1158X (0.81 %), S466X (0.81%), G1349G (0.81%), N1303K (0.81%), and CFTRdele2,3(21kb)

Table 4. Genotype variants and their frequency

Genotype variants	Frequency number (N=)	Percentage %
F508del/F508del	87	75.00
F508del/non F508del	23	19.82
F508del/G85E	4	3.44
F508del/E822X	3	2.58
F508del/621+1G>T	3	2.58
F508del/R1066C	3	2.58
F508del/G1349D	2	1.72
F508del/G542X	2	1.72
F508del/ N1303K	2	1.72
F508del/R1070Q	1	0.86
F508del/ S466X-R1070Q - in cis	1	0.86
F508del/ R1158X	1	0.86
F508del /E831X	1	0.86
non F508del/non F508del	6	5.17
G542X/621+1G>T	1	0.86
G85E/ R1158X	1	0.86
G542X/ E822X	1	0.86
621+1G>T/621+1G>T	1	0.86
CFTR dele 2.3/ 1811+1G->C	1	0.86
S466X-R1070Q in cis / E822X	1	0.86

N: number of genotypes; %: percentage rounded up to max. 2 digits after the full stop (thus may not add up exactly to 100%).

(0.40 %), E831X (0.40 %), do not surpass 1% of the CF patients included.

According to their effect on the synthesis and/or function of the CFTR protein, 87.29 % (213/244 alleles) of the mutations pertain to class II with four different mutations, explained by F508del being by far the most frequent mutation in this cohort.

Following class II, the second most frequent mutation class was class I with 8.60 % (21/244 alleles) and eight different mutations. Three mutations are unclassified 1.22% (3/244 alleles).Two mutations belong to class III 0.81% (2/244 alleles). One mutation belongs to class V 0.40% (1/244 alleles). Mutations belonging to class IV and VI were not present in our patients. The mutation classes are presented in Table 3. Only one mutation (1/244 alleles) has a varying clinical expression (consequences) [8].

Genotype Frequencies

Table 4 shows the genotypes of the 116 patients for whom the two mutations were identified, as well as their frequency expressed in numbers and percentages.

As it is noted from the frequency of genotypes in the Table 3, the most frequent genotype was F508del/F508del (75%; 87/116) followed by F508del/non F508del genotype (19.82%; 23/116) and non F508del/non F508del (5.17%; 6/116).

From 6 patient non F508del/non F508del genotypes resulted six different ones as follows: G542X/621+1G>T (0.86%); G85E/R1158X (0.86%); G542X/ E822X (0.86%); 621+1G>T/621+1G>T (0.86%); CFTR dele 2.3/1811+1G->C (0.86%); S466X-R1070Q in cis / E822X (0.86%).

DISCUSSION

This study aims to shed some light on the current situation about CF mutation pattern, presenting data from patients with CF followed during 2019 in the Department of Pediatrics in the UHCMT. Practically, UHMCT is the only center which diagnoses and follows cystic fibrosis patients in Albania. It is important to note that in Albania no CF national neonatal screening has been applied until now. It is assumed that the incidence would be higher if neonatal screening program would take place.

The Albanian population is composed of an ethnic group originating in the Balkan peninsula. There are approximately five million Albanians in this region, with roughly half living in Albania and the other half in Kosovo, Northern Macedonia, Montenegro and smaller populations in Croatia and Serbia. Significant numbers of the Albanian population are also found in Greece and smaller insignificant communities in Bulgaria and Romania as well. Albanians also make up a significant diaspora, spread all over the world, especially in North America, Europe and Oceania.

According to the latest census of the National Institute of Statistics Albania (INSTAT), the current population of Albania is 2,876,591 inhabitants [7].

The most common CFTR mutation is F508del, either in homozygous or heterozygous form. In Europe, this mutation is met in 82% of patients with CF (41% homozygous and 41% heterozygous) [9]. Among European countries, there are different variations of this CFTR mutation prevalence, from Denmark 83.2% [10], to a minimal prevalence in Turkey of only 24.5% [11]. This confirms the northwest to southeast gradient in the F508del distribution in Europe [11]. The Albanian population expresses a high prevalence of F508del mutation, in comparison to other populations in the region. Thus, in neighboring Italy is 43.9% [12], in North Macedonia is 75.9% [13], in Serbia and Montenegro it is 72.28 % [14].

In Albania, there is a high prevalence of this mutation which is characteristic of Central, Northern and Northeastern Europe. This fact is well known and previously explained due to early migrations of the Caucasian population towards Northwestern Europe and Southeastern Europe, and thereafter the migration through the Mediterranean routes from Middle East and Africa towards Europe [11]. The second migration seems to have left the Albanian population unaffected.

The second most prevalent mutation found in the Albanian population is 621+1G>T (2,56%). This mutation is most prevalent in Southern Europe, found more frequently in our neighboring country Greece (6.4%) [10], while in North Macedonia this mutation is under 2% [15].

The third most prevalent mutations found in the Albanian population are G85E (2.13%) and E822X (2.13%). The G85E mutation, is most frequent in Israel (2.6%) [10].

E822X, R1066C, G1349D, S466X, 1811+1G->C, E831X, CFTRdele2-3(21kb) mutations were newly found in the patients of our study. The latter, the CFTRdele2-3(21kb) mutation, was found in only one patient in this study, is found mainly in Slavic and Eastern and Central European populations [16]. The most frequent result of this mutation is in Belarus (11.2%) [10]

There are 14 CFTR mutations and 18 confirmed genotypes. To define the optimal treatment for an individual patient, genotyping of CFTR is clearly the first step [17].

CONCLUSIONS

This information suggests that most of these patients can benefit from new modulatory therapies targeting CFTR mutations, which translates to very hopeful prospects for these patients [18, 19]. The Albanian population would benefit from Cystic Fibrosis neonatal screening, since outcomes can be improved by early diagnosis.

Despite a lack of discussion on the topic of a neonatal screening program, it is crucial to improve diagnosis and allowing optimal treatment of patients with CF before overt disease progression sets in.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

REFERENCES

1. Sharma N, et al. Defining the disease liability of variants in the cystic fibrosis transmembrane conductance regulator gene. Nat Genet. 2013 Oct;45(10):1160-7.
2. Guo J, Garratt A, Hill A. Worldwide rates of diagnosis and effective treatment for cystic fibrosis. J Cyst Fibros. 2022 May;21(3):456-462. doi: 10.1016/j.jcf.2022.01.009. Epub 2022 Feb 4. PMID: 35125294
3. Bell SC, DeBoeck K, D AmaralM. New Pharmacological Approaches for Cystic Fibrosis: Promises, Progress, Pitfalls. Pharmacol Ther. 2015 Jan; 145:19-34
4. The molecular genetic epidemiology of cystic fibrosis. Human Genetics Program, Chronic Diseases and Health Promotion, World Health Organization: Report of a joint meeting of WHO/ECFTN/ICF(M)A/ECFS; 2002.
5. O'Sullivan BP, Freedman SD. Cystic fibrosis. Lancet. 2009 May 30; 373(9678):1891-904.
6. www.ecfs.eu/ecfspr
7. Institute of Statistics Albania www.instat.gov.al
8. CFTR2 31 August 2018. www.cftr2.org/mutation list
9. European Cystic fibrosis society. Patient Registry. At-A-Glance report 2016
10. European Cystic fibrosis society. Patient Registry. At-A-Glance report 2021
11. Bobadilla JL1, Macek M Jr, Fine JP, Farrell PM. Cystic fibrosis: a worldwide analysis of CFTR mutations--correlation with incidence data and application to screening. Hum Mutat. 2002 Jun; 19(6):575-606.
12. https://www.registroitalianofibrosicistica.it/documenti/servizi/rifc_at_a_glance_2021.pdf
13. Terzic M, Jakimovska M, Fustik S, Jakovska T, Sukarova-Stefanovska E, Plaseska-Karanfilska D. Cystic fibrosis mutation spectrum in North Macedonia: A step toward personalized therapy. Balkan Journal of Medical Genetics. 2019 Aug 28;22(1):35-40.
14. Radivojevic D, Djurisic M, Lalic T, Guc-Scekic M, Savic J, Minic P. Spectrum of cystic fibrosis mutations in Serbia and Montenegro and strategy for prenatal diagnosis. Genet Test. 2004;8(3):276–280. et al.
15. Fustik S, Plaseska Karanfilska D, Terzikj M, Spirevska L, Stamatova A. Spectrum of CFTR mutations in Republic of North Macedonia European Respiratory Journal 2020 56: 2768; DOI: 10.1183/13993003.congress-2020.2768,

16. T. Dörk, M. Macek Jr., F. Mekus, B. Tümmler, J. Tzountzouris, T. Casals, *et al.* Characterization of a novel 21-kb deletion, CFTRdele2,3 (21 kb), in the *CFTR* gene: a cystic fibrosis mutation of Slavic origin common in Central and East EuropeHum Genet, 106 (2000), pp. 259-268
17. Ikpa PT, Bijvelds MJ, De Jonge HR. Cystic fibrosis: toward personalized therapies. Int J Biochem Cell Biol 2014; 52:192–200
18. Rubin JL1, O'Callaghan L2, Pelligra C3, Konstan MW4, Ward A3, Ishak JK5, Chandler C3, Liou TG6. Modeling long-term health outcomes of patients with cystic fibrosis homozygous for F508del-CFTR treated with lumacaftor/ivacaftor. TherAdvRespir Dis. 2019 Jan-Dec; 13:175
19. Ponzano S, Nigrelli G, Fregonese L, Eichler I, Bertozzi F, Bandiera T, Galietta LJV3, Papaluca M1. A European regulatory perspective on cystic fibrosis: current treatments, trends in drug development and translational challenges for CFTR modulators. EurRespir Rev. 2018 Apr 13; 27(148).

BJMG 27 (1), 2024 • 37-42
DOI: 10.2478/bjmg-2024-0010
sciendo

ORIGINAL ARTICLE

MEANING AND CLINICAL INTEREST OF MINOR MALFORMATIONS AND NORMAL VARIANTS IN NEONATOLOGY

Grubeša Raguž S[1,*], Jerković Raguž M[1,3], Brzica J[4], Džida S[2], Mikulić S[2], Kolobarić A[2], Galić T[2]

[*]**Corresponding Author:** Svjetlana Grubeša Raguž, med MD, specialist in pediatrics, Clinic for Children's Diseases, Clinical Department of Neonatology, University Clinical Hospital Mostar;
E-mail: gsvjetlana@gmail.com Phone: 0038763952832; Fax: 0038736341970

ABSTRACT

Congenital malformations can be found in all organ systems of a newborn. Almost two-thirds of congenital malformations have an unknown cause. There are minor (mM) and major (MM) congenital malformations. Searching for minor malformations has its vital place in everyday neonatology practice. Minor malformations are defined as physical variants that have no medical consequences and are mostly located on the face and distal parts of the extremities and are easily noticed. Minor malformations occur in approximately 15% of newborns. Minor congenital malformations are of great importance because they can be an indicator of the existence of major congenital malformations and syndromes. In a one-year retrospective study that analyzed the occurrence of 38 minor malformations through the year 2023 at the University Clinical Hospital of Mostar, there was an incidence of 10.59% of minor malformations. The most frequently recorded minor malformation was deep a sacral dimple at 44.72%, then poorly modeled ears at 15.08%, and moderate rectal diastasis at 14.58%. Three or more minor congenital malformations indicate one or more major congenital malformations. Major congenital malformations are severe structural defects of tissues and organs that endanger life, create serious functional disturbances and hinder the development of the child. In our country, there is currently a recorded incidence of 8.04%. The search for minor malformations in the newborn period is of great importance to children and the whole family, and the search must not be neglected.

Key words: minor malformations, major malformations, newborn, search

INTRODUCTION

Malformations are macroscopically visible defects in the shape (morphology) of a certain part of the body, an organ or its part that occurs during organogenesis, i.e. from the second to the twelfth gestational week, and which are visible at birth (1). Deformations are tissue and organ changes that occurred because of intrauterine damage - usually of a mechanical nature. Disruptions are morphological defects caused by the destruction of tissues that were originally developed normally. A syndrome implies a group of malformations that are the result of a specific cause (2). Congenital malformations are structural changes that occurred prenatally and are visible in the newborn at birth. Structurally, malformations can be divided into minor and major malformations. Minor malformations occur in approximately 15% of newborns (3). They do not impair health but should be seen as a possible indicator of the existence of major malformations. Major congenital malformations are severe structural defects of tissues and organs that endanger life, create serious functional disturbances, and hinder the development of the child (4). Most studies have shown that minor malformations occur more often in children with low birth weight and less gestational age than in term newborns (5). Congenital malformations occur as a result of genetic diseases, but also as a result of the interaction of numerous environmental factors with genes (6). There are many environmental factors such as teratogenic drugs and chemicals, alcohol, maternal infections, ionizing radiation, but also maternal diseases such as unregulated diabetes, epilepsy, phenylketonuria. Each of these exogenous factors will then

[1] Clinic for Children's Diseases, Clinical Department of Neonatology, University Clinical Hospital Mostar

[2] Clinic for Children's Diseases, University Clinical Hospital Mostar, 88000 Mostar, Bosnia and Herzegovina

[3] Faculty of Medicine, University of Mostar, 88000 Mostar, Bosnia and Herzegovina

[4] Health center, 88340 Grude, Bosnia and Herzegovina

cause a disruption (7, 8). Major malformations are those that have an unfavorable effect on the function of a certain organ, organ system or the social acceptance of the child, and later on the person as an adult. These malfunctions are often recognized immediately at birth, and sometimes intrauterine. On the other hand, minor malformations usually have no major physiological or cosmetic significance, and examples include preauricular appendages, syndactyly and others (7). Minor malformations are often present in the normal population and are physical variants that, based on the frequency of occurrence in the general population, can be divided into minor malformations and normal variants (9). The normal variant is present in more than 4%, and minor malformations appear in less than 4% of the normal population. They are most often found in the facial area and the distal parts of the extremities (3). Minor malformations have no major clinical significance (apart from aesthetic ones), but according to Mehes' scheme of minor malformations, three or more minor malformations require additional diagnostic treatment (table 1) (10).

In previous studies, the incidence of minor malformations is higher in prematurity, and the aim of this study at the Clinic for Children's Disease is to show the frequency of minor malformations and normal variants in term newborns.

MATERIALS AND METHODS

A one-year retrospective study was conducted. The research was carried out at the Clinic for Gynecology and Obstetrics of UCH Mostar's "Department for Newborns". Data was collected from children's records and the mother's medical history, and at the Clinic for Pediatrics, Department for Neonatology and Intensive Care from discharge letters and transfer lists of newborns. All newborns were involved in the study, including term newborn who were born at a gestational age from $37^{+0/7}$ to $41^{+6/7}$ weeks and newborns born with lower birth weight and chromosomal abnormalities. The study included all newborns who met the above criteria as of January 1, 2023 until January 1, 2024. The parameters considered the newborn child's gender, gestational age, Apgar score, birth weight, birth length, and minor anomalies - malformations such as: preauricular appendages, low-laid ears, high-arched palate, small chin, simian line, antimongoloid shaped eye slits, partial syndactyly of 2 and 3 fingers, accessory wart, umbilical hernia, moderate rectus diastasis and other minor malformations. Also, the parameters considered the mother's age, number of pregnancies, births, abortions, method of conception, course of pregnancy, pathological conditions during pregnancy (hypertension, diabetes, infections prior to delivery, hypothyroidism), method of delivery, medications during pregnancy, and other available data from medical records. For the neonatological examination, the most practical was that of Mehes' scheme, with 38 mM items (Table 1). We adhered to this scheme (10). All term newborns were examined during the first 24 hours of life, and if minor malformations were noticed, they were recorded in the clinical status of the newborn's temperature chart. Major malformations were registered according to EUROCAT recommendations (11). The newborn's birth weight is determined with a digital scale manufactured by Momert, model MM6475, immediately after birth, and the progress of the neonate was monitored by daily weighing with the aforementioned scale. The gestational age of the newborn is calculated based on the date of birth and the date of birth of the pregnant woman.

Statistical analysis

R Studio (RStudio Team 2021) was used for statistical analysis and graphical display of data: Integrated Development Environment for R. (RStudio, PBC, Boston, MA URL http://www.rstudio.com/) and Microsoft Excel for Microsoft 365 MSO (Version 2111. Microsoft Corporation, Redmond, WA, USA). For the nominal variables in the research, the frequencies of occurrence were stated, and the differences between the frequencies were tested with the Chi-squared test.

RESULTS

In the period from January 1, 2023 until January 1, 2024, a total of 1,880 children were born (1,872 live births, 8 stillbirths), and some minor malformations were observed

Table 1. Mehes' scheme of minor malformations (10)

Head and neck: Small mandible, Prominent forehead, Flat occiput, Prominent occiput, Extra posterior cervical skin	**Foot:** Partial syndactyly 2nd and 3rd toes, Wide distance between 1st and 2nd toes, Broad hallux, Hallux dorsiflexion, Prominent heal
Eye: Epicanthic folds, Mongoloid slant, Antimongoloid slant, Short palpebral fissures, Hypertelorism, Ptosis	**Thorax:** Short sternum, Accessory nipples, Wide set nipples
Ears: Small ears, Asymmetrical size, Poorly modeled ears, Low-set ears, Preauricular tags, Preauricular fistula	**Abdomen:** Umbilical hernia, Inguinal hernia, Moderrate rectal diastasis
Mouth: Small oral opening, Large tongue, High-arched palate, Bifid uvula	**Skin:** Raised and large hemangioma(s) Large pigmented nevi, Deep sacral dimple
Hand: Simian crease, Clinodactyly, Single crease on 5th fingers	

in 199 (10.59%) newborns. In total, 16 newborns out of 199 (8.04%) had one of the major malformations. More than 53% of mothers whose child had one of the malformations gave birth between the ages of 30 and 39, while the least represented age group was of mothers under 20. The largest number of mothers gave birth naturally, 138 (69.35%) of them. The highest number of children with some type of malformation was registered in first pregnancies, 93 (46.73%). As many as 197 (98.99%) of women giving birth, had natural insemination, and only 2 (1.01%) had medically assisted insemination. 183 (91.96%) of women who gave birth had no previous abortions, while 12 (6.03%) of them had 1 abortion. In the categories where mothers had at least one abortion, no statistically significant differences were noticed between the frequencies of the observed types of malformation. All pregnant women were monitored for certain pathological conditions during pregnancy. The largest number of women giving birth were without pathological conditions, 153 (74.63%). Among women in labor who had at least one pathological condition, the largest number of them had hypothyroidism 32 (15.61%). The largest number of mothers did not use any medication during pregnancy, 157 (77.34%), and of those who used drugs, the largest number of them used levothyroxine sodium, 31 (15.27%). Malformation of the oral cavity was the most frequent malformation in pregnant mothers who did not use any therapy, 45.72%, and in pregnant mothers who used levothyroxine sodium, 10.55%. In groups where mothers used antidiabetic or antihypertensive drugs, all malformations were equally represented. As stated earlier, a total of 199 children were born, of which 116 (58.29%) were boys and 83 (41.71%) were girls ($\chi2 = 5.47$, df = 1, $p < 0.05$). The largest number of boys and girls had a birth weight between 2500 and 3500 grams, and a length between 53 and 56 centimeters. The largest number of male and female children were born between the 37th and 42nd week. Additionally, the study analyzed how many children have up to three and how many more than 3 stigmata. In total, 168 (84.42%) of the children had less than 3 stigmata, while 31 (15.58%) of them had three or more stigmata ($\chi2 = 94.31$, df = 1, $p < 0.01$). From the above mentioned data, it is clearly visible that those children who had some type of malformation on the eyes ($\chi2 = 5.54$, df = 1, $p < 0.05$), head and neck ($\chi2 = 6.26$, df = 1, $p < 0.05$) mostly had at least 2 additional malformations. All subjects who had one of the malformations on their hand also had at least 2 other malformations. In malformations of the abdomen ($\chi2 = 13.33$, df = 1, $p < 0.01$) and oral cavity ($\chi2 = 40.33$, df = 1, $p < 0.01$), a greater number of children had up to three malformations. Children with skin malformations had no additional malformations. In malformations of the chest, feet, and ears, no statistically significant differences were observed between the frequencies of children who had up to 3 malformations and those children who had more than 3 malformations. Statistically significant differences were noticed between the observed frequencies of different types of malformations ($\chi2 = 182.31$, df = 9, $p < 0.01$). Thus, the most frequent malformation was the deep sacral dimple, where as many as 89 (44.72%) of children had this type of malformation. Of the other malformations, the most common were poorly modeled ears observed in 30 (15.08%), and moderate rectus diastasis observed in 29 (14.57%) (Table2). In total, there were 16 (8.04%) of the children with major malformations. Among 199 children, cytogenetic analysis confirmed Down syndrome in 6 cases (3.02%), and cleft palate was identified in 8 (4.02%). Out of the children who had one of the major malformations, the largest number of them had a malformation of the cardiovascular system, 4.02% ($\chi2 = 9.63$, df = 3, $p < 0.05$). No statistically significant differences were noticed between the observed frequencies of different types of minor malformations in children who had one of the major malformations ($\chi2 = 20.22$, df = 23, $p = 0.63$). In 16 children with one of the major malformations, 8 of them (50.00%) had more than three minor malformations, and the same number of children had up to three malformations (Table 3).

Table 2. Ten most frequent minor malformations

Minor malformations	N	%	χ2	df	p
Deep sacral dimple	89	44,72	182.31	9	<0.01
Poorly modeled ears	30	15,08			
Moderate rectal diastasis	29	14,57			
Hypertelorism	18	9.05			
Wide distance between st and 2nd toes	18	9.05			
High-arched palate	18	9.05			
Small mandible	17	8.54			
Low-set ears	17	8.54			
Preauricular tags	12	6.03			
Extra posterior cervical skin	12	6.03			

Table 3. Frequency of minor malformations in newborns with some type of major malformation

Minor malformations	N	%	χ2	df	p
Wide distance between 1st and 2nd toes	7	43,75	20.22	23	0.63
Extra posterior cervical skin	6	37.5			
High-arched palate	6	37,5			
Hypertelorism	5	31,25			
Small mandible	5	31,25			
Small ears	5	31,25			
Epicanthic folds	4	25			
Prominent heal	4	25			
Clinodactyly	4	25			
Simian crease	4	25			

DISCUSSION

The clinical features of genetic diseases are varied. Various malformations and congenital abnormalities that are already present intrauterine or postpartum can arouse the suspicion that it is a genetic disease. Therefore, clinical features, i.e. the clinical examination of the child, is an extremely important part of diagnostics. Suspicion of a genetic disease can be aroused by various forms of facial dysmorphic disorder: wide face, coarse facial features, protruding lateral parts of the frontal bone, widely spaced eyes, mongoloid or anti-mongoloid shaped eyes, microphthalmia, epicanthus, wide and high nose root, low-laid and malformed ears, microtia, macrotia and anotia, macrostomia and microstomia, cleft lip and cleft palate, as well as high-laid palate. Any morphological change with an incidence greater than 4% in the population is a normal variation in development (12). Some authors state that normal variation is any morphological category with an incidence greater than 6% (13).

We conducted a one-year survey from 2023 until 2024 and compared it with a similar survey from 28 years ago, conducted in the same geographic area and in the same hospital, on almost the same number of births (14, 15). Šumanović D. et al, state in their research through 1995/1996, that the incidence of minor malformations stood at 23.7% (14, 15). This research was carried out at the end of the war in Bosnia and Herzegovina. In our research, after almost 28 years, the incidence of minor malformations was halved to 10.59%. Most studies have shown that minor malformations occur more often in children with low birth weight and less gestational age than in term newborns (4), which is not the case in our study. Most of our newborns with minor malformations were born after the 37th week of gestational age and with proper weight for gestational age. The results may be different for each population because the studies were conducted in relatively distant and different geographical regions. Neonatologists and medical staff are the first to notice minor malformations in newborns. Thus, Sawardekar states the incidence of minor malformations in a regional hospital in Oman was at 12.4% per 1000 births (16). A study from Egypt by El Awady H. et al., reported an incidence of 21.6% (17). A study from Congo reported that 34.8% of newborns had one minor malformation, 11.6% had two, and 4.3% had three (18). The presence of three or more minor malformations was associated with a 4.5 times higher risk of death (19). 25.4% of newborns born to mothers who used prescription opioids during pregnancy were diagnosed with major or minor congenital malformations (20). Some characteristics in one population may be minor malformations, and in another a normal variant of development. Thus, Tsai et al. report the simian line and mongoloid-shaped eye slits as a normal variant for Chinese newborns (21). In other ethnic groups, however, these signs serve as predictive markers for some chromosomal aberrations and specific syndromes (10).

Down syndrome and cleft palate accounted for 56% of oral cavity malformations in our study population of 199 children. Of these, six children had Down syndrome and eight had cleft palate. Common minor oral malformations in these children included high-arched palate, bifid uvula, small oral opening, large tongue. Hod M. et al., investigated the prevalence of minor congenital malformations in newborns of mothers with gestational diabetes, and this prevalence was between 19.4% and 20.5%. In our study, however, it was an almost imperceptible percentage of 3.45% (22). In this study, the most common pathological condition of the mother was hypothyroidism 32/15.61%. According to Kolobarić et al. (23), women with hypothyroidism had significantly higher rates of gestational diabetes (15%) and preeclampsia (3.5%). Gestational diabetes itself carries the prevalence of minor congenital malformations. In our research, the most common minor malformation was a deep sacral dimple in as much as 44.72%, which is also confirmed by research almost three decades ago in the same area of ours (15). This is in favor of the normal variants of our geographical area. Pediatric neurosurgeons concur that imaging studies are not required for newborns and infants presenting with simple sacral dimple (24). Immediately after the birth of the newborn, already in the delivery room, an orientation examination should be done in which we look for malformations. Some of the congenital malformations are immediately noticeable and do not necessarily endanger the child's life. An example of such malformations can be ear deformations, which in our research is the second most frequent malformation: poorly modeled ears at 15.08%. Bader D. et al. (25) report a frequency for malformation of the ears of 43.1% and state that the male gender is more frequently affected by this, coinciding with our research. However, there are malformations that are not immediately visible and can endanger the child's life, such as congenital anomalies of the heart. Almost three decades ago, the aforementioned study from our geographical area (15) stated the incidence of major malformations of 57.5%, and in our study the prevalence of major malformations was 8.04%. We can contribute this big drop to better prenatal care of pregnant women, the improvement of prenatal diagnostics and the improvement of the socio-economic conditions of pregnant women. El Awady H. et al. report a major incidence at 78.4%, and the cardiovascular system was most often affected in 32.4%. This coincides with our research (17). Newborns with congenital heart disease exhibit a broad

spectrum of dysmorphism (26). The great importance of minor malformations is that in 90% of all newborns who have 3 or more minor malformations, a major malformation also exists. Thus, minor malformations indicate the possible presence of major malformations (27). The risk of major malformations increases with the number of minor malformations. Any newborn with 3 or more minor malformations must be clinically treated and major malformations such as cardiac, renal or spinal anomalies must be sought. Newborns who were discharged after 24 hours from the maternity hospital and who had 3 minor malformations were recommended further diagnostic and ultrasound treatment. Newborns who were transferred to the Clinical Department of Neonatology with three or more minor malformations were subjected to diagnostic processing.

CONCLUSION

Awareness of the presentation and frequency of minor malformations in the population is crucial for neonatologists, who must distinguish between abnormal findings and normal variations. Recognizing minor malformations in a newborn, especially when their number exceeds three, should prompt a diagnostic search for the presence of major malformations. Neonatologists, as pediatricians managing the youngest population, have a unique opportunity to be the first to identify abnormalities in children and to make clinical judgments on the health risks they may pose. The results of our study strongly suggest that any newborn with three or more minor malformations should undergo an extended clinical evaluation due to the higher risk of associated major malformations.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

REFERENCES:

1. Duško Mardešić i suradnici. Pedijatrija. 8. Prerađeno i dopunjeno izd. Zagreb: Školska knjiga, 2016. p. 323. 33. 314. 315. 108. 80. 81. 109. 829. 911.
2. Grković S. i sur. Klinička genetika i genetsko savjetovalište. Hrvatskog društva za humanu genetiku hrvatskog liječničkog zbora. Zagreb, 1991.
3. Marden PM, Smith DW, McDonald MJ. Congenital anomalies in the newborn infant, including minor variations. A study of 4,412 babies by surface examination for anomalies and buccal smear for sex chromatin. J Pediatr. 1964;64:357-71.
4. Tomašević J. Etiologija kongenitalnih anomalija kod djece male tjelesne porođajne mase. Subspecijalistički rad; 1985.
5. Škrinjarić I, Jukić J, Škrinjarić K, Ulovec Z. Minor anomalije u djece sa smetnjama u razvoju. Paediatr Croat. 2002;46:115-21.
6. Corsello G, Giuffrè M. Congenital malformations. J Matern Fetal Neonatal Med. 2012 Apr;25 Suppl 1:25-9.
7. Turnpenny P, Ellard S. (2011) Kongenitalne anomalije i dismorfični sindromi. U: Bulić Jakuš F, Barišić I, ur., Emeryjeve osnove medicinske genetike. Zagreb. Medicinska naklada, str. 249-261.
8. Feldkamp ML, Carey JC, Byrne JLB, Krikov S, Botto LD. Etiology and clinical presentation of birth defects: population based study. BMJ. 2017 May 30;357:j2249.
9. Pereza N, Ostojić S, Zergollern-Čupak Lj, Kapović M, Peterlin B. Klinička dismorfologija i razvojne anomalije. Medicina 2010, Vol. 46, No. 1, p. 5-18.
10. Méhes K, Mestyán J, Knoch V, Vincellér M. Minor malformations in the neonate. Helv Paediatr Acta. 1973 Nov;28(5):477-83.
11. https://eu-rd-platform.jrc.ec.europa.eu/
12. Zergollern Lj. Nasljedne bolesti i mane pri porodu. U Zergollern Lj. i sur. Pedjatrija 1. Naprijed, Zagreb, 1994;241.
13. Mihić DB, Bojanić ZS. Kongenitalne minorne malfomacije. Medicinska knjiga Beograd – Zagreb, 1987.
14. Šumanović D. Minor malformacije i njihov značaj u neonatologiji. Paediatr Croat. 2004; 48(Supl 1):206.
15. Šumanović D, Božić T, Brkić V, Robović A, Saraga-Karačić V. Minor malformations: Neonatal or Anthropological Story? Coll. Antropol. 33(2009) Suppl2:31-35.
16. Sawardekar KP. Prevalence of isolated minor congenital anomalies in a regional hospital in Oman. Saudi Med J. 2005 Oct;26(10):1567-72.
17. El Awady H, AlGameel A, Ragab T, Hassan N. Congenital anomalies in neonates in Fayoum Governorate, Egypt. East Mediterr Health J. 2021 Aug 26;2788):790-797.
18. Mobungu G, Lumaka A, Mvuama N, Tshika D, Makay P, Tshilobo PL et al. Morphological characterization of newborn in Kinshase, DR Congo: Common variants, minor, and major anomalies. Am J Med Genet A. 2020 Apr;182(4):632-639.

19. Mubungu G, Makay P, Lumaka A, Mvuama N, Tshika D, Tady BP et al. Dysmorphism and major anomalies are a main predictor of survival in newborns admitted to the neonatal intensive care unit in the Democratic Republic of Congo. Am J Med Genet A. 2021 Feb;185(2):453-460.
20. Wen X, Belviso N, Murray E, Lewkowitz AK, Ward KE, Meador KJ. Association of Gestational Opioid Exposure and Risk of Major and Minor Congenital Malformations. JAMA Netw Open. 2021 Apr1;4(4):e215708.
21. Tsai FJ, Tsai CH, Peng CT, Wu JY, Lien CH, Wang TR. Different race, different face: minor anomalies in Chinese newborn infants. Acta Paediatr. 1999 Mar;88(3):323-6.
22. Hod M, Merlob P, Friedman S, Litwin A, Mor N, Rusecki Y et al. Prevalence of minor congenital anomalies in newborn of diabetic mother. Eur J Obstet Gynecol Reprod Biol. 1992 Apr21;44(2):111-6.
23. Kolobarić A, Delić L, Jerković Raguž M. Can the pathological conditions of the thyroid gland during pregnancy affect the course and outcome of pregnancy and the parameters of the newborn? Annals of Biomedical and Clinical Research. 2023;2:108-118.
24. Aby JL, Rocque BG, Loyal J. Management of asymptomatic cutaneous lumbosacral findings in newborns: a neurosurgical perspective. J Neurosurg Pediatr.2023 Feb 24;31(5):484-487.
25. Bader D, Grun M, Riskin-Mashiah S, GrunfenLd A, Kugleman A, Chistyakov I et al. Auricular mild errors of morphogenesis: epidemiological analysis, local correlations and clinical and clinical significance. Ann Genet. 2004 Jul-Sep;47(3):225-34.
26. Falsaperla R, Giacchi V, Aguglia MG, Mailo J, Longo MG, Natacci F et al. Monogenic Syndromes with Congenital Heart Diseases in Newborns (Diagnostic Clues for Neonatologists): A Critical Analysis with Systematic Literature Review. J Pediatr Genet. 2021 Sep;10(3):173-193.
27. Leppig KA, Werler MM, Cann Cl, Cook CA, Holmes LB. Predictive value of minor anomalies. I. Association with major malformations. J Pediatr 1987;110:531-7.

BJMG 27 (1), 2024 • 43-50
DOI: 10.2478/bjmg-2024-0009
sciendo

ORIGINAL ARTICLE

HIGH-RESOLUTION HLA-DRB1 ALLELE FREQUENCIES IN A ROMANIAN COHORT OF STEM CELL DONORS

Caragea MA[1,2], Ursu IR[3,*], Visan DL[2], Maruntelu I[1,2], Iordache P[4], Constantinescu A[1], Tizu M[1,2], Tălăngescu A[1,2], Constantinescu I[1,2]

[*]**Corresponding Author:** Lect., MD, PhD Radu-Ioan Ursu, Carol Davila University of Medicine and Pharmacy, Department of Medical Genetics, Bucharest, Romania, Str. Batistei 12, Bucharest, Romania; tel.: 0040736167020; e-mail: dr.radu.ursu@gmail.com

ABSTRACT

The goal of the current study was to determine the high-resolution frequencies of the HLA-DRB1 alleles among the analyzed Romanian cohort of healthy stem cell donors. Using Next Generation Sequencing (NGS), we estimated class II HLA-DRB1 allele frequencies to a 6-digit resolution through HLA typing in a Romanian cohort of healthy individuals. The study for HLA genotyping included 420 willing donors from the National Registry of Voluntary Hematopoietic Stem Cell Donors (RNDVCSH). In 2020 and 2021, peripheral blood samples were collected and transported to the Fundeni Clinical Institute. We used the Immucor Mia Fora NGS MFlex kit for HLA genotyping. Forty-one different alleles were detected in 420 analyzed samples, out of which the most frequent HLA-DRB1 alleles were DRB1*16:01:01 (12.6%), DRB1*11:04:01 (12.1%) and DRB1*03:01:01 (12%). The HLA-DRB1*11:01:02 and -DRB1*08:04:01, -DRB1*05:01:01, -DRB1*13:05:01, -DRB1*14:07:01, -DRB1*09:01:02, -DRB1*11:02:01, -DRB1*04:07:01, -DRB1*15:03:01, -DRB1*03:02:01, -DRB1*04:06:02, -DRB1*04:08:01, -DRB1*14:05:01 were identified only once. The results revealed similarities with countries belonging to the Eastern Europe, the Balkans and the Caucasus regions. Further studies on larger Romanian cohorts are needed for confirming the current results.

Keywords: HLA-DRB1, allele frequencies, transplantation immunogenetics

INTRODUCTION

The Major Histocompatibility Complex (MHC) has been the subject of much scientific interest in recent years especially due to the numerous studies on the HLA genes, part of the MHC [1-2]. The human leukocyte antigen system (HLA) is one of the most polymorphic genetic systems in the human genome [1,3]. Accurate HLA allele identification is essential for both anthropological research and for the field of organ and stem cell transplantation. Because more and more HLA alleles are being discovered through multiple studies, Next Generation Sequencing (NGS) HLA genotyping is necessary [3-4]. Using NGS for HLA typing has two advantages, one being able to resolve allelic ambiguities and the other in establishing updated allele frequencies [3-4]. These advantages will support the use of HLA types in research and clinical medicine in more precise and thorough ways [1-4].

HLA-DRB1 is one of HLA class II's beta chain paralogues. Alpha (DRA) and beta (DRB), both of which are anchored in the membrane, form the heterodimer that is the class II molecule [5-7]. Presenting peptides derived from extracellular proteins, it plays a pivotal role within the immune system. Antigen-presenting cells, or APCs (B lymphocytes, dendritic cells, and macrophages), express class II molecules [6-10]. Professional antigen-presenting cells (APCs) in complex with the alpha chain HLA-DRA present antigenic peptides for recognition by the alpha-beta

[1] Carol Davila University of Medicine and Pharmacy, Bucharest, Romania

[2] Center for Immunogenetics and Virology, Fundeni Clinical Institute, Bucharest, Romania

[3] Carol Davila University of Medicine and Pharmacy, Department of Medical Genetics, Bucharest, Romania

[4] Carol Davila University of Medicine and Pharmacy, Department of Epidemiology, Bucharest, Romania

T cell receptor (TCR) on HLA-DRB1-restricted CD4-positive T cells [6-10]. This directs the actions of T-helper effectors that are specific to antigens, thereby facilitating the elimination of infectious agents and transformed cells through antibody-mediated immune response and macrophage activation [8-10].

Genetic variants have been proven to play an essential role in most of the common human disorders (i.e. obesity, type II diabetes, hypertension, neurological disease, cancer, etc.) [11-12], research conducted on HLA-DRB1 frequencies on a variety of populations has also linked HLA-DRB1 alleles with the susceptibility and clinical response for many disorders, such as rheumatoid arthritis, sarcoidosis, Goodpasture syndrome and multiple sclerosis [13–20]. In order to comprehend the risk for these disorders, it is crucial first to determine the frequencies of the various HLA-DRB1 alleles in the Romanian population.

The goal of the current study was to determine the high-resolution frequencies of the HLA-DRB1 alleles among a healthy Romanian cohort of healthy stem cell donors whose data was analyzed.

MATERIAL AND METHODS

The study for HLA typing included 405 healthy voluntary donors (Romanians/Europeans, 61% male, age 43.3 ± 7.7 years) who were registered in the National Registry of Voluntary Hematopoietic Stem Cell Donors (RNDVCSH).

This study was conducted at the Fundeni Clinical Institute in the Medical Analysis Laboratory 2, and it involved healthy donors who voluntarily registered for stem cell donation in the RNDVCSH between 2020 and 2021.

In accordance with the Declaration of Helsinki, written consent from willing donors was requested for the processing of personal data and evidence. This study was reviewed and approved by the Ethics Committee of the Fundeni Clinical Institute (no. Ten Points: 7916/10.02. 2021).

Each donor's medical file contained medical data that the project's research team extracted, processed, and statistically examined. We included willing donors between the ages of 20 and 50 in the study who had no underlying health issues. We looked through each donor's personal medical record to see if their medical history was in compliance with the national protocol. After they donated blood, we also looked at their viral status and biochemical parameters.

Peripheral blood collected in vacutainers containing the anticoagulant EDTA (ethylene-diamino-tetra-acetic acid) provided the DNA used in this investigation. DNA was extracted from blood using the manual technique. DNA was extracted using the QIAmp DNA Blood Mini® extraction kit (QIAGEN, Hilden, Germany). The purification of total DNA (genomic, mitochondrial) from bone marrow, cell cultures, leukocyte concentrate, and whole blood was made possible by this rapid and easy technique based on silicon dioxide membranes.

Each blood sample was thoroughly vortexed, mixed with protease and lysis buffer, and heated in a thermoblock for 10 minutes at 56 degrees Celsius to facilitate rapid lysis. The DNA was still free in the lysate after the cell membranes broke down, so we added 80% alcohol to make it precipitate. The lysate was placed into tubes with silicon membranes, to which DNA adheres, because the two materials had different electrical charges. The DNA was purified by multiple washings and separated from the silicon membrane following the addition of the elution buffer, which neutralizes the electrical charges.

Prior to usage, the DNA was divided into tubes and stored at -18 °C. An IMPLEN nanophotometer was used to measure the concentration and purity of the DNA using an A260nm/A280nm ratio between 1.7 and 1.9, certifying solution purity and a DNA concentration at >20 ng/µL.

The Mia Fora NGS MFlex kit from Immucor was used to genotype the HLA class II alleles (HLA-DRB1, DRB3/4/5) at a 6-digit resolution. Using Next-Generation Sequencing techniques, HLA genotyping was carried out using the MIA FORA NGS MFlex HLA kit (MIA FORATM NGS MFlex) from Immucor. The three main components of this process are long-range PCR, library construction, and sequencing and data analysis. In the long-range PCR step, the most pertinent HLA genes were amplified. After fragmented probes are used to build libraries, adenine nucleotides are added to the ends of each fragment to enhance the ligation of the unique index adapters. Each fragment is barcoded to make identification easier during sequencing. To ensure adequate cluster generation, a final amplification of the size-selected library was then required. By using the Pippin Prep system, DNA fragments containing 500–900 base pairs were selected. Before the final library preparation, the concentration was measured using a Qubit® fluorometer (Thermo Fisher Scientific) and adjusted according to the protocol.

Using Illumina reagents, the NGS sequencing library was prepared and then loaded into an Illumina MiniSeq sequencer. Making use of the MIA FORA NGS FLEX program (Sirona Genomics, Inc. Sirona Genomics and IMGT databases, two reference databases, were used to interpret data after sequencing was completed. To confirm the allele frequency distribution among the analyzed population, the Hardy-Weinberg equilibrium was used.

RESULTS

A total of 41 different HLA-DRB1 alleles were detected in our cohort (table 1).

Table 1. The alleles with the highest frequencies for the HLA-DRB1 locus were represented by the DRB1*16:01:01 (12.6%), DRB1*11:04:01 (12.1%), DRB1*03:01:01 (12%), DRB1*07:01:01 (9.3%), DRB1*01:01:01 (7.3%), DRB1*11:01:01 (6.3%) and DRB1*13:01:01 (6%) (table 1). Out of the total of 41 detected alleles, 3 had frequencies higher than 10% (table 1), identified out of a total of 297 individuals. The top 3 alleles amount to more than one third of all the identified alleles (36.67%) (table 1). All these 3 alleles had frequencies of 12% - 13% [DRB1*16:01:01 (12.6%), DRB1*11:04:01 (12.1%), DRB1*03:01:01 (12%)], with the top frequency at 12.6% (table 1, figure 1).7 of the 41 HLA-DRB1 alleles had frequencies higher than 5%.

Table 1. HLA-DRB1 alleles identified in the Romanian cohort (6-digits)

HLA-DRB1 alleles	No.	AF (%)
*16:01:01	102	12.59
*11:04:01	98	12.10
*03:01:01	97	11.98
*07:01:01	75	9.26
*01:01:01	59	7.28
*11:01:01	51	6.30
*13:01:01	49	6.05
*15:01:01	35	4.32
*13:02:01	24	2.96
*04:01:01	21	2.59
*04:02:01	17	2.10
*04:03:01	17	2.10
*13:03:01	15	1.85
*14:54:01	15	1.85
*10:01:01	14	1.73
*15:02:01	13	1.60
*04:04:01	11	1.36
*16:02:01	11	1.36
*12:01:01	10	1.23
*08:01:01	9	1.11
*01:02:01	9	1.11
*14:03:01	9	1.11
*14:01:01	9	1.11
*04:05:01	8	0.99
*14:04:01	8	0.99
*11:03:01	6	0.74
*08:03:02	3	0.37
*02:02:01	2	0.25
*11:01:02	1	0.12
*08:04:01	1	0.12
*05:01:01	1	0.12
*13:05:01	1	0.12
*14:07:01	1	0.12
*09:01:02	1	0.12
*11:02:01	1	0.12
*04:07:01	1	0.12
*15:03:01	1	0.12
*03:02:01	1	0.12
*04:06:02	1	0.12
*04:08:01	1	0.12
*14:05:01	1	0.12
Total general	**810**	**100**

AF – Allele Frequency; No. – Number of alleles

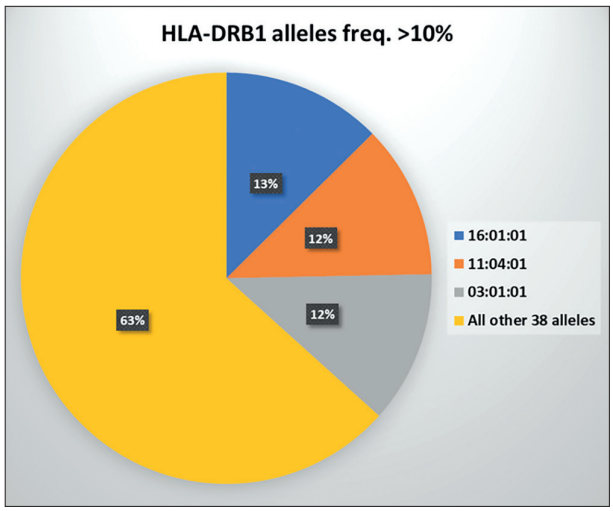

Figure 1. HLA-DRB1 alleles with frequencies > 10% (6-digits)

In total, the added frequencies of these 7 alleles represent almost two thirds (65.55%) of all detected HLA-DRB1 variants, identified in 531 individuals. HLA-DRB1 alleles with low frequencies (<5%) stand for 35% (279 individuals) of all observed variants (table 1).

Out of the entire cohort and all detected HLA-DRB1 variants, 18 alleles showed frequencies lower than 1% (table 1). 13 of these rare variants were detected in just one individual each (0.12%), while the HLA-DRB1*02:02:01 allele was observed in 2 individuals (0.24%) and DRB1*08:03:02 in 3 (0.37%), all with frequencies lower than 0.5%.

3 of the 18 rare alleles had frequencies of 0.5%-1% (DRB1*11:03:01 and DRB1*14:04:01, both in 8 individuals, 0.99% each) and DRB1*11:03:01 (6 persons, 0.74%).

Apart from the aforementioned 18 rare HLA-DRB1 alleles, 4 others had frequencies close to 1% (1.11%), being detected in 9 individuals each. These 4 alleles represent together 4.44% of all resulted HLA-DRB1 variants.

In total, 22 HLA-DRB1 alleles had frequencies of a maximum of 1.11%, totalizing 9.38% of all detected variants and being identified in 76 individuals. The rest of the 734 HLA-DRB1 alleles represent 90.62% of all variants.

When adding the identified frequencies of all the variants for each HLA-DRB1 allele, the top alleles, with

Table 2. HLA-DRB1 allele frequencies (2-digits)

HLA-DRB1 alleles	AF (%)
DRB1*11	19.38
DRB1*16	13.95
DRB1*03	12.10
DRB1*13	10.99
DRB1*04	9.51
DRB1*07	9.26
DRB1*01	8.40
DRB1*15	5.93
DRB1*14	5.31
DRB1*10	1.73
DRB1*12	1.23
DRB1*02	0.25
DRB1*05	0.12
DRB1*09	0.12

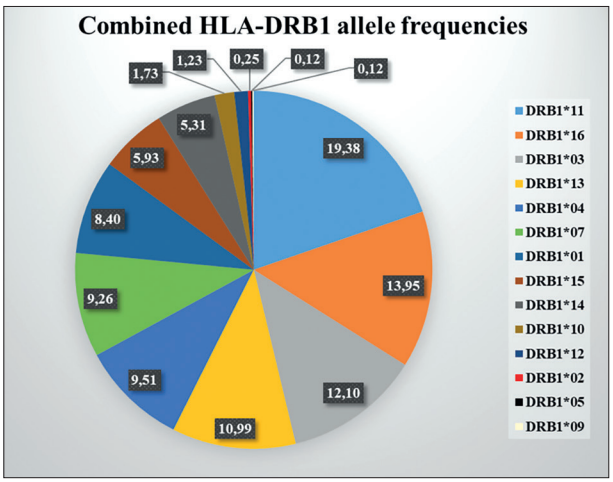

Figure 2. Combined HLA-DRB1 allele frequencies (2-digits)

frequencies higher than 10%, were DRB1*11 (19.38%), DRB1*16 (13.95%), DRB1*03 (12.10%), DRB1*13 (10.99%) (table 2, figure 2).

Table 2. 6 other alleles revealed average frequencies, of 5% - 10% (DRB1*04, DRB1*07, DRB1*01, DRB1*15 and DRB1*14), while 2 were found in the 1%-5% frequency range and 3 had low frequencies, of less than 1% (DRB1*02, DRB1*05, DRB1*09) (table 2).

DISCUSSIONS

The most frequent HLA-DRB1 alleles identified through the current research (frequencies >10%) were HLA-DRB1*16:01:01 (12.6%), HLA-DRB1*11:04:01 (12.1%), DRB1*03:01:01 (12%). The other more common variants detected (frequencies 5% - 10%) were HLA-DRB1*07:01:01 (9.3%), HLA-DRB1*01:01:01 (7.3%) and HLA-DRB1*11:01:01 (6.3%) and HLA-DRB1*13:01:01 (6%), while HLA-DRB1*15:01:01 was observed in approx. 4.4% of all tested individuals and HLA-DRB1*13:02:01 and HLA-DRB1*04:01:01 being at approximately a 3% frequency.

When adding the frequencies of all the variants for each HLA-DRB1 allele, the most common alleles are DRB1*11 (19.38%), DRB1*16 (13.95%), DRB1*03 (12.10%), DRB1*13 (10.99%), DRB1*04 (9.51%), DRB1*07 (9.26%), DRB1*01 (8.40%), DRB1*15 (5.93%), DRB1*14 (5.31%), while the other 5 detected HLA-DRB1 alleles (DRB1*10, DRB1*12, DRB1*02, DRB1*05, DRB1*09) had low combined frequencies (1.73% or lower).

In Europe, on average, the most common HLA-DRB1 variants are HLA-DRB1*07:01, followed by the DRB1*03:01, DRB1*15:01, DRB1*01:01, DRB1*11:01, DRB1*13:01, DRB1*04:01 and DRB1*16:01 alleles (listed in the descending order of their frequencies) [7].

The frequency observed for the most common HLA-DRB1 allele identified in our analyzed Romanian cohort (HLA-DRB1*16:01:01, 12.6%), if confirmed through further studies on wider populations, would be the second highest detected in all populations worldwide, only Bulgarians reveal higher frequencies for this allele (AF 15.5%) [7, 14]. The next population group, in descending order of allele frequencies for this HLA-DRB1 variant, would be Polish (8%) and then certain Russian populations (Belgorod and Nizhny Novgorod Russians and Bashkortostan Bashkirs) (AF 3.3% - 4.9%), but with much lower percentages for this allele [7].

Similarly, to HLA-DRB1*16:01:01, the frequency detected for the second most common allele in our cohort (HLA-DRB1*11:04:01, 12.1%), would also, if confirmed, be the second highest worldwide, being surpassed, again, only by the Bulgarian population (AF 15.5%) [7, 20]. The next high frequencies can be observed in the Paraguayan/Argentinian Guarani population (AF 10%) and different Russian populations (Russians from the Belgorod and Vologda regions and Tatars from Bashkortostan) (AF 5.54% - 5.88%), but also the Portuguese Madeira population (5.5%) [7].

The other more common HLA-DRB1*11 variant, HLA-DRB1*11:01, was observed in only approx. 6.3% of all analyzed Romanian individuals, almost half the frequency of HLA-DRB1*11:04. Similar findings were observed in continental and Crete island Greeks, Bulgarians, Macedonians, Croatians, Turks, and Italians, where HLA-DRB1*11:04 predominated over HLA-DRB1*11:01 [7, 21-30]. In contrast, the two alleles displayed equal or opposite frequencies in Central and Western European

countries, where the HLA-DRB1*11:01 allele is widely more prevalent [7].

Both the HLA-DRB1*16:01:01 and the HLA-DRB1*11:04:01 variants appear to be more specific for the populations belonging to or historically tracing back from the Eastern European, Caucasus, Black Sea and Balkan regions.

HLA-DRB1*16:01:01 is the most frequent in Macedonia (AF 14.9%), the second most prevalent in Bulgaria (after HLA-DRB1*11:04:01), Kosovo (12.9%) and Greece (HLA-DRB1*16:01, 4 digits, AFs 7.8% – 13.7%), the fourth in Slovenia, while the HLA-DRB1*16:01 allele (low resolution) is the third most frequent is among the population on the Croatian island of Krk (AF 11.2%) (and the HLA-DRB1*16 has the second highest prevalence with an AF of 11.8%). This is also possible in high percentages in Albania, where the second most commonly detected (low frequency) HLA-DRB1 variant is HLA-DRB1*16 (AF 12.4%), as is in Kosovo (AF 13.75%) [7, 21-30].

The HLA-DRB1*11:04:01 is possibly an even more ancient populational indicator, being observed in high frequencies in cohorts from Macedonia (second most frequent allele, AF 13.9%), Bulgaria (most common variant), Greece (HLA-DRB1*11:04 AFs 13.9% – 19.3%), Turkey (the most frequent HLA-DRB1 allele), also on the Croatian island of Krk (13.2%), Israeli Jews of Georgian descent (24.2%) and Polish Jews (17.8%), Armenia's combined Regions (11.5%), in Lebanon's Kafar Zubian (23.7%) and Niha el Shouff (17%) (where the DRB1*11:04 allele is the highest prevalent), Israeli Jews of Libyan descent (17.4%), Polish (17.8%) and Moroccan Jews (16.4%), or in Kosovo (10.5%), (HLA-DRB1*11:04 the second most common), parts of Black Sea Russia, Middle Eastern regions, and including Spanish Basque areas of Cantabria and Rome in Italy (the most prevalent variant) [7, 21-30].

A difference, nevertheless, can be observed between Eastern European countries (where both of these alleles are highly common) and the other above mentioned populations, (as are the Krk Island Croatians, the Armenians, the Spanish Cantabrian Basques and the Italians), where only HLA-DRB1*11:04:01 (or HLA-DRB1*11:04, depending on resolution) is the top or the second allele, but the HLA-DRB1*16:01:01 is one of the least frequent variants detected.

Both these similarities and differences could find an explanation in the historical genetic lineage of the peoples living in these areas, most of them being connected to the Pelasgians/Thracians/Getae/Dacians populations originating from these geographical areas (Turkey, Black Sea countries, Romanian regions, Bulgaria, parts of Albania and Macedonia, Kosovo, etc.). Also, the presence of the HLA–DRB1*11:04:01 allele in certain populations only (Basques, Armenians, Italians) can be explained by common ancient ancestors (Pelasgians/Thracians/Etruscans) of these peoples with the populations from the Eastern Europe and Balkan areas, the HLA-DRB1*16:01:01 variant appearing to be a mutational event which occurred after the separation of these groups, with a high specificity for Eastern Europe/Balkans.

Having reviewed related work, the frequencies of HLA-DRB1*16:01:01 and HLA-DRB1*11:04:01 were at a very high frequency compared to the prevalence in most Central and Western European populations.

In research articles from this European geographical area, and in the Romanian population as resulted from previous studies, the most common HLA-DRB1 variants are HLA-DRB1*03:01:01 and HLA-DRB1*07:01:01 [7,18,21-25]. The frequencies of the HLA-DRB1*07:01:01 and HLA-DRB1*03:01:01 alleles in the tested cohort were, by contrast, lower than those found in all populations from Central and Western Europe and also in past Romanian studies.

The third most common HLA-DRB1 variant in our studied population, HLA-DRB1*03:01:01 (11.98%), is one of the frequently identified alleles in various populations globally. Similar frequencies have been observed in populations such as USA Caucasians (12.47%), Portuguese Madeirans (12.2%), Israel Yemenite Jews (12%), Morocco Settat Chaouya (11.7%) and Atlantic Coast Chaouya (11.6%), Algerian Oran (11.6%), American citizens of Italian descent (11.2%) and Polish populations (10.9%). The highest frequencies worldwide for this variant can be seen in the Morocco Nador Metalsa (20.2%), Spanish Canary Islands (Gran Canaria island) (16.3%) and Northern Ireland (15.4%) populations [7].

Apart from the more frequently detected alleles in other populations throughout the globe, the current study also revealed the presence of 4 rare alleles (described in a maximum of 3 cohorts in published research results: HLA-DRB1*1:03:01 – 1 previous report; HLA-DRB1*11:01:02 – 2 reports; HLA-DRB1*04:08:01 – 2 reports; HLA-DRB1*13:0:01 – 3 reports) and also one new variant, not previously described in the literature (HLA-DRB1*04:06:02).

Further studies on larger Romanian cohorts need to be undertaken as to confirm these results and to understand their clinical, epidemiological, diagnostic, therapeutic or molecular implications.

CONCLUSIONS

The current research provides important data on the HLA-DRB1 alleles frequencies among the Romanian population.

As expected and observed in previous studies, there are similarities and disparities when it comes to the more common and less frequent alleles between our analyzed cohort and the various European and non-European populations. Important similarities can be observed with different populations from Eastern Europe, the Balkans and Caucasus regions, possibly indicating a common genetic ancestry (documented by archaeological and historical data), while the Central and Western European countries show a constantly different constellation of HLA-DRB1 variants.

Further studies are needed for confirming and strengthening the findings of this research for the Romanian population.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

REFERENCES

1. Delves PJ, Martin SJ, Burton DR, Roitt IM. Part. 1. Fundamentals of Immunology. In: Roitt's Essential Immunology, 13th ed.; Delves, P.J., Martin, Eds.; John Wiley and Sons, Ltd.: Oxford, UK, 2017, 1–291.
2. Rich RR, Fleisher TA, Shearer WT, Schroeder Jr, HW, Frew AJ, Weyand CM Part One: Principles of immune response. Clinical Immunology. Principles and practice. Fourth edition; Rich, R.R; Elsevier Saunders: UK, 2013, 3–183.
3. Tan MS, Jiang T, Tan L, Yu JT. Genome-wide association studies in neurology. Ann Transl Med, 2014, 2(12):124. doi: 10.3978/j.issn.2305-5839.2014.11.12 PMID: 25568877 PMCID: PMC4260053.
4. Uçar F, Karkucak M, Alemdaroğlu E, Capkin E, Yücel B, Sönmez M, et al. HLADRB1 allele distribution andits relation to rheumatoid arthritis in eastern Black Sea Turkish population. Rheumatol Int, 2012, 32:1003-1007.
5. Gourraud PA, Pappas DJ, et al. High-resolution HLA-A, HLA-B, and HLA-DRB1 haplotype frequencies from the French Bone Marrow Donor Registry. Human Immunology, 2015, 76(5):381-384. https://doi.org/10.1016/j.humimm.2015.01.028.
6. Grubic Z, Burek Kamenaric M, Mikulic M, Stingl Jankovic K, Maskalan M, Zunec R. HLA-A, HLA-B and HLA-DRB1 allele and haplotype diversity among volunteer bone marrow donors from Croatia. Int J Immunogenet, 2014, 41(3):211-21. doi: 10.1111/iji.12117 PMID: 24762167.
7. Allele Frequency Net Database. Available at http://www.allelefrequencies.net/default.asp (accessed on 09 January 2024)
8. Constantinescu I, Boscaiu V, Cianga P, Dinu AA, Gai E, Melinte M, Moise A. The frequency of HLA alleles in the Romanian Population. Immunogenetics 2016, 68(3), 167-78. doi: 10.1007/s00251-015-0891-9. Epub 2015 Dec 28. PMID:26711124.
9. Online Mendelian Inheritance in Men, OMIM. Available at www.omim.org (accessed on 09 January 2024)
10. Genecards. Available at www.genecards.org (accessed on 09 January 2024)
11. Ursu RI, Cucu N, Ursu GF, et al. Frequency study of the FTO and ADRB3 genotypes in a Romanian cohort of obese children. Rom Biotechnol Lett. 2016, 21(3), 11610–11620.
12. Ursu RI, Iordache P, Radoi VE, et al. Novel loci associated with hypertension in a Romanian cohort of elder male individuals. European Heart Journal 2017, 38 (suppl_1), 559. https://doi.org/10.1093/eurheartj/ehx502.P2631
13. Rossman MD, Thompson B, Frederick M, Maliarik M, Iannuzzi MC, Rybicki BA, Pandey JP, Newman LS, Magira E, Beznik-Cizman B, Monos D, ACCESS Group. HLA-DRB1*1101: a significant risk factor for sarcoidosis in blacks and whites. Am J Hum Genet, 2003, 73: 720-735 PubMed: 14508706.
14. Stumpf AN, van der Meijden ED, van Bergen CA, Willemze R, Falkenburg JH, Griffioen M. Identification of 4 new HLA-DR-restricted minor histocompatibility antigens as hematopoietic targets in antitumor immunity. Blood, 2009, 114(17):3684-3692 doi: 10.1182/blood-2009-03-208017 PMID: 19706888.
15. International Multiple Sclerosis Genetics Consortium, Wellcome Trust Case Control Consortium 2. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature, 2011, 476: 214-219 PubMed: 21833088
16. Shams H, Klucar P, Weis SE, Lalvani A, Moonan PK, Safi H, Wizel B, Ewer K, Nepom GT, Lewinsohn DM, Andersen P, Barnes PF. Characterization of a Mycobacterium tuberculosis peptide that is recognized by human CD4+ and CD8+ T cells in the context of multiple HLA alleles. J Immunol, 2004, 173(3):1966-77 doi: 10.4049/jimmunol.173.3.1966 PMID: 15265931.
17. Fujiki F, Oka Y, Kawakatsu M, Tsuboi A, Nakajima H, Elisseeva OA, Harada Y, Li Z, Tatsumi N, Kamino E, Shirakata T, Nishida S, Taniguchi Y, Kawase I, Oji Y, Sugiyama H. A WT1 protein-derived, naturally processed 16-mer peptide, WT1(332), is a

promiscuous helper peptide for induction of WT1-specific Th1-type CD4(+) T cells. Microbiol Immunol, 2008, 52(12):591-600 doi: 10.1111/j.1348-0421.2008.00080.x PMID: 19120973.

18. Ooi JD, Petersen J, Tan YH, Huynh M, Willett ZJ, Ramarathinam SH, Eggenhuizen PJ, Loh KL, Watson KA, Gan PY, Alikhan MA, Dudek NL, Handel A, Hudson BG, Fugger L, Power DA, Holt SG, Coates PT, Gregersen JW, Purcell AW, Holdsworth SR, La Gruta NL, Reid HH, Rossjohn J, Kitching AR. Dominant protection from HLA-linked autoimmunity by antigen-specific regulatory T cells. Nature, 2017, 545(7653):243-247. doi: 10.1038/nature22329 PMID: 28467828 PMCID: PMC5903850.
19. Vica ML, Matei HV, Bondor CI, et al. HLA Polymorphisms and Haplotype Diversity in Transylvania, Romania. J Immunol Res. 2019, 30, 1342762. doi: 10.1155/2019/1342762. PMID: 32083138; PMCID: PMC7012258.â
20. Suarez-Trujillo F, Rey D, Bendukidze N, Juarez I, Sanchez-Orta A, Palacio-Gruber J, Manuel Martin-Villa J, Arnaiz-Villena A. Class II HLA in Georgia Caucasus Tbilisi Georgians and their Mediterranean ancestry: The Usko Mediterranean languages. Hum Immunol. 2022 Nov;83(11):739-740. doi: 10.1016/j.humimm.2022.08.005. Epub 2022 Aug 18. PMID: 35987703.
21. Ivanova M, Rozemuller E, Tyufekchiev N, Michailova A, Tilanus M, Naumova E. HLA polymorphism in Bulgarians defined by high-resolution typing methods in comparison with other populations. Tissue Antigens, 2002, 60(6):496-504 doi: 10.1034/j.1399-0039.2002.600605.x PMID: 12542743.
22. Arnaiz-Villena A, Dimitroski K, Pacho A, Moscoso J, Gómez-Casado E, Silvera-Redondo C, Varela P, Blagoevska M, Zdravkovska V, Martínez-Laso J. HLA genes in Macedonians and the sub-Saharan origin of the Greeks. Tissue Antigens, 2001, 57(2):118-127 doi: 10.1034/j.1399-0039.2001.057002118.x PMID: 11260506.
23. Petlichkovski A, Efinska-Mladenovska O, Trajkov D, Arsov T, Strezova A, Spiroski M. High-resolution typing of HLA-DRB1 locus in the Macedonian population. Tissue Antigens, 2004, 64(4):486-491 doi: 10.1111/j.1399-0039.2004.00273.x PMID: 15361127.
24. Papassavas EC, Spyropoulou-Vlachou M, Papassavas AC, Schipper RF, Doxiadis IN, Stavropoulos-Giokas C. MHC class I and class II phenotype, gene, and haplotype frequencies in Greeks using molecular typing data. Hum Immunol, 2000, 61(6):615-623 doi: 10.1016/s0198-8859(00)00115-4. Erratum in: Hum Immunol, 2000, 61(8):843 PMID: 10825590.
25. Tokić S, Žižkova V, Štefanić M et al. HLA-A, -B, -C, -DRB1, -DQA1, and -DQB1 allele and haplotype frequencies defined by next generation sequencing in a population of East Croatia blood donors. Sci Rep, 2020, 10, 5513 https://doi.org/10.1038/s41598-020-62175-9.
26. Sanchez-Velasco P, Gomez-Casado E, Martinez-Laso J, Moscoso J, Zamora J, Lowy E, Silvera C, Cemborain A, Leyva-Cobián F, Arnaiz-Villena A. HLA alleles in isolated populations from North Spain: origin of the Basques and the ancient Iberians. Tissue Antigens. 2003 May;61(5):384-92. doi: 10.1034/j.1399-0039.2003.00041.x. PMID: 12753657.
27. Sulcebe G, Shyti E. HLA-A, -B, -C, -DRB1 and -DQB1 allele and haplotype frequencies in a population of 432 healthy unrelated individuals from Albania. Hum Immunol. 2016 Aug;77(8):620-621. doi: 10.1016/j.humimm.2016.06.001. Epub 2016 Jun 2. PMID: 27262454.
28. Matevosyan L, Chattopadhyay S, Madelian V, Avagyan S, Nazaretyan M, Hyussian A, Vardapetyan E, Arutunyan R, Jordan F. HLA-A, HLA-B, and HLA-DRB1 allele distribution in a large Armenian population sample. Tissue Antigens. 2011 Jul;78(1):21-30. doi: 10.1111/j.1399-0039.2011.01668.x. Epub 2011 Apr 19. PMID: 21501120.
29. Yantır E, Gündüz E, Çolak E. HLA Alleles, Genotype and Haplotype Analyzes from Central Anatolia Region of Turkey. Balkan Med J. 2023 Sep 11;40(5):358-366. doi: 10.4274/balkanmedj.galenos.2023.2023-4-55. Epub 2023 Aug 16. PMID: 37584525; PMCID: PMC10500136.
30. Nestorowicz K, Bogacz A, Bukowska A, Chraplak M, Czerwiński J, Góralski M, Gronkowski M, Jopek K, Kniżewski Ł, Kolasiński M, Kowalski ML, Nowak J, Sowiński M, Wróblewska-Kabba S, Tymoniuk B, Dudkiewicz M. High-resolution allele frequencies for NGS based HLA-A, B, C, DQB1 and DRB1 typing of 23,595 bone marrow donors recruited for the Polish central potential unrelated bone marrow donor registry. Hum Immunol. 2020 Feb-Mar;81(2-3):49-51. doi: 10.1016/j.humimm.2019.12.005. Epub 2020 Jan 2. PMID: 31902540.

BJMG 27 (1), 2024 • 51-58
DOI: 10.2478/bjmg-2024-0007
sciendo

ORIGINAL ARTICLE

THE IMPACT OF THE COVID-19 PANDEMIC ON INDIVIDUALS WITH DOWN SYNDROME: A CROATIAN SURVEY

Barišić A[1], Ergović Ravančić M[2], Majstorović D[3], Vraneković J[4,*]

[*]**Corresponding Author:** Assoc. Prof. Jadranka Vraneković, Faculty of Medicine, University of Rijeka, Department of Medical Biology and Genetics, B. Branchetta 20, 51000 Rijeka, Croatia;
tel: +385 (0)51 651 131, fax: +385 (0)51 678 896. Email: jadranka.vranekovic@medri.uniri.hr

ABSTRACT

Background: Severe acute respiratory syndrome coronavirus-2 infection has spread uncontrollably worldwide. Among the most vulnerable groups in society are populations with multiple comorbidities, including individuals with Down syndrome (DS).

Aim: Our aim was to conduct an online survey to assess the impact of COVID-19 on DS individuals in Croatia. We also explored the views of their parents and caregivers about the challenges they faced during COVID-19.

Methods: The anonymous online survey was launched in March 2022 and remained open until October 2022. Participants were conducted online through closed group on Facebook. The survey included questions about participant characteristics, medical information, clinical presentation of COVID-19, and challenges faced by the parents during COVID-19.

Results: A total of 268 surveys were collected and analysed. We found that age and body mass index of DS individuals were significantly and positively correlated with the clinical presentation of COVID-19. Lack of social activities, cancelled therapies, and psychological problems were the most frequently cited challenges during the pandemic.

Conclusion: Clinicians and caregivers should primarily be alert to the same COVID-19 signs and symptoms that occur in the general population (fever, cough, shortness of breath). Ongoing therapies, social activities, and psychological support should be cited as indispensable for maintaining physical health and emotional well-being in DS individuals.

Keywords: COVID-19; Croatia, Down syndrome; health; pandemic

INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection has spread uncontrollably worldwide. Among the most vulnerable groups in society are populations with multiple comorbidities, including individuals with Down syndrome (DS) [1]. Down syndrome, an expression of chromosome 21 trisomy (Chr21), is the most common genetic disorder known to date. It is observed in 1 in 400-1500 newborns worldwide [2]. Individuals with DS suffer varying degrees of cognitive disability, morphogenetic abnormalities, and a number of specific comorbidities. In addition, DS is often characterized by upper respiratory tract anatomical differences, immune dysfunction, and cardiovascular disease, which may promote coinfection and increase the risk for more severe clinical outcomes of COVID-19 [3,4]. In addition, Chr21 contains genes directly involved in the cell entry of SARS-CoV-2 (e.g. *TMPRSS2, APP, SYNJ1, ITSN1*) [5], and multiple genes involved in orchestrating immune response (e.g. four interferon receptors, which serve as receptor subunits for the interleukins [6].

To gain insight into the susceptibility, manifestation, and impact of COVID-19 and DS, the Trisomy 21 Research Society (T21RS) conducted the largest survey of individuals with DS who had COVID-19. The survey was designed to describe the epidemiologic and clinical characteristics of COVID-19 and Down syndrome, including risk factors for severe disease progression, compared to those in

[1] Department of Gynaecology and Obstetrics, Clinical Hospital Centre Rijeka, Rijeka, Croatia

[2] Faculty of Tourism and Rural Development in Požega, Croatia

[3] Faculty of Medicine, Juraj Dobrila University of Pula, Pula, Croatia

[4] Department of Medical Biology and Genetics, Faculty of Medicine, University of Rijeka, Rijeka, Croatia

the general population. Preliminary results that are based on >1000 individuals with Down syndrome suggest a more severe manifestation of SARS-CoV-2 infection, with more severe medical complications and a higher mortality rate in individuals with DS compared with individuals without [7]. Since 2021 several studies have examined the impact of the COVID-19 pandemic on health in people with DS from diverse countries [8,9,10,11,12,13,14,15,16,17].

Considering this, we conducted an online survey between March and October of 2022 to assess the impact of COVID-19 on DS individuals in Croatia. In addition, we explored the views of their parents and caregivers about the challenges they faced during COVID-19 and their experience with medical support.

MATERIALS AND METHODS

Study design

This anonymous online survey was launched on March 22, 2022, and remained open until October 10, 2022. It was approved by the Institutional Review Board of the Biomedical Research Ethics Committee of the Faculty of Medicine, University of Rijeka (Ref. No 2170-24-04-3/1-22-13). The survey targeted parents and caregivers of DS individuals in Croatia.

Participants

Participants were recruited online through a closed Facebook support group DS – the strength of chromosome 21 (576 members). The group offered education and support for parents and caregivers of DS individuals. Potential participants received a link to the survey website. Inclusion criteria were a willingness to participate in the study and a completed questionnaire. An information sheet for participants was provided at the beginning of the survey.

Survey

The questionnaire was originally developed in Croatian by the authors for the current survey. It included 18 questions on participant characteristics (six questions), medical information (three questions), clinical presentation of COVID-19, treatment, and vaccination (six questions), as well as challenges parents faced during COVID-19, the impact on the child, and medical support for them (three questions). To preserve the anonymity of the survey, no internet log addresses were collected. The full questionnaire can be found in the Supplementary material. Additionally, participant characteristics were compared with data of T21RS.

Data analysis

Statistical analysis was performed using SPSS version 13.3 (StatSoft, Inc., Tulsa, OK, USA), for Windows. Descriptive statistics were used to summarize the data. Categorical data were described as frequencies (percentages), and quantitative variables were expressed as means (SD). A chi-square test examined the differences between categorical variables to determine if they were related. Post hoc tests were applied to detect specific differences between groups when results were statistically significant. One-way ANOVA was used to compare independent and dependent variables, whereas Pearson correlation measured the statistical relationship between the variables.

Results

We collected 268 surveys from parents or caregivers of individuals with DS from March to October 2022. Results stratified by category are shown below.

Participant characteristics

Participant characteristics are listed in Table 1. The most common age category was 5-12 years (38.4%), with a mean age 8.7± 8.1 (mean±SD) years. The proportion of male children was slightly higher (54.1%). Our study showed that 60.8% of DS individuals had body mass index (BMI) <18.5 kg/m^2. There was a statistically significant association between BMI and age (r=0.811). The majority of cases (74.7%) had full trisomy 21 and a moderate level of intellectual disability (34.7%). Most of the cases lived at home with their family (95.5%). In comparison with high-income countries (HICs), all analysed participant characteristics (except gender) were significantly different ($p<0.05$).

Medical information

We catalogued 18 different comorbidities that are common in individuals with DS. Congenital heart defect (CHD) was the most common comorbidity (41.4%), followed by thyroid disorder (19.5%) and allergies (8.6%). Among CHDs, the most common type was atrioventricular septal defect (AVSD) (32.4%). Overall, 69.8% of cases reported not taking any supplements, whereas the others who reported their daily intake, mainly used probiotics (34.0%), multivitamins (29.8%), and vitamin D (25.6%) (Table 2). There was no statistically significant correlation between CHDs/supplementation with symptom severity (r= -0.190; r=0.001).

Clinical presentation of COVID-19, treatment and vaccination

About half of the cases reported being SARS-CoV-2 positive (50.7%). The main signs and symptoms associated with COVID-19 in individuals with DS were fever, cough, and shortness of breath - alone or in combination (Table 3). The chi-square test for a relationship between

Table 1. Participant characteristics (N=268) in comparison with high income countries (T21RS data)

	Count (N)	Percent (%)	Count (N)	Percent (%)	
	CROATIA		HIC		P – value*
AGE, mean (SD)	8.7 (8.1)		33.5 (19.1)		
0-1	49	18.3			<0.001
2-4	55	20.5			
5-12	103	38.4			
13-19	32	11.9			
>20	29	10.9			
GENDER					
Female	123	45.9	355	44.7	0.773
Male	145	54.1	436	54.9	
Other	0	0.0	3	0.4	
BMI, mean (SD)	19.1 (6.2)		not reported		
<18.5	163	60.8			
18.5-25	56	20.9			
25-30	27	10.1			
>30	14	5.3			
Missing	8	2.9			
TYPE OF TRISOMY 21					
Full/standard	201	74.7	274	88.4	<0.001
Mosaic	11	4.1	29	9.4	
Translocation	17	6.3	5	1.6	
Don't know	39	14.9	2	0.6	
LEVEL OF INTELLECTUAL DISABILITY					
Borderline/mild	91	34.0	126	18.3	<0.001
Moderate	93	34.7	404	58.7	
Severe/profound	19	7.0	158	23.0	
Don't know	65	24.3	0	0.0	
LIVING SITUATION					
Living at home with family	256	95.5	425	55.5	<0.001
Living at home with caregivers	2	0.8	10	1.3	
Residential care facility	6	2.2	181	23.6	
Other	4	1.5	149	19.5	

*tested with $\chi 2$ test (categorical variables) or t-test (continuous variables); BMI=body mass index, HIC=high income countries

age and symptoms of COVID-19, showed a statistically significant positive association ($\chi 2$=23.35; P=0.025). A post hoc test revealed that this difference originated from age group 3 (5-12 years), which was found to have the lowest number of symptoms, compared with all other groups ($\chi 2$=12.28; P=0.006). BMI was significantly and positively correlated with clinical presentation of COVID-19 (F(1.13)=5.44; P=0.021). The most common medications were paracetamol, azithromycin, and natural remedies - alone or in combination. 4.4% of cases reported medical complications due to COVID-19 and were hospitalised. Vaccination against SARS-CoV-2 was reported in 11.2% of DS individuals (Table 3).

Challenges for parents during COVID-19, impact on the child and medical support for parents

Overall, 144 (53.7%) of parents reported one or more challenges during COVID-19. The lack of social activities, cancelled therapies, and psychological problems were most frequently mentioned (Table 4). Significant impairment of the child's physical health and/or emotional well-being was reported in 19.7% of cases. Parents' level of satisfaction with medical support ranged from 'not satisfied' (12.7%) to 'very satisfied' (17.9%), with the highest proportion in the 'moderately satisfied' group (21.7%) (Figure 1, Table 4).

Table 2. Medical information (N=268)

	Count (N)	Percent (%)
COMORBIDITIES		
Allergy	23	8.6
Blood cancer	2	0.8
Celiac disease	8	2.9
Chronic lung disease	3	1.2
Congenital heart defect	111	41.4
Epilepsy/seizures	7	2.6
Gastroesophageal reflux	6	2.2
Immune-compromised	10	3.7
Obesity	11	4.1
Obstructive sleep apnoea	5	1.9
Thyroid disorder	52	19.5
Other	8	2.9
No comorbidities	22	8.2
Congenital heart defect type		
Aortic stenosis	2	1.8
Atrial septal defect	24	21.6
Atrioventricular septal defect	36	32.4
Combined heart defect	10	9.0
Ductus arteriosus persistens	6	5.4
Tetralogy Fallot	2	1.8
Ventricular septal defect	22	19.8
Don't know	9	8.2
SUPPLEMENTATION		
No	187	69.8
Yes, one	47	17.5
Yes, two or more	34	12.7
Supplements		
Multivitamins	28	29.8
Omega 3	10	10.6
Probiotics	32	34.0
Vitamin D	24	25.6

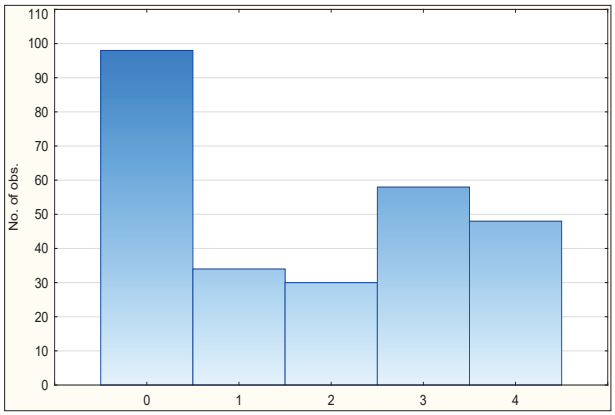

Figure 1. Parents' level of satisfaction with medical support (0=N/A 1=Not satisfied, 2= Slightly satisfied, 3- moderately satisfied, 4= Very satisfied)

Table 3. Clinical presentation of COVID-19, treatment and vaccination (N=268)

	Count (N)	Percent (%)
SARS-CoV-2		
-	132	49.3
+	136	50.7
NUMBER OF SYMPTOMS		
0	13	9.5
1	60	44.2
2	40	29.4
≥3	23	16.9
Symptoms		
Cough	6	4.8
Runny nose	9	7.3
Fever ≥38°C	45	36.6
Fever ≥38°C + cough	21	17.1
Fever ≥38°C + shortness of breath	4	3.3
Fever ≥38°C + muscle or joint pain	4	3.3
Fever ≥38°C + nasal signs	6	4.9
Fever ≥38°C + cough + shortness of breath	9	7.3
Fever ≥38°C + cough + nasal signs	8	6.5
Other	11	8.9
TREATMENT		
At home	130	95.6
In hospital	6	4.4
MEDICATIONS		
Azithromycin	7	5.1
Natural remedies	17	12.5
Paracetamol	49	36.1
Paracetamol + azithromycin	4	2.9
Paracetamol + natural remedies	7	5.1
Nothing	47	34.6
Other	5	3.7
MEDICAL COMPLICATIONS DUE COVID-19		
-	130	95.6
+	6	4.4
VACCINATION AGAINST SARS-CoV-2		
-	238	88.8
+	30	11.2

DISCUSSION

It is known that individuals with DS have specific co-morbidities and immune response dysfunctions that lead to a significantly higher risk of developing severe symptoms of infectious disease, in this specific case, COVID-19 [4]. While the difference in severity of COVID-19 between

Table 4. Challenges for parents during COVID-19, impact on the child and medical support for parents (N=268)

	Count (N)	Percent (%)
NUMBER OF CHALLENGES		
0	124	46.3
1	72	26.9
2	58	21.6
≥3	14	5.2
Challenges		
Cancelled therapy	20	13.9
Cancelled therapy + lack of social activity	22	15.3
Cancelled therapy + lack of social activity + psychological problems	9	6.3
Lack of social activity	32	22.2
Lack of social activity + psychological problems	24	16.7
Psychological problems	16	11.1
Other	21	14.5
IMPACT ON THE CHILD		
No impact	125	46.6
Emotional well-being	32	11.9
Physical health	10	3.7
Physical health + emotional well-being	11	4.2
N/A	90	33.6
MEDICAL SUPPORT FOR PARENTS		
Not satisfied	34	12.7
Slightly satisfied	30	11.2
Moderately satisfied	58	21.7
Very satisfied	48	17.9
N/A	98	36.6

individuals with and without DS is well established, the question is whether we can apply the conclusions from the international T21RS online survey to different cultural and demographic groups [7,18]. To investigate this, we conducted an online survey in Croatia to collect COVID-19 information on the clinical presentation of COVID-19 and disease progression in individuals with DS and the challenges for their parents during the COVID-19 pandemic.

Our group of DS individuals were children with a mean age of 8.7± 8.1 years. This is important to note because previous studies suggest that children do not have the same risk of COVID-19 related mortality as older adults [19]. However, compared with children without intellectual and developmental disabilities, the mortality rate from COVID-19 was reported as increased in individuals with DS [20]. Our results showed a statistically significant positive association between age and the number of symptoms. This may serve as one of the markers for the severity of COVID-19. Interestingly, post-hoc analysis showed that the least affected age group was that of 5-12-year-olds. The reasons for the differences in clinical manifestations between children and adults are likely age-related comorbidities, along with age-related factors that may modulate the antiviral immune response: a more vigorous innate response that promotes more efficient viral clearance, a stronger local innate IFN response in the airways mediated by cells producing IL-17A and IFN-γ, higher baseline innate activity in nasal mucosae, and increased frequency of naïve T cells, depletion of natural killer (NK) cells, and lower frequency of cytotoxic T cells in peripheral blood immune cells [21,22].

Moreover, our analysis showed a significant and positive correlation between BMI and clinical presentation of COVID-19. Obesity is the other known risk factor for more severe cases of COVID-19, and obesity is common in individuals with DS [23]. The proposed mechanism that leads to COVID-19 is immune system dysregulation leading to chronic meta-inflammation that can blunt the host antiviral response [24]. In addition, obesity is associated with upper airway obstruction, obstructive sleep apnoea, lower lung capacity and reserve, which can make ventilation more difficult, especially with DS, where this is exacerbated by specific anatomical differences. The T21RS survey highlights obesity as a significant risk factor for hospitalisation in paediatric COVID-19 patients with DS [7]. Another statistically significant pattern found in our study is the increase in BMI with age, which is consistent with data from the literature [24, 25]. The unusual distribution is seen in the different types of trisomy 21. Indeed, the full/standard type is represented by only 74.7%, while the other types (mosaic; translocated) have the expected distribution (4.1%; 6.3%). This can be explained by the high percentage of parents (14.9%) who do not know the type of T21, which is probably regular and therefore, consistent with the expected numbers and the reports from HICs. Similar pattern can be observed in the levels of intellectual disabilities [7].

As expected, the most common comorbidity in our group was CHD. Structural heart defects were reported in approximately 40% of DS individuals, with AVSDs, being the most common, which is consistent with the literature [26,27]. In DS individuals, the interplay of complicated cardiovascular and respiratory anatomy and pathophysiology may lead to increased severity and mortality of respiratory infections [28]. Nevertheless, we did not find any statistically significant correlation between CHDs and symptom severity in our group.

Dietary supplementation as the main reason of improved immunity has continued to increase, especially during COVID-19 pandemics [29]. In addition, there is

considerable clinical interest in whether children with DS benefit from therapeutic supplementation to improve their development, cognitive decline, and overall health [30]. Accordingly, we analysed this field and found that the most commonly used supplements were probiotics, multivitamins and vitamin D. However, there was no significant association with the milder COVID-19 disease symptoms.

As with the general population, fever, cough and shortness of breath were the most common signs and symptoms associated with COVID-19 in individuals with DS [31]. Interestingly, less than 10% of DS individuals were hospitalized and developed complications due to infection with COVID-19 in our study, while T21RS reported 38.8% hospitalised DS patients [7].

Accordingly, the medications used for treatment were predominantly paracetamol, azithromycin, and natural remedies. Possible explanations for this phenomenon include non-participation of older adults with DS, the most vulnerable group, or underrepresentation of patients from the lowest socioeconomic groups who may have been at increased risk for poor outcomes. It would also be interesting to consider whether intellectual disability and young age are also associated with underdiagnoses of symptoms.

The final part of the survey addressed parents' (caregivers') experiences with health services and support during the pandemic. Overall, the largest proportion of parents reported being moderately satisfied with the medical support they received. This is consistent with the European survey of parents' experiences with health services related to COVID -19 and children with congenital anomalies. Reports for Croatia were in a similar range [32]. In addition, many participants reported disruptions in their child's routine care that appeared to affect the physical health and emotional well-being of some children. Most of the disruptions were related to discontinued therapies, lack of social activities, and psychological problems, similar to the European survey reports [32].

Our study has some limitations. Since we included only DS patients, we could not consider some specific differences compared to DS the rest of the population infected with COVID-19. In addition, our sample was limited to Croatian individuals and was therefore smaller compared to data from some other T21RS countries. Most of the respondents were parents of children with DS, so we lacked data from the DS adult group. This is a possible reason for the small sample of hospitalized patients, which prevented us from inferring any severe complications of COVID-19.

CONCLUSION

Our study examined the effects of COVID-19 on individuals with DS in Croatia. We found that age and BMI of DS individuals significantly and positively correlated with clinical presentation of COVID-19. Given the limited data, the present study also suggests that younger individuals are unlikely to develop severe disease. Clinicians and caregivers should primarily look for the same signs and symptoms that occur in the general population (e.g., fever, cough, shortness of breath). Considering the views of parents and caregivers of DS individuals and the challenges they faced during COVID-19 and their experiences with medical support, ongoing therapies, social activities, and psychological support should be cited as inevitably important for maintaining physical health and emotional well-being in DS individuals.

REFERENCES

1. Callea M, Cammarata-Scalisi F, Galeotti A, Villani A, and Valentini D. COVID-19 and Down syndrome. Acta Paediatr. 2020;109(9): 1901-1902.
2. Kazemi M, Salehi M, and Kheirollahi M. Down Syndrome: Current Status, Challenges and Future Perspectives. Int J Mol Cell Med. 2016;5(3): 125-133.
3. Antonarakis SE, Skotko BG, Rafii MS, Strydom A, Pape SE, Bianchi DW, *et al*. Down syndrome. Nat Rev Dis Primers. 2020;6(1): 9.
4. De Toma I, and Dierssen M. Network analysis of Down syndrome and SARS-CoV-2 identifies risk and protective factors for COVID-19. Sci Rep. 2021;11(1): 1930.
5. Illouz T, Biragyn A, Frenkel-Morgenstern M, Weissberg O, Gorohovski A, Merzon E, *et al*. Specific Susceptibility to COVID-19 in Adults with Down Syndrome. Neuromolecular Med. 2021;23(4): 561-571.
6. Kantar A, Mazza A, Bonanomi E, Odoni M, Seminara M, Verde ID, *et al.* COVID-19 and children with Down syndrome: is there any real reason to worry? Two case reports with severe course. BMC Pediatr. 2020;20(1): 561.
7. Emes D, Hüls A, Baumer N, Dierssen M, Puri S, Russell L, *et al.* COVID-19 in Children with Down Syndrome: Data from the Trisomy 21 Research Society Survey. *Journal of Clinical Medicine*. 2021; 10(21): 5125.
8. Embregts P, Heerkens L, Frielink N, Giesbers S, Vromans L, and Jahoda A. Experiences of mothers caring

for a child with an intellectual disability during the COVID-19 pandemic in the Netherlands. J Intellect Disabil Res. 2021;65(8): 760-771.

9. Amatori S, Sisti D, Perroni F, Brandi G, Rocchi MBL, and Gobbi E. Physical activity, sedentary behaviour and screen time among youths with Down syndrome during the COVID-19 pandemic. J Intellect Disabil Res. 2022;66(12): 903-912.
10. Shetty J, Shetty A, Mundkur SC, Dinesh TK, and Pundir P. Economic burden on caregivers or parents with Down syndrome children-a systematic review protocol. Syst Rev. 2023;12(1): 3.
11. Sideropoulos V, Kye H, Dukes D, Samson AC, Palikara O, and Van Herwegen J. Anxiety and Worries of Individuals with Down Syndrome During the COVID-19 Pandemic: A Comparative Study in the UK. J Autism Dev Disord. 2023;53(5): 2021-2036.
12. Rubenstein E, Kyprianou N, Kumar PS, Kriova AM, Sokoloff A, Hillerstrom H, et al. Healthcare and Behavior Changes for Adults With Down Syndrome 1-Year Into COVID-19. Am J Intellect Dev Disabil. 2023;128(4): 273-281.
13. Meguid NA, Nashaat NH, Abdallah HR, Hemimi M, Elnahry A, El-Hariri HM, et al. Influence of COVID-19 pandemic lockdown on a sample of Egyptian children with Down syndrome. Egypt J Med Hum Genet. 2022;23(1): 68.
14. Santoro SL, Baker S, Blake JM, Cabrera MJ, Caughman K, Chung J, et al. Views on the impact of the COVID-19 pandemic on health in people with Down syndrome from diverse backgrounds. Am J Med Genet A. 2023;191(8): 2045-2056.
15. Fujino H, and Itai M. Disinfection behavior for COVID-19 in individuals with Down syndrome and caregivers' distress in Japan: a cross-sectional retrospective study. J Dev Phys Disabil. 2023;35(1): 81-96.
16. Pagnamenta E, Hodgkinson P, Davidson R, and Joffe VL. The impact of COVID-19 (Coronavirus) on children and young people with Down syndrome in the United Kingdom. Front Psychol. 2023;14: 1175636.
17. Hartley SL, Fleming V, Piro-Gambetti B, Cohen A, Ances BM, Yassa MA, *et al*. Impact of the COVID-19 pandemic on daily life, mood, and behavior of adults with Down syndrome. Disabil Health J. 2022;15(3): 101278.
18. Hüls A, Feany PT, Zisman SI, Costa ACS, Dierssen M, Balogh R, *et al*. COVID-19 Vaccination of Individuals with Down Syndrome—Data from the Trisomy 21 Research Society Survey on Safety, Efficacy, and Factors Associated with the Decision to Be Vaccinated. Vaccines. 2022; 10(4): 530.
19. Viner RM, Mytton OT, Bonell C, Melendez-Torres GJ, Ward J, Hudson L, *et al*. Susceptibility to SARS-CoV-2 Infection Among Children and Adolescents Compared With Adults: A Systematic Review and Meta-analysis. JAMA Pediatr. 2021;175(2): 143-156.
20. Turk MA, Landes SD, Formica MK, and Goss KD. Intellectual and developmental disability and COVID-19 case-fatality trends: TriNetX analysis. Disabil Health J. 2020;13(3): 100942.
21. Pierce CA, Preston-Hurlburt P, Dai Y, Aschner CB, Cheshenko N, Galen B, *et al*. Immune responses to SARS-CoV-2 infection in hospitalized pediatric and adult patients. Sci Transl Med. 2020;12(564): eabd5487.
22. Yoshida M, Worlock KB, Huang N, Lindeboom RGH, Butler CR, Kumasaka N, *et al*. Local and systemic responses to SARS-CoV-2 infection in children and adults. Nature. 2022;602(7896): 321-327.
23. Hatch-Stein JA, Zemel BS, Prasad D, Kalkwarf HJ, Pipan M, Magge SN, *et al*. Body Composition and BMI Growth Charts in Children With Down Syndrome. Pediatrics. 2016;138(4): e20160541.
24. Newman AM, Jhaveri R, Patel AB, Tan TQ, Toia JM, and Arshad M. Trisomy 21 and Coronavirus Disease 2019 in Pediatric Patients. J Pediatr. 2021228: 294-296.
25. Pinku H, Hüls A, Feany PT, Baumer N, Dierssen M, Bargagna S *et al*. Differences in clinical presentation, severity, and treatment of COVID-19 among individuals with Down syndrome from India and high-income countries: Data from the Trisomy 21 Research Society survey. J Glob Health. 2022;12: 05035
26. Santoro M, Coi A, Spadoni I, Bianchi F, and Pierini A. Sex differences for major congenital heart defects in Down Syndrome: A population based study. Eur J Med Genet. 2018;61(9): 546-550.
27. Pfitzer C, Helm PC, Rosenthal LM, Berger F, Bauer UMM, and Schmitt KR. Dynamics in prevalence of Down syndrome in children with congenital heart disease. Eur J Pediatr. 2018;177(1): 107-115.
28. Krishnan US, Krishnan SS, Jain S, Chavolla-Calderon MB, Lewis M, Chung WK, *et al*. SARS-CoV-2 Infection in Patients with Down Syndrome, Congenital Heart Disease, and Pulmonary Hypertension: Is Down Syndrome a Risk Factor? J Pediatr. 2020;225: 246-248.
29. Hamulka J, Jeruszka-Bielak M, Górnicka M, Drywień ME, and Zielinska-Pukos MA. Dietary Supplements

during COVID-19 Outbreak. Results of Google Trends Analysis Supported by PLifeCOVID-19 Online Studies. Nutrients. 2020;13(1): 54.

30. Ani C, Grantham-McGregor S, and Muller D. Nutritional supplementation in Down syndrome: theoretical considerations and current status. Dev Med Child Neurol. 2000;42(3): 207-13.

31. Docherty AB, Harrison EM, Green CA, Hardwick HE, Pius R, Norman L, *et al*. Features of 20 133 UK patients in hospital with covid-19 using the ISARIC WHO Clinical Characterisation Protocol: prospective observational cohort study. BMJ. 2020;369: m1985.

32. Latos-Bieleńska A, Marcus E, Jamry-Dziurla A, Rankin J, Barisic I, Cavero-Carbonell C, *et al*. 2022, 'COVID-19 and children with congenital anomalies: a European survey of parents' experiences of healthcare services, BMJ Open. 2022. 12(7): e061428.

BJMG 27 (1), 2024 • 59-64
DOI: 10.2478/bjmg-2024-0008
sciendo

CASE REPORT

ULTRA-EARLY DIFFUSE LUNG DISEASE IN AN INFANT WITH PATHOGENIC VARIANT IN TELOMERASE REVERSE TRANSCRIPTASE (*TERT*) GENE

Visekruna J[1], Basa M[1], Grba T[1], Andjelkovic M[2], Pavlovic S[2], Nathan N[3,4], Sovtic A[1,5,*]

[*]**Corresponding Author:** Ass. Prof. Aleksandar Sovtic, Department of Pulmonology, Mother and Child Health Institute of Serbia, Belgrade, Serbia, School of Medicine, University of Belgrade, Serbia; Email: aleksandar.sovtic@med.bg.ac.rs; Address: 6 Radoja Dakica, 11070 Belgrade, Serbia; Phone: +381 11 3108 158

ABSTRACT

The pathogenic variants in the telomerase reverse transcriptase (*TERT*) gene have been identified in adults with idiopathic pulmonary fibrosis, while their connection to childhood diffuse lung disease has not yet been described. Within this study, we present a case of a five-month-old, previously healthy infant, with early-onset respiratory failure. The clinical suspicion of diffuse lung disease triggered by cytomegalovirus (CMV) pneumonitis was based on clinical and radiological presentation. Multiorgan involvement was not confirmed. Considering the possible connection between CMV pneumonitis and early-onset respiratory failure, clinical exome sequencing was performed and a novel variant, classified as likely pathogenic in the *TERT* gene (c.280A>T, p.Lys94Ter) was detected. After segregation analysis yielded negative results, the *de novo* status of the variant was confirmed. Respiratory support, antiviral and anti-inflammatory therapy offered modest benefits, nevertheless, eighteen months after the initial presentation of disease, an unfavourable outcome occurred. In conclusion, severe viral pneumonia has the potential to induce extremely rare early-onset diffuse lung disease accompanied by chronic respiratory insufficiency. This is linked to pathogenic variants in the *TERT* gene. Our comprehensive presentation of the patient contributes to valuable insights into the intricate interplay of genetic factors, clinical presentations, and therapeutic outcomes in cases of early-onset respiratory failure.

Key words: cytomegalovirus (CMV), diffuse lung disease, early-onset respiratory failure, the *TERT* gene variants.

INTRODUCTION

Diffuse lung disease in children is characterized by a heterogeneous clinical presentation and radiological and prognostic features [1-3]. In adults, idiopathic pulmonary fibrosis (IPF) can be a consequence of telomere-related gene mutations, including telomerase reverse transcriptase (TERT) gene mutations which are associated with short telomere syndromes [4]. Telomeres are nucleoprotein structures with DNA repetitive sequences that protect chromosome ends and maintain chromosome stability, limit progressive shortening during cell replication, and prevent recombination at chromosome ends [5]. Telomere shortening leads to genomic instability, inducing DNA damage responses such as apoptosis and cell senescence [5].

IPF associated with pathogenic variants in the *TERT* gene is typically an age-dependent disease with clinical expression by the age of 50 years or later. The anticipation phenomenon refers to the earlier presentation of symptoms in younger generations [4].

In addition to previously described germline mutations in the *TERT* gene in children with malignant diseases, no association with childhood-onset pulmonary disease has been observed [6]. Here we present the case of a child with early onset respiratory failure associated with a pathogenic variant in the *TERT* gene.

[1] Department of Pulmonology, Mother and Child Health Institute of Serbia, Belgrade, Serbia

[2] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

[3] AP-HP, Sorbonne Université, Pediatric Pulmonology Department and Reference Centre for Rare Lung Disease RespiRare, Armand Trousseau Hospital, Paris, France

[4] Sorbonne Université, Inserm UMR_S933 Laboratory of Childhood Genetic Diseases, Armand Trousseau Hospital, Paris, France

[5] School of Medicine, University of Belgrade, Serbia

CASE PRESENTATION

The data of the patient were collected at the Department of Pulmonology at the Mother and Child Health Care Institute of Serbia "Dr. Vukan Cupic". This is a tertiary-level institution, recognized as the reference centre for rare diseases. This study was approved by the Ethics Committee of the Mother and Child Health Care Institute of Serbia "Dr. Vukan Cupic" in Belgrade, Serbia (Decision 8/106). Written informed consent was obtained for publication.

A full-term male infant was born to healthy non-consanguineous parents. His birth weight was 4020 g, and his APGAR score was 9. The patient met early developmental milestones. At the age of five months, the child was admitted to a regional hospital with fever, cough, tachypnoea, cyanosis, and increased breathing work. A chest radiography revealed diffusely decreased lung transparency with diffuse alveolar opacification. Therefore, a course of parenteral antibiotics and systemic corticosteroids as well as inhaled bronchodilators was administered. The respiratory viral PCR panel of the nasopharyngeal swab tested negative. A few days later, the patient was intubated due to clinical deterioration and transferred to our hospital. Upon admission, bilateral late inspiratory crackles were observed. A chest CT showed bilateral consolidation of the lung parenchyma with coarse intralobular thickening, minor ground-glass areas, and volume loss (Fig. 1A).

The genetic results for primary immunodeficiency, cystic fibrosis, and metabolic disorders were negative. Flexible bronchoscopy revealed bronchomalacia. Bronchoalveolar fluid (BAL) analysis revealed significant lymphocytosis (12%) and neutrophilia (20%), while PCR was positive for cytomegalovirus (CMV). Therefore, parenteral ganciclovir was initiated. Immunophenotypic analysis of BAL showed <1% CD1+ cells with a normal CD4/CD8 ratio. The complete blood count and liver function test results were normal. The immunophenotype of lymphocytes in the peripheral blood showed a slightly decreased CD4 count and a CD4:CD8 ratio of 1.2.

One week later, the child developed life-threatening cardiac dysrhythmias requiring a pacemaker implantation. Echocardiographic findings were normal without pulmonary hypertension. A combination of respiratory insufficiency and cardiac arrhythmias arose clinical suspicion for central congenital hypoventilation syndrome. Genetic analysis for the *PHOX2B* gene mutations was negative.

Considering the possible connection between CMV pneumonitis and early-onset respiratory failure, clinical exome sequencing (CES) was performed using the TruSight One (TSO) panel (Illumina, San Diego, CA, USA). This panel includes all known disease-associated genes described in the OMIM database as of 2013 and is designed to cover all exons and flanking intronic regions of 4,813 genes (~62,000 exons). All genes in the TSO panel where pathogenic, likely pathogenic, or variants of uncertain significance (VUS) were detected were further analysed. Variant Interpreter (Illumina) software was used for systematic interpretation of detected variants, and the variants were classified according to the recommendations of the American College of Medical Genetics and Genomics (ACMG) [7]. A novel heterozygous nonsense variant, c.280A>T, p.Lys94Ter (p.K94*), was detected in the *TERT* gene (NM_198253.3). This variant introduces a premature STOP codon in the second of the 16 exons in the gene (Fig. 2A), leading to protein truncation and degradation via nonsense-mediated mRNA decay. The variant was classified as "likely pathogenic" according to the ACMG classification recommendations based on the

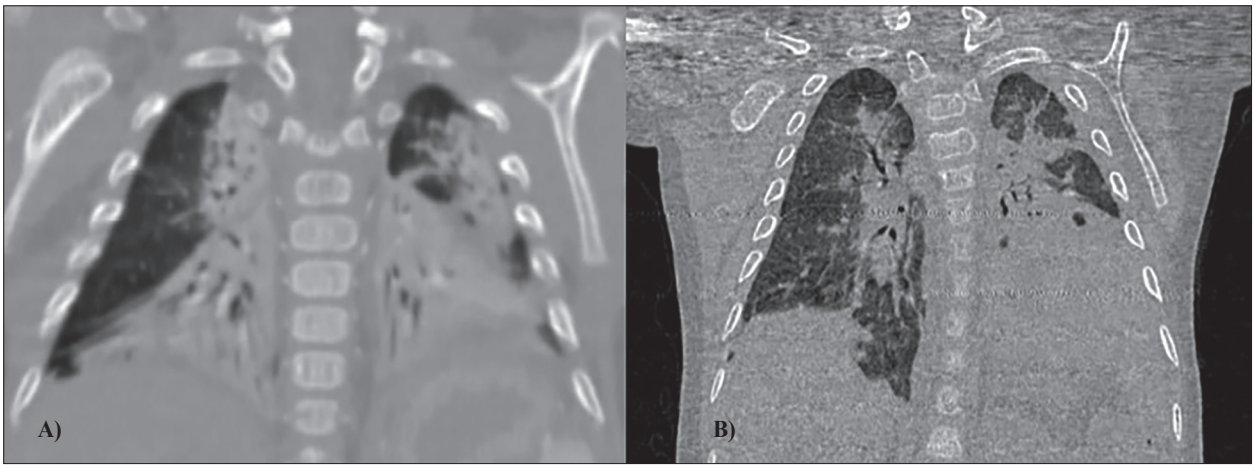

Figure 1. Chest CT scan. A) Initial finding – consolidations, intralobular thickening and mirror ground-glass opacifications. B) A follow up CT scan six months later – bilateral consolidations with fibroindurative lesion or plate-like atelectasis, and mild septal thickening.

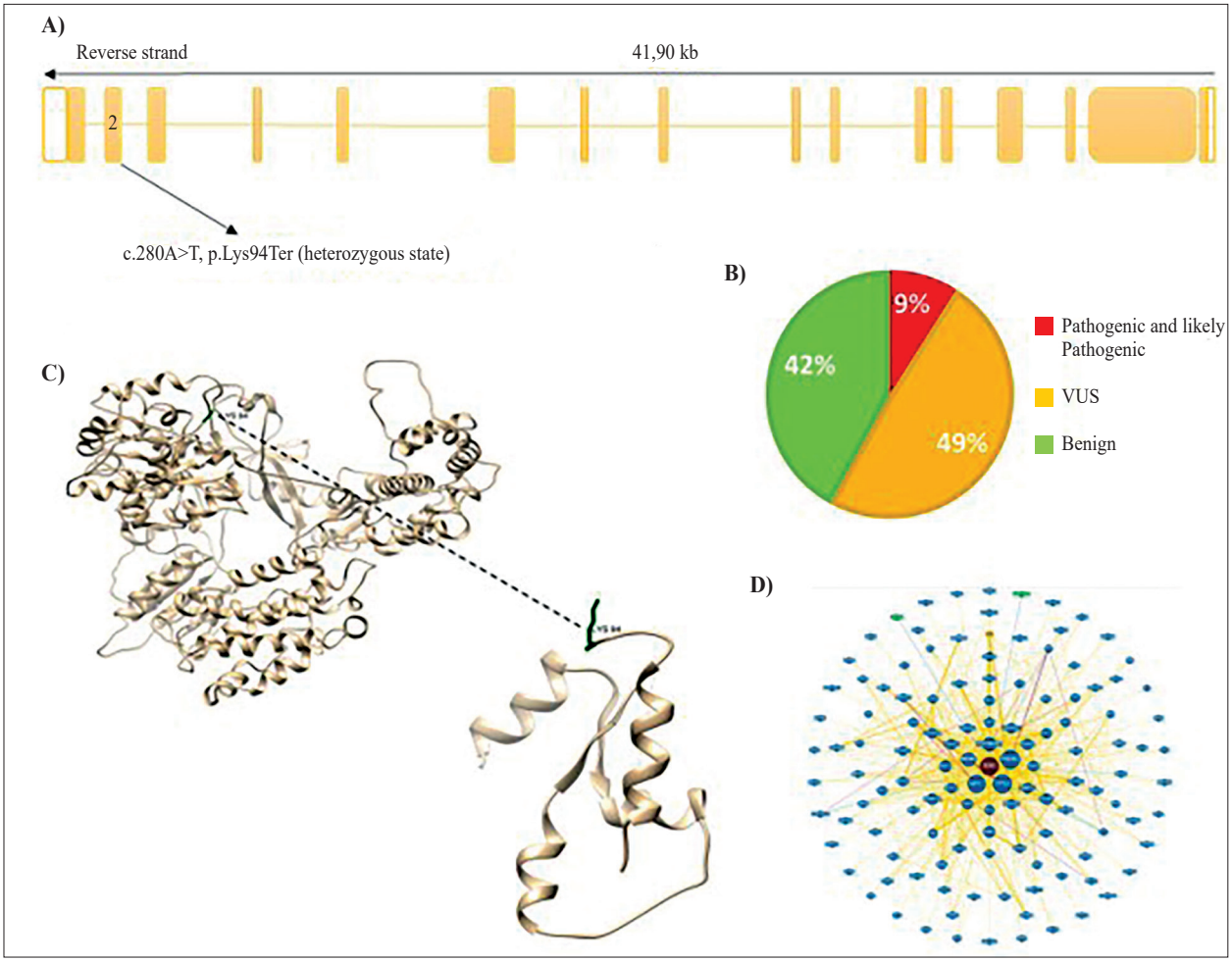

Figure 2. ***In silico*** **analysis of the impact of the detected variant in the TERT gene.** A) Schematic representation of sixteen exons in the *TERT* gene and marked detected loss of function variant in exon 2. B) Percentage ratio of pathogenic, likely pathogenic, VUS, and benign variants in the coding region of the *TERT* gene. C) Three-dimensional molecular models of *TERT* wild-type and mutated proteins. D) The protein-protein interaction network of *TERT* and interactors.

following criteria: PVS1 (Very Strong): Loss-of-function (LOF) variants in the *TERT* gene are a known mechanism of disease, with 76 reported pathogenic LOF variants; PM2 (Supporting): The variant has not been previously recorded in the GnomAD Exomes or GnomAD Genomes population databases, indicating it is rare. The results of the segregation analysis showed that neither parent was a carrier of the detected variant, indicating its *de novo* origin.

Pulse doses of methylprednisolone were initiated, with prednisone between doses and hydroxychloroquine and azithromycin, showing modest clinical benefits. Unfortunately, failure to wean off MV in a further course led to a tracheotomy, and MV was continued at home. An open lung biopsy was not performed. A control chest CT scan six months later revealed progression of the lung disease (Fig. 1B). Head CT, performed before steroid therapy commenced, showed supratentorial parenchymal volume loss with compensatory enlargement of CSF spaces. Eighteen months after the initial presentation, a new severe bilateral pneumonia led to multi-organ failure and death. The parents did not provide consent for autopsy.

DISCUSSION

The proposed mechanism of pulmonary involvement emphasises the importance of triggers such as smoking, stress, obesity, and inflammation [8]. CMV infection could be the trigger, as it has been described as a presenting feature in some *TERT*-variant associated disorders, such as dyskeratosis congenita (DC) [6]. The propensity of patients with confirmed pathogenic variants in the *TERT* gene to develop pneumonitis has been previously demonstrated [9]. Additional evidence suggests an impaired T cell immunologic response to CMV in lung transplant recipients with short telomere syndromes [10]. Although the relationship between viral infection and pulmonary fibrosis in adults

is not fully understood, direct damage caused by a virus and immune-mediated injury are proposed mechanisms [11]. However, in this case, the reason for the early onset of the disease remains unclear since ventilation-induced lung injury or CMV-associated acute respiratory distress syndrome could have similar consequences.

The typical clinical course in adults diagnosed with *TERT* mutation-associated conditions is insidious, with typical onset after the fourth decade of life [4,12]. Although the phenomenon of genetic anticipation based on the progressive shortening of telomeres is a characteristic of *TERT* gene mutations, no childhood presentation has currently been reported [4,13].

According to the literature and databases, each of the *TERT* mutations is referred to as "private mutations", and a total of 2625 clinically known and classified variants within the coding region of the *TERT* gene, of which only 9% are classified as pathogenic/likely pathogenic (Fig. 2B). Within the coding region of the *TERT* gene, loss of function (LOF) variants are very rare and have been detected only in the heterozygous state, thus indicating that the *TERT* gene is almost completely intolerant to LOF variants [14]. The detected heterozygous variant, c.280A>T (p.Lys94Ter) in the patient, was an LOF variant. The results of *in silico* modelling of wild-type and mutated TERT demonstrated that amino acid changes and consequential downstream introduction of the STOP codon led to protein truncation and consequent removal of protein-binding sites (Fig. 2C). Since TERT interacts with 122 different interactors (Fig. 2D) in cells, its function is further disrupted due to the absence of accurate amino acids for protein-protein interactions. Zaug et al. described families suffering from pulmonary fibrosis due to *TERT* mutations with a highly variable degree of telomerase functional impairment. The results of their study showed that the degree of functional impairment of telomerase was highly variable and many *TERT* mutations were shown to retain high—near normal—telomerase enzyme activity [15]. A limitation of this study was the absence of telomere length measurements or telomerase enzyme activity, which could have provided insights into the association between the detected germline variants, telomere length, and unusual early onset diseases.

In conclusion, the identification of a pathogenic variant in the *TERT* gene underlines the importance of genetic testing in paediatric patients presenting with respiratory failure, especially when confronted with atypical clinical features. The association between CMV infection and *TERT* mutations sheds light on potential disease mechanisms involving impaired telomerase function and immune response dysregulation and may lead to extremely rare early onset lung disease with chronic respiratory insufficiency and an unfavourable final outcome.

ACKNOWLEDGEMENT

The authors are grateful to Prof. Aurelien Justet and Prof. Caroline Kannengiesser for their valuable suggestions on diagnostics, treatment, and critical revision of the manuscript. We thank Bojana Gojsina, Milan Rodic and Vesna Topic for clinical contributions.

Conflict of interest: Authors declare no conflict of interest.

Funding: This research did not receive any specific grants from funding agencies in the public, commercial, or not-for-profit sectors.

Ethical approval: This study was approved by the Ethics Committee of the Mother and Child Health Care Institute of Serbia "Dr. Vukan Cupic" in Belgrade, Serbia (Decision 8/106) and written informed consent was obtained for publication.

REFERENCES

1. Spagnolo P, Bush A. Interstitial Lung Disease in Children Younger Than 2 Years. Pediatrics. 2016 Jun;137(6):e20152725.doi: 10.1542/peds.2015-2725. PMID: 27245831.
2. Kurland G, Deterding RR, Hagood JS, et al. An official American Thoracic Society clinical practice guideline: classification, evaluation, and management of childhood interstitial lung disease in infancy. Am J Respir Crit Care Med. 2013 Aug 1;188(3):376–94.doi: 10.1164/rccm.201305-0923ST. PMID: 23905526.
3. Antoniou KM, Margaritopoulos GA, Tomassetti S, et al. Interstitial lung disease. Eur Respir Rev. 2014 Mar 1;23(131):40-54. doi: 10.1183/09059180.00009113. PMID: 24591661.
4. Turner KJ, Vasu V, Griffin DK. Telomere Biology and Human Phenotype. Cells. 2019 Jan 19;8(1):E73.5.doi: 10.3390/cells8010073. PMID: 30669451.
5. Watkins TBK, Lim EL, Petkovic M, et al. Pervasive chromosomal instability and karyotype order in tumour evolution. Nature. 2020 Nov;587(7832):126–32. doi: 10.1038/s41586-020-2698-6. Epub 2020 Sep 2. PMID: 32879494.
6. Srinivas N, Rachakonda S, Kumar R. Telomeres and Telomere Length: A General Overview. Cancers. 2020 Feb 28;12(3):E558. doi: 10.3390/cancers12030558. PMID: 32121056.

7. Ellard S, Baple E, Callaway A, et al. ACGS best practice guidelines for variant classification in rare disease 2020. 2020. Available from: Retrieved from https://www acgs uk com/media/11631/uk-practice-guidelines-for-variant-classificationv4-01-2020 pdf.
8. Arora A, Singh R, Dogra M. Bilateral cytomegalovirus retinitis as the presenting feature of Dyskeratosis Congenita. Eur J Ophthalmol. 2023 May;33(3):NP122–5. doi: 10.1177/11206721221086154. Epub 2022 Mar 4. PMID: 35243907.
9. Arish N, Petukhov D, Wallach-Dayan SB. The Role of Telomerase and Telomeres in Interstitial Lung Diseases: From Molecules to Clinical Implications. Int J Mol Sci. 2019 Jun 19;20(12):E2996.doi: 10.3390/ijms20122996. PMID: 31248154.
10. Popescu I, Mannem H, Winters SA, et al. Impaired Cytomegalovirus Immunity in Idiopathic Pulmonary Fibrosis Lung Transplant Recipients with Short Telomeres. Am J Respir Crit Care Med. 2019 Feb 1;199(3):362–76.doi: 10.1164/rccm.201805-0825OC. PMID: 30088779.
11. Huang WJ, Tang XX. Virus infection induced pulmonary fibrosis. J Transl Med. 2021Dec 7;19:496. doi: 10.1186/s12967-021-03159-9. PMID: 34876129
12. Van der Vis JJ, van der Smagt JJ, Hennekam FAM, et al. Pulmonary Fibrosis and a TERT Founder Mutation with a Latency Period of 300 Years. Chest. 2020 Aug;158(2):612–9.doi: 10.1016/j.chest.2020.03.069. Epub 2020 Apr 19. PMID: 32315675.
13. Sousa SR, Caetano Mota P, Melo N, et al. Heterozygous TERT gene mutation associated with familial idiopathic pulmonary fibrosis. Respir Med Case Rep. 2019;26:118–22.doi: 10.1016/j.rmcr.2018.12.005. PMID: 30603600.
14. TERT | Gene [Internet]. [cited 2023 Sep 14]. Available from:https://varsome.com/gene/hg19/TERT
15. Zaug AJ, Crary SM, Jesse Fioravanti M, et al. Many disease-associated variants of hTERT retain high telomerase enzymatic activity. Nucleic Acids Res. 2013 Oct;41(19):8969-78. doi: 10.1093/nar/gkt653. Epub 2013 Jul 30. PMID: 23901009; PMCID: PMC3799428.

BJMG 27 (1), 2024 • 65-68
DOI: 10.2478/bjmg-2024-0001
sciendo

CASE REPORT

THE IMPORTANCE OF MOLECULAR BIOLOGICAL ANALYSIS FOR THE LABORATORY DIAGNOSTIC OF HOMOZYGOUS HAEMOGLOBIN MALAY

Bahar R[1,2], Zulkafli Z[1,2*], Zulkeflee RH[1,2], Hassan MN[1,2], Rahman Wan S Wan Ab[2,3], Noor NH M[1,2], Ramli M[1,2], Hussin A[1,2], Abdullah AD[1,2], Iberahim S[1,2], Abdullah M[1,2], Yusoff S M[1,2]

[*]**Corresponding Author:** Dr Zefarina Zulkafli, School of Medical Sciences, Universiti Sains Malaysia, 16150, Kubang Kerian, Kelantan. Email address: zefarinazulkafli@gmail.com

ABSTRACT

Haemoglobin (Hb) Malay is variant haemoglobin with a β^{++} thalassemia phenotype. The prevalence of Hb Malay in the Malaysian population was 5.5%. We describe a 58-year-old male who presented with symptomatic anaemia to the Hospital Universiti Sains Malaysia. Further history revealed that the patient had anaemia since the age of 28, and on regular follow-up at other hospital. Physical examination revealed pallor, jaundice and hepatosplenomegaly. The full blood count and peripheral blood smear showed hypochromic microcytic anaemia with anisopoikilocytosis, and many target cells. High-performance liquid chromatography results showed a β thalassemia trait. However, the diagnosis does not alight with the patient's condition. Bone marrow aspirate was completed and showed reactive changes and erythroid hyperplasia. A molecular test was then performed for β globin gene mutation detection using Multiplex Amplification Refractory Mutation System (M-ARMS) PCR method. This revealed the result as homozygous codon 19 mutation or Hb Malay. Therefore, in this case report we would like to highlight the laboratory approaches, the challenges faced by the usual haematological investigations and the importance role of molecular testing in the diagnosis of severe anaemia.

Keywords: Hb Malay, thalassaemia, haemoglobinopathies, anaemia

[1] School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan.

[2] Hospital USM, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan.

[3] School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan.

INTRODUCTION

Almost 300 beta (β)-globin gene mutations have now been characterized (http://globin.cse.psu.edu). Some mutations completely inactivate the β gene, resulting in the absence of β-globin production that leads to β^0 thalassemia. Other types of mutations allow the production of some β globin and cause β^+- or β^{++} ("silent") thalassemia whereas β^{++} has more β globin production compared to β^+. β^0 / β^+ /β^{++} thalassemia phenotype depends on the site and nature of the mutation.[1] Therefore, the clinical and haematological spectrum of beta-thalassemia ranges from silent carrier to clinically manifested conditions, including severe transfusion dependent beta-thalassemia major and beta-thalassemia intermedia.[2]

The standard screening method for β-thalassemia includes full blood count (FBC), including the level of (MCV) < 80 fL and/or (MCH) <than 27 pg (being used as a cutoff level for a positive thalassemia screening result). The full blood picture (FBP) in thalassemia disease shows typical RBC morphology, consisting of microcytosis, hypochromia, and anisopoikilocytosis. Beta thalassemia major typically shows markedly elevated HbF (30-95%) levels with elevated HbA2. The proportion of HbA2 is dependent on the precise mutation of the β globin gene cluster.[3,4] Therefore, the minimal deficit of β-globin production is not associated with any consistent haematological changes and are the limitation of standard screening method for β-thalassemia in carriers of very mild-or-silent types of β-thalassemia.[3,4] Hence, the challenges faced during laboratory approaches and the importance of molecular genetic testing to confirm the diagnosis are discussed in this case report.

CASE REPORT

A 58-year-old male presented with symptomatic anaemia. Further history revealed that the patient has had

anaemia since the age of 28 and on regular transfusion and follow-up at other hospital. However, bleeding did not manifest. The patient has no history of trauma or fever. The patient also has no family history of haematological disorders. The physical examination revealed pallor, jaundice and hepatosplenomegaly (liver 13 cm and spleen 16 cm below costal margin) but no lymphadenopathy.

Laboratory results at presentation showed haemoglobin (Hb) 5.9 g/dL, total leukocyte count 6.43 X 10^3/μL, MCV 48.9 fl, MCH15.4 pg MCHC31.5 g/dL and platelet count350X 10^9/L. Other investigations, including coagulation profile (activated partial thromboplastin time, prothrombin time and fibrinogen), liver and renal function tests were all within normal ranges. The peripheral blood smear showed hypochromic microcytic anaemia with anisopoikilocytosis, and many target cells. Occasional nucleated red blood cell. No eosinophilia or basophilia (Figure 1).

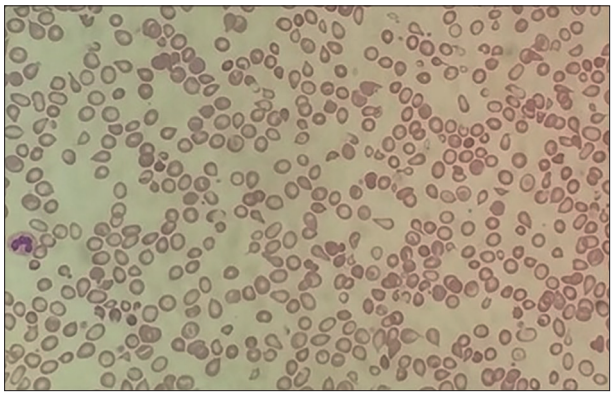

Figure 1. FBP stained with Wright Stain (X20 magnification)

Other investigations, including Hb analysis, high performance liquid chromatography (HPLC) showed A window (81.9%) with the presence of prominent peak at F (10.6%) and A2/E (7.5%) normal range is 2% to 3.2%. Alkaline gel electrophoresis showed prominent A2 band. The impression of Hb Analysis is a β thalassemia trait. The patient's bone marrow aspirate (BMA) showed reactive changes and erythroid hyperplasia. There was no evidence of acute leukaemia and other haematological malignancy. Later, Multiplex Amplification Refractory Mutation System (M-ARMS) PCR revealed homozygous codon 19 mutation/ Hb Malay.

DISCUSSION

Hb Malay was first described in 1989, being a β^{++} thalassemia phenotype with A → G mutation in codon 19, as detected in this case.[5,6] The prevalence of Hb Malay in the Malaysian population was 5.5%.[7] Homozygous Hb Malay usually presented with an average Hb of 7 to 8g/dL. Previously, it was reported that there was an increased production of Hb F between 9-25% in cases of homozygous Hb Malay and compound heterozygous Hb E/Malay.[6] This was also seen in this case, where the Hb F level in homozygous Hb Malay was 10.6%. Hb Malay (5.5%) was detected in northeast Thailand[8].

To date, many Hb variants have been discovered and can be detected by current screening methods for beta thalassemia; electrophoretic and HPLC methods. However, these techniques still have some limitations. It is because the available screening method is still unable to detect certain Hb variants with neutral substitutions.[6,7] It is difficult to diagnose a variant causing silent β-thalassemia, especially heterozygous Hb Malay because the haematological parameters and Hb A2 levels remain within a normal range. [9,10] Furthermore, as seen in this case, even though the Hb level is reduced, it is still challenging to confirm homozygous Hb Malay because both HPLC and capillary zone electrophoresis cannot differentiate between Hb A and Hb Malay. Hb Malay migrates as Hb A.[6-10] Therefore, the definitive diagnosis of Hb Malay can only be made via molecular analysis; M-ARMS PCR. Based on this case, the presence of a variant causing silent β-thalassemia should be considered and emphasized in unexplained clinical presentation typical of thalassemia.[6–9] Hence, it is a challenge or difficulty for the hospital or medical centre with no molecular technique facility to diagnose of Hb Malay. The hospital should therefore identify the nearest centre that has this service and send the sample to them for confirmation. Identification of this variant haemoglobin is important to prevent the birth of β-thalassemia major or intermedia children. Furthermore, for the couples at risk of conceiving a baby with β-thalassemia major or intermedia should be given genetic.[6]

CONCLUSION

In conclusion, well organized information, consisting of complete red cell indices and a Hb analysis result, together with a detailed history, including ethnic background, physical examination, and then followed up with molecular techniques such as M-ARMS PCR, can be used as a guideline for an effective tool in the investigation, detection, and confirmation of the diagnosis of Hb Malay. This is particularly important in the multi-ethnic populations of Malaysia as well as for proper clinical management of the patients.

REFERENCES

1. Thein SL. The molecular basis of β-thalassemia. Cold Spring Harb Perspect Med. 2013;3(5):1–24.
2. Traivaree C, Boonyawat B, Monsareenusorn C. Molecular analysis of beta-globin gene mutations among Thai beta-thalassemia children: results from a single center study. Appl Clin Genet. 2014;253.
3. Lee YK, Kim HJ, Lee K, Park SH, Song SH, Seong MW, et al. Recent progress in laboratory diagnosis of thalassemia and hemoglobinopathy: A study by the Korean Red Blood Cell Disorder Working Party of the Korean Society of Hematology. Blood Res. 2019;54(1):17–22.
4. Brancaleoni V, Di Pierro E, Motta I, Cappellini MD. Laboratory diagnosis of thalassemia. Int J Lab Hematol [Internet]. 2016 May 1 [cited 2022 Feb 20];38 Suppl 1:32–40. Available from: https://pubmed.ncbi.nlm.nih.gov/27183541/
5. Amran HS, Sarijan N, Sathar J, Md Noor S. Case series of homozygous and compound heterozygosity of Hb Malay, the diagnostic features and transfusion requirements. J Biomed Clin Sci [Internet]. 2017;3(2):10–7. Available from: http://apps.amdi.usm.my/journal/index.php/jbcs/article/view/180
6. George E, Huisman THJ, Yang KG, Kutlari F, Wilson JB, Kutlar A, et al. First observation of haemoglobin Malay. Med J Malaysia. 1989;44(3):259–62.
7. Yusoff YM, Hamid FS, Esa E, Aziz NA, Hassan S, Anoar SZ, et al. Molecular and Haematological Characterisation of Hb Malay in Malaysian Population. Asian J Med Biomed [Internet]. 2018 [cited 2022 Feb 20]; Available from: https://www.semanticscholar.org/paper/Molecular-and-Haematological-Characterisation-of-Hb-Yusoff-Hamid/5fd503a15bf5e5a039200a868e47b1a2c8b09fdf
8. Yamsri S, Singha K, Prajantasen T, Taweenan W, Fucharoen G, Sanchaisuriya K, Fucharoen S. A large cohort of β(+)-thalassemia in Thailand: molecular, hematological and diagnostic considerations. Blood Cells Mol Dis. 2015 Feb;54(2):164-9. doi:
9. Sharma S, Sehgal S, Das R, Gulati S. Phenotypic heterogeneity of delta-beta thalassemia. Indian J Pathol Microbiol [Internet]. 2019 Jan 1 [cited 2022 Feb 20];62(1):185–6. Available from: https://pubmed.ncbi.nlm.nih.gov/30706898/
10. Karim MUA, Moinuddin, Babar SU. Cap +1 mutation; an unsuspected cause of beta thalassaemia transmission in Pakistan. Turkish J Hematol. 2009;26(4):167–70.

BJMG 27 (1), 2024 • 69-74
DOI: 10.2478/bjmg-2024-0005
sciendo

CASE REPORT

NOVEL DGAT1 MUTATIONS IDENTIFIED IN CONGENITAL DIARRHEAL DISORDER 7: A CASE REPORT WITH THERAPEUTIC EXPERIENCE

Shi C[1#], Liu XL[2#], Li XN[1], Zhao YJ[3*]

[*]**Corresponding Author:** Yunjing Zhao, PhD, Department of Pediatrics, Shengjing Hospital of China Medical University, No.36 Sanhao Street, Shenyang 110004, China; Email: zhaoyunjing@sj-hospital.org

[#] Ce Shi and Xiaoliang Liu contributed equally to this work

ABSTRACT

Congenital diarrheal disorders (CDD) are a group of rare inherited intestinal disorders, among which CDD7 was recently identified to be associated with only 24 mutations in gene coding for diacylglycerol-acyltransferase 1 (*DGAT1*).

We report on a female patient who presented with diarrhea, vomiting, hypoalbuminemia, and failure to thrive after birth. Two novel variants of c.1215_1216delAG and c.838C>T were found in the *DGAT1* gene by whole exome sequencing, which was confirmed to be compound heterozygous by Sanger sequencing. Her symptoms and nutritional status improved significantly after 1 year of a fat-restricted enteral diet. Weight for age and weight for length increased from -5.0 SDS and -4.0 SDS at 3 months to +0.08 SDS and +1.75 SDS at 15 months, respectively.

This report expanded the mutation spectrum of *DGAT1*-related CDD7 and enriched our knowledge of the clinical features. Moreover, early fat-restricted enteral diet intervention was suggested for the treatment of such patients.

Keywords: congenital diarrheal disorders, diacylglycerol-acyltransferase 1, nutrition

INTRODUCTION

Congenital diarrheal disorders (CDD) are rare inherited intestinal disorders characterized by diarrhea, nutrient malabsorption, and sometimes life-threatening [1]. Genetic background of CDD is heterogeneous. Recent studies have shown that mutations in diacylglycerol-acyltransferase 1 (*DGAT1*, OMIM* 604900), a gene encoding a protein involved in lipid metabolism, were associated with CDD7 (OMIM# 615863). DGAT1 is a microsomal enzyme that is highly expressed in several organs, such as the small intestine, adrenal medulla, adrenal cortex, and testes [2]. DGAT1 and its isozyme diacylglycerol-acyltransferase 2 (DGAT2) are responsible for the conversion of diacylglycerol and fatty acyl-CoA to triacylglycerol in humans [1]. The human intestine might be particularly vulnerable to DGAT1 deficiency, as the human intestine expresses *DGAT1* exclusively and *DGAT2* is mainly expressed in the liver [1], whereas mice and other mammalian intestines express both DGAT1 and DGAT2 [3-4]. CDD7, caused by bi-allelic variants of the *DGAT1* gene, mostly develops in the neonatal period with severe diarrhea, vomiting, hypoalbuminemia, and failure to thrive. To date, few mutations of the *DGAT1* gene have been reported on.

Herein, we present an infant with CDD7 caused by two novel *DGAT1* mutations. The clinical features and physical growth parameters were analyzed and followed up at 12 months. The efficacy of nutritional therapy is instructive for pediatricians to consider as early treatment for such patients.

CASE REPORT

A girl aged 3 months was admitted with the main complaint of slow weight gain. She was born by vaginal delivery without complications at 40 weeks' gestation.

[1] Department of Pediatrics, The Fourth Affiliated Hospital of China Medical University, Shenyang, China

[2] Department of Clinical Genetics, Shengjing Hospital of China Medical University, Shenyang, China

[3] Department of Pediatrics, Shengjing Hospital of China Medical University, Shenyang, China

The birth weight was 2.75 kg (weight for age: -1.13 SDS), and the birth length was 49.0 cm (length for age: -0.05 SDS). Her parents were not consanguineous and denied any familial history of genetic diseases. She was the first child of her parents. She had mixed feeding (breastfed and regular formal) and she presented watery diarrhea (twice a day) 2 days after birth, accompanied by occasional vomiting and slow weight gain. At 3 months, her height was 53.0 cm (length for age: -3.24 SDS). She was underweight and emaciated with the weight of 2.8 kg (weight for age: -5.0 SDS; weight for length: -4.0 SDS). The stool routine indicated increased fat globules without red or white blood cells. The serum 25-hydroxyvitamin D was as low as 3.29 ng/mL (normal range: 20-40 ng/mL). Both albumin (25.0 g/L, normal range: 35-53 g/L) and prealbumin (0.107 g/L, normal range: 0.18-0.45 g/L) were significantly reduced. The levels of serum potassium (3.48 mmol/L, normal range: 3.5-5.5 mmol/L) and sodium (130 mmol/L, normal range: 136-145 mmol/L) were slightly low. Aspartate aminotransferase was elevated (60 U/L, normal range: 5-34 U/L). Blood sugar, hemoglobin, and plasma amino acids were within the normal ranges. The urine routine and urinary organic acids tests were also normal. She was diagnosed with severe malnutrition and hospitalization was recommended, which was not accepted by the parents. Take-home high-energy formula was thus provided to improve nutrition. However, the diarrhea worsened rapidly after 2 days of Nutricia formula feeding (5-6 times per day), and she was admitted to the Intensive Care Unit (ICU) for dehydration and low serum bicarbonate (13.6 mmol/L, normal range: 22-28 mmol/L) ten days later. The stool routine was normal, and the virus and bacterial pathogens were negative. Immunoglobulin G was significantly decreased (IgG 0.99 g/L, normal range: 5.19-10.79 g/L), and lymphocyte subset analysis was normal. Ultrasonography showed gallbladder stones. During that hospitalization, she received albumin, intravenous immunoglobulin infusions, red blood cell transfusion, and parenteral nutrition. Treatment with extensively hydrolyzed formula was effective with no more diarrhea. She was discharged from the hospital after 20 days.

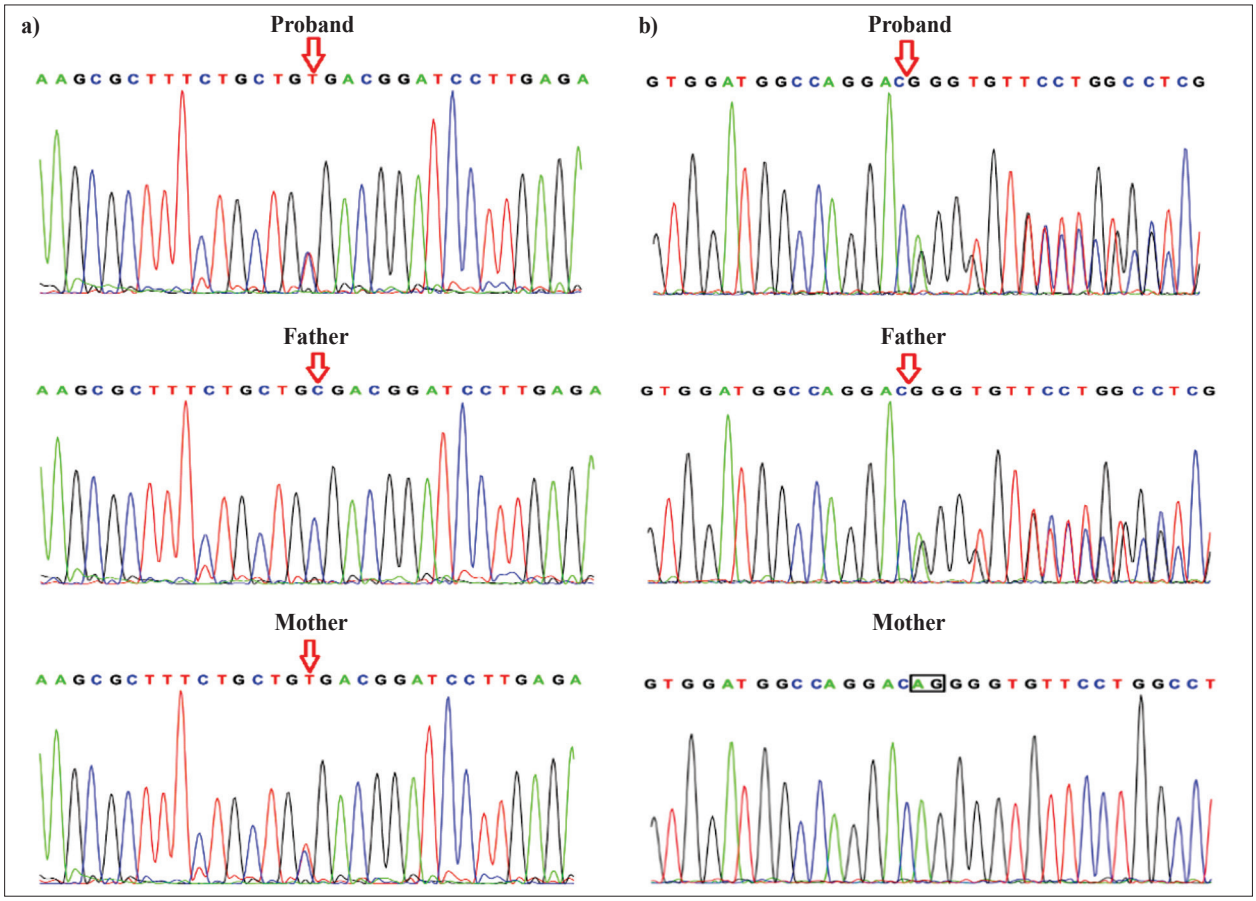

Figure 1. Sanger sequencing results show mutations in *DGAT1* detected in the proband`s family. (a) The proband was maternally inherited the variant of c.838 C>T in the *DGAT1* gene. (b) The proband was paternally inherited the variant of c.1215 _ 1216 del AG in the *DGAT1* gene.

Due to the unknown etiology of diarrhea and slow weight gain, a whole exome sequencing (WES) was performed using peripheral blood samples from the proband after obtaining written informed consent from the parents. Compound heterozygous mutations in *DGAT1* were identified by WES and confirmed by Sanger sequencing (Figure 1a, b): a paternally inherited variant of c.1215_1216delAG (p.Phe408fsTer74) and a maternally inherited variant of c.838C>T (p.Arg280*). Neither of the mutations has been reported in the human gene mutation database (HGMD), in the literature, nor found in the public or in-house databases. The variant of c.838C>T (p.Arg280*) was evaluated as "likely pathogenic" according to the American College of Medical Genetics and Genomics (ACMG) guidelines with evidence of PVS1+PM2. The variant of c.1215_1216 del AG (p.Phe408fsTer74) was evaluated as "pathogenic" according to the ACMG guideline with evidence of PVS1+PM2+PM3. No other variants in the WES data were found to be related to digestion and absorption. By reviewing the literature and disease database, a total of 26 *DGAT1* variants (including ours) have been reported on in patients with CDD7. The schematic presentation of the *DGAT1* mutation spectrum is depicted in Figure 2a. By constructing a three-dimensional molecular model, using Pymol software, these variants caused an abnormal DGAT1 protein structure, which might be destructive for the normal function of protein (Figure 2b). The major clinical features of these patients are summarized in Table 1.

The patient was referred to the developmental pediatrics for feeding guidance and physical monitoring since discharge. At first, an extensive hydrolyzed formula (a limited fat to 45.2% of total calories) was provided. There were no more complaints about diarrhea, yet there was also no weight gain observed for 2 months (Figure 3). She was recommended to consume a fat-restricted diet based on the definitive genetic diagnosis, with several explorations and modifications according to previous reports [1,3,5]. Treatment with adult low-fat milk powder (a limited fat of 3.6% of total calories) was subsequently chosen as an alternative. The consumption and ratio of milk powder and water was intensively calculated based on the weight and energy requirements. The patient's growth parameters rapidly improved during regular follow-up (Figure 3). At of 15 months of age, her malnutrition was corrected with a catch-up with a weight of 9.7 kg (weight for age: +0.08 SDS, weight for length: +1.75 SDS). The short stature was slightly ameliorated (recumbent length 70.6 cm, length for age: -2.56 SDS). The absorptive parameters were satisfactory, with normal albumin and fat-soluble vitamin levels (Vitamin A, D, E, K). Triglycerides was also slightly elevated (1.7 mmol/L,

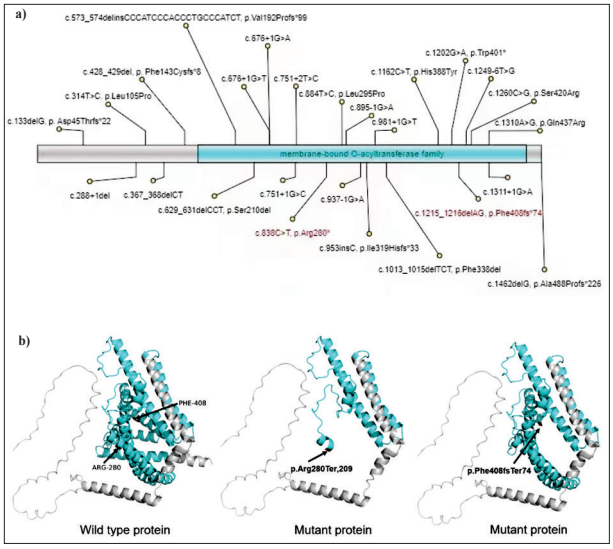

Figure 2. (a) The schematic presentation of the *DGAT1* mutation spectrum in previous studies, and the mutations identified in this study are shown in red. (b) DGAT1 structure model with *DGAT1* mutations identified in this study. Note: these pictures are drawn by using DOG2.0 (http://dog.biocuckoo.org/) and PyMOL (www.pymol.org). Reference database for protein domain (https://www.ebi.ac.uk/interpro/protein/UniProt/O75907/entry/pfam/#table).

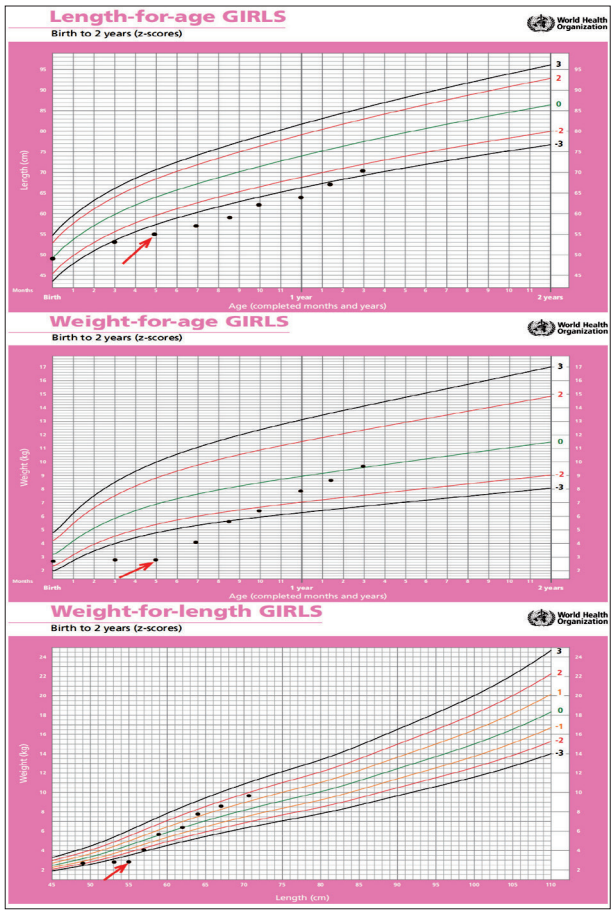

Figure 3. The growth curve of the proband. Red arrows indicate the time when the proband started a fat-restricted diet.

Table 1. Characteristics of published DGAT1 deficiency patients

Descent	DGAT1 mutation site	Protein position	Onset age	Phenotype
Turkish [1]	c.1202G>A	p. Trp401Ter	Birth (diarrhea)	FTT, vomiting, hypoalbuminemia, hypogammaglobulinemia, edema
Turkish [1]	c.573_574delAGinsCCCAT CCCACCCTGCCCATCT	-	3 weeks (diarrhea)	Vomiting, hypoalbuminemia, edema, FTT, hypogammaglobulinemia, hypertriglyceridemia
Turkish [1]	c.937-1G>A	-	2 months (diarrhea)	FTT, diarrhea, vomiting, hypoalbuminemia, hypogammaglobulinemia
Turkish [1]	c.953insC	p. Ile319Hisfs*31	Before the age of 2.5 months (diarrhea)	FTT, vomiting, diarrhea, hypoalbuminemia, hypogammaglobulinemia
Caucasian [1] and NM [6]	c.629_631delCCT	p. Ser210del	Before the age of 2 weeks (diarrhea, vomiting)	Vomiting, diarrhea, FTT, malnutrition, hypoalbuminemia.
NM [5]	c.288+1del c.629_631del	p.? p. Ser210del	Before the age of 17 day (vomiting, diarrhea, weight loss)	FTT, vomiting, diarrhea, hypoalbuminemia
NM [5]	c.428_429del c.629_631del (paternal)	p. Phe143Cysfs*8 p. Ser210del	The early postnatal period (feed intolerance)	FTT, vomiting, diarrhea
Mexican [6]	c.676+1G>A	-	11 days (vomiting)	Vomiting, Diarrhea, feeding difficulties, FTT, mild developmental delay
NM [6]	c.1311+1G>A c.1462delG	- p. Ala488Profs*226	1 week (vomiting, tired, and sleepy during a feed)	FTT, diarrhea, vomiting, nutritional microcephaly, anemia, arachnodactyly and mild dysmorphic facial features, hypogammaglobulinemia
NM [6]	c.1310A>G (de novo) c.981+1G>T(maternal)	p. Gln437Arg -	3 weeks (poor feeding and vomiting)	FTT, poor feeding, vomiting, short stature, rickets, abnormal brain MRI, anemia, hypoglycemia.
Ashkenazi Jewish [3] and Hispanic [7]	c.751+2T>C	-	Before the age of 7 weeks (vomiting)	Diarrhea, vomiting, malnutrition, hypertriglyceridemia, or triglyceride levels were normal, and hypoalbuminemia
South Asian [8]	c.314T>C	p. Leu105Pro	Shortly after birth (diarrhea)	Diarrhea, FTT, hypertriglyceridemia, hypoalbuminemia
Chinese [11]	c.895-1G>A (paternal) c.751+1G>C (maternal)	- -	Soon after birth (vomiting)	Vomiting, diarrhea, hypoalbuminemia, hypertriglyceridemia
Chinese [12]	c.895-1G>A	-	Birth (diarrhea, vomiting)	Diarrhea, vomiting, Malnutrition, hypoalbuminemia, intestinal lymphangiectasia,
Chinese [12]	c.1249-6T>G	-	30 months (edema)	Malnutrition, hypoalbuminemia, lymphopenia, edema
Arab-Muslim [4]	c.884T>C	p. Leu295Pro	2 months (diarrhea)	Diarrhea, hypoalbuminemia, hypogammaglobulinemia, FTT, edema, anemia
Chinese [13]	c.676+1G>T(maternal) c.367_368delCT(paternal)	- -	Birth (diarrhea, vomiting)	FTT, diarrhea, vomiting, hypoalbuminemia, and triglyceride levels were normal
Latin America [14]	c.1162C>T c.838C>T	p. His388Tyr p. Arg280*	2 months (diarrhea)	Diarrhea, growth retardation, anemia, hypoalbuminemia, thrombocytosis, hypogammaglobulinemia.
Caucasian [15]	c.1013_1015delTCT(maternal) c.1260C>G (paternal)	p. Phe338del p. Ser420Arg	1 months (vomiting)	FTT, vomiting, diarrhea, malnutrition, hypoalbuminemia, rickets
Chinese [16]	c.133delG	p. Asp45Thrfs*22	20 days (vomiting)	FTT, feeding difficulties, vomiting, diarrhea, hypoalbuminemia, hypertriglyceridemia
Chinese (This study)	c.1215_1216 del AG (paternal) c.838C>T (maternal)	p. Phe408fsTer74 p. Arg280*	Birth (diarrhea)	FTT, feeding difficulties, vomiting, hypoalbuminemia, hypertriglyceridemia

FTT: Failure to thrive; NM: not mentioned

normal range: 0.4-1.69 mmol/L). Our therapeutic experience was supportive for early fat-restricted enteral diet in *DGAT1*-related CDD7.

DISCUSSION

CDDs are a group of uncommon, clinically varying enteropathies that are often missed or misdiagnosed, and usually present with persistent diarrhea in the first few months of life [6]. If unrecognized, patients can suffer from malnutrition, failure to thrive, and even death [7]. CDD7 is a rare autosomal recessive condition caused by loss of function mutations in the *DGAT1* gene. Since the first case with *DGAT1* mutation was described in 2012 [3], only 32 patients with 24 *DGAT1* mutations have been identified, with varied severity in the disease phenotype [7]. More data about the clinical features are needed to enhance our awareness of the disease. All of the patients with *DGAT1* mutations suffer from diarrhea or vomiting within the first week of life, as well as hypoalbuminemia, and failure to thrive. The severity of the disease is correlated with the amount of residual DGAT1 activity [8]. Our patient had an

early disease onset at 2 days and aroused the attention of the parents as late as 3 months. Moreover, a wrong treatment of high energy formula was provided to deteriorate the diarrhea. The necessity of early genetic diagnosis is critical for the correct breeding strategy of these patients.

To date, 26 variants (including ours) in the *DGAT1* gene have been identified (Table 1), including various types of nonsense, missense, splicing, frameshift, and insertion-deletion. We added two novel mutations to the *DGAT1* mutation spectrum, both of which were supposed to be loss-of-function by creating frame-shift and premature termination codon. The symptoms of our patient were also more severe than in the previously reported cases. It is interesting to see that the construction of DGAT1 deficient mice were lean and resistant to obesity but did not recapitulate the diarrhea observed in human patients [9-10]. The etiology of diarrhea due to DGAT1 deficiency is still unknown [3]. One of the hypotheses is that increased levels of DGAT1 lipid substrates from the diet in the intestine mucosa or lumen could result in cellular dysfunction due to lipotoxic stress in enterocytes [3]. In addition, toxicity to enterocytes could also lead to protein-losing enteropathy which occurs in all patients. Furthermore, a deficiency of DGAT1 could affect bile acid metabolism, and bile acid malabsorption can cause diarrhea [3]. Mild hypertriglyceridemia occurred in some affected patients. Some reasons may be from overcompensation of hepatic DGAT2 or the interruption of bile acid absorption in the distal small intestine [3]. However, not all patients with *DGAT1* mutations present with hypertriglyceridemia. In addition, hypertriglyceridemia also did not appear to be associated with homozygous or heterozygous mutations in *DGAT1*. Therefore, more clinical cases and experiments are needed to clarify this question.

Our patient was promptly switched to a fat-restricted diet as soon as the genetic diagnosis was obtained. We carefully explored the amount of dietary fat, which was on one hand tolerable to the patient, and on the other hand satisfactory for growth. A limited fat to 2%-10% of total calories intake was found effective. This constitution was similar to previous experience suggested by Eldredge et al. [5]. Moreover, the patient was suggested to be fed with small amounts of fat multiple times, which was supposed to increase the tolerance of dietary fat in these patients [8]. Patients with a fat-restricted diet must be monitored for the levels of essential fatty acids, fat-soluble vitamins, serum lipid, and total protein levels. As described in previous literature, most patients develop catch-up growth and normal development after diet modification [11]. Our experience provides an alternative method using calculated adult low-fat milk powder for children who are unable to obtain low-fat infant formula. Our follow-up data showed a satisfactory rate of weight gain and normal metabolic parameters. However, the increase in length was less satisfactory. We speculated that it may be related to the short treatment and follow-up period or the fact that the two novel mutations in the *DGAT1* gene in the child might cause short stature. Therefore, more cases and longer follow-up times will need to be studied in the future.

CONCLUSIONS

We report the clinical presentation, diagnosis, and treatment of an infant with CDD7 caused by two novel variants in the *DGAT1* gene. Our data expanded the mutation spectrum, emphasized the importance of early genetic diagnosis, and shared our successful experience in diet therapy, which might be instructive for pediatricians to better understand the rare *DGAT1*-related CDD7.

ACKNOWLEDGMENTS

The authors would like to thank the patient and the patient's family for participating in this study.

DECLARATIONS

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Declaration of Participate Consent

All procedures performed in this study involving human participants were following with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written consent was obtained from the parents of the proband for the collection of samples and for DNA sequencing.

Declaration of Patient Consent

The authors certify that informed consent for publication of identifying images or the clinical details was obtained from the parents or legal guardians of any participant under the age 18.

Authors' contributions

Zhao Y. designed the research. Li X., Liu X. and Zhao Y. conducted the research. Shi C., Liu X. and Zhao Y. collected and analyzed the data. Shi C. and Liu X. were major contributors to writing the manuscript. Zhao Y. had the primary responsibility of the final content. The authors offered critical comments, read, and approved the final manuscript.

Funding

This study was supported by grant from the Natural Science Foundation of Liaoning Province (2022-MS-208), and the 345 Talent of Shengjing Hospital of China Medical University (C0101).

Availability of data and materials

All datasets generated or analyzed during the current study are included in this published article and available from the corresponding author on reasonable request.

REFERENCES

1. van Rijn JM, Ardy RC, Kuloğlu Z, Härter B, van Haaften-Visser DY, van der Doef HPJ, et al. Intestinal failure and aberrant lipid metabolism in patients with DGAT1 deficiency. Gastroenterology. 2018;155(1):130-143.e15.
2. Cases S, Smith SJ, Zheng YW, Myers HM, Lear SR, Sande E, et al. Identification of a gene encoding an acyl CoA: diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. Proc Natl Acad Sci U S A. 1998;95(22):13018-13023.
3. Haas JT, Winter HS, Lim E, Kirby A, Blumenstiel B, DeFelice M, et al. DGAT1 mutation is linked to a congenital diarrheal disorder. J Clin Invest. 2012;122(12):4680-4684.
4. Stephen J, Vilboux T, Haberman Y, Pri-Chen H, Pode-Shakked B, Mazaheri S, et al. Congenital protein losing enteropathy: an inborn error of lipid metabolism due to DGAT1 mutations. Eur J Hum Genet. 2016;24(9):1268-1273.
5. Eldredge JA, Couper MR, Barnett CP, Rawlings L, Couper RTL. New pathogenic mutations associated with diacylglycerol O-acyltransferase 1 deficiency. J Pediatr.2021;233:268-272.
6. Gupta A, Dsouza NR, Zarate YA, Lombardo R, Hopkin R, Linehan AR, et al. Genetic variants in DGAT1 cause diverse clinical presentations of malnutrition through a specific molecular mechanism. Eur J Med Genet. 2020;63(4):103817.
7. Schlegel C, Lapierre LA, Weis VG, Williams JA, Kaji I, Pinzon-Guzman C, et al. Reversible deficits in apical transporter trafficking associated with deficiency in diacylglycerol acyltransferase. Traffic.2018;19(11):879-892.
8. Gluchowski NL, Chitraju C, Picoraro JA, Mejhert N, Pinto S, Xin W, et al. Identification and characterization of a novel DGAT1 missense mutation associated with congenital diarrhea. J Lipid Res. 2017;58(6):1230-1237.
9. Yen CL, Stone SJ, Koliwad S, Harris C, Farese RV Jr. Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. J Lipid Res. 2008;49(11):2283-2301.
10. Chen HC, Farese RV Jr. Inhibition of triglyceride synthesis as a treatment strategy for obesity: lessons from DGAT1-deficient mice. Arterioscler Thromb Vasc Biol.2005;25(3):482-486.
11. Xu L, Gu W, Luo Y, Lou J, Chen J. DGAT1 mutations leading to delayed chronic diarrhoea: a case report. BMC Med Genet. 2020;21(1):239.
12. Ye Z, Huang Y, Wang Y, Lu J, Wu J, Yu Z. Phenotype and genotype of a cohort of Chinese children with early-onset protein-losing enteropathy. J Pediatr. 2019;208:38-42.e3.
13. Cheng Q, Fang YH, Luo YY, Lou JG, Chen J. Congenital diarrhea disorders caused by diacylglycerol acyl transferase 1 gene mutation. Zhonghua Er Ke Za Zhi. 2020;58(12):1018-1020.
14. Valentini MA, Fedrizzi VA, Krochik AG, Abbate S, Ferrari M, Contreras MB, et al. DGAT1 mutation in two sisters with failure to thrive: a case report. Arch Argent Pediatr. 2023;121(1):e202202606.
15. Ratchford TL, Kirby AJ, Pinz H, Patel DR. Congenital diarrhea from DGAT1 mutation leading to electrolyte derangements, protein-losing enteropathy, and rickets. J Pediatr Gastroenterol Nutr. 2018;66(3):e82-e83.
16. Wang S, Wang J, Li T, Wang D, Zeng L. Congenital diarrhea and enteropathies caused by DGAT1 gene mutation:a case report and literature review. Chin J Obstet Gynecol Pediatr (Electron Ed).2022;18(2):192-197. (Article in Chinese)

INFORMATION FOR AUTHORS

1. Aims and scope of the Journal

Balkan Journal of Medical Genetics (BJMG) was founded in 1998, and encompasses all aspects of human genetics: cytogenetics, molecular genetics, clinical genetics, immunogenetics, oncogenetics, pharmacogenetics, population genetics, genetic screening and diagnosis of monogenic and polygenic diseases, prenatal and preimplantation genetic diagnosis, genetic counselling, advances in the treatment and prevention of genetic disease

The Journal is supported by an International Editorial Board.

The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence, and will publish original articles, case studies, reviews, surveys, opinions, commentaries and essays.

2. Identification Statement

The Journal is published twice a year in June and November by the Macedonian Academy of Sciences and Arts, Skopje, Republic of North Macedonia.

The Journal is published in print form and online form on www.bjmg.edu.mk, www.sciendo.com and www.ncbi.nlm.nih.gov/pmc/

Accepted manuscripts for publication are published online ahead of the archival print issue, ensuring their public availability and can be cited as soon as possible after their acceptance for publication. The online publication is identical to the printed version and is not a preliminary, unedited version, thus maintaining the journal's tradition of excellence and the integrity of the published record.

Impact factor:
2018: 0,769
Clarivate Analytics Journal Citation Report 2019

3. Publication fees

- Page charges: 50 Euros/per page for the first six pages and 75 Euros/ per page for each page in excess of six
- Page with color figure(s): 100 Euros/ page
- Supplemental material fee: 50 Euros/unit

Balkan Journal of Medical Genetics does not apply article submission charges.

By signing the "License to publish" and "Publication cost approval" at the time of submission, the authors agree to bear responsibility for the publication of page charges.

4. Subscription

Print subscription price: 200 Euros for public institutions and 100 Euros for individuals.

Payments can be made by check and/or bank transfer to the following bank account:

Bank details:
National Bank of Republic of North Macedonia
SWIFT BIK NBRM MK 2A
St. Kuzman Josifovski Pitu, 1, 1000 Skopje,
Republic of Macedonia

Final Beneficiary:
IBAN MK07 1007 0100 0002789
Macedonian Academy of Sciences and Arts; ICGIB, Skopje, Republic of Macedonia

The transfer costs (taxes and commissions) are not included in the above mentioned price.

Bank certificate should be sent by e-mail to confirm the payment.

5. Submission of manuscripts:

Manuscripts written in English should be submitted online on www.bjmg.edu.mk or by an e-mail as an attached file to the Editorial Office:

Dijana Plaseska-Karanfilska (Editor)
Macedonian Academy of Sciences and Arts,
Av. Krste Misirkov 2, POB 428
1000 Skopje, Republic of North Macedonia
E-mail: bjmg@manu.edu.mk

The text files should be prepared using MS Word or Rich Text Format (RTF) and should be clearly labeled with the first author's name. Each manuscript must be accompanied by a statement that it has not been published or submitted for publication elsewhere. A "License to publish" and "Publication cost approval" (www.bjmg.edu.mk) must be signed and send together with the submitted article. If the submission contains any identifiable patient images or other protected health information, the corresponding author must submit a signed written statement, indicating that they have permission from the patient or the patient`s parent or guardian. Authors are responsible for obtaining permission to reproduce copyrighted material from other sources and are required to sign an agreement for the copyright transfer to the publisher. All accepted manuscripts, artwork, and photographs become the property of the publisher.

All persons making significant contributions to the work of the manuscript should be considered as co-authors. If an author discovers a significant error in the published work, the author is obligated to inform the journal editor in order to have the paper corrected or retracted.

6. Manuscript Review

Manuscripts are evaluated by two or more expert reviewers based on Editorial Review and/or Blind Peer Review process. Authors are encouraged to suggest the names and contacts (including e-mail addresses) of prospective reviewers. Manuscripts will be treated as confidential documents, not shown or discussed with other interested parties without the authorization from the editor.

7. Preparation of the manuscript

- All new manuscripts must be accompanied by a cover letter from the corresponding author stating that the manuscript represents original, unpublished material not under editorial consideration elsewhere, and that ethical guidelines were followed in the conduct of the research.
- All manuscripts must be written in English (the Vancouver style) and the text typed double spaced, with wide margins of at least 2.5 cm on all four sides. Uniform requirements for manuscripts may be found in the International Committee of Medical Journal Editors (ICMJE) Recommendations (http://www.icmje.org/about-icmje/faqs/icmje-recommendations/).
- The title page should contain: title of article, abbreviated running title of no more than 30 characters, author(s) name, initial(s), department, institution, city and country, name and full postal address, telephone, fax and e-mail of the corresponding author.
- An abstract of no more than 250 words should start on page 2. A list of 3-6 key words or short phrases should be provided in alphabetic order below the abstract.
- The main text should include the following components arranged in a fixed order: Introduction, Materials and Methods, Results, Discussion, Acknowledgments, references, Figures legends and Tables. Figures should be presented in a separate file.
- **Declaration of Interest.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article. (This statement should follow Acknowledgments.)
- Cite each reference, figure and table in strict numerical order: [1-5]; (Table 1); (Figure 1).
- References (not exceeding 30-40 in number) should be numbered in the order in which they are first mentioned in the text. Please cite all authors if there are only six; cite the first six authors followed by *et al.* if there are more than six authors listed. Prepare the reference list in accordance with the American Medical Association (AMA) Manual of Style, 9th ed. Examples: The surnames of all authors followed by their initials should be given. Abbreviate journal names in accordance with the Index Medicus system (http://www.nlm.nih.gov /pubs/factsheets /constructitle.html).

Example:

Journal:

Du Manoir S, Speicher **MR**, Joos S, **Schröck** E, Popp S, **Döhner** H *et al.* Detection of complete and partial chromosome gain and losses by comparative genomic in situ hybridization. Hum Genet. 1993; 90**(6)**: 590-610..

Book:

Huisman THJ, Carver MFH, Efremov GD. A Syllabus of Human Hemoglobin Variants **(Second Edition)**. Augusta, GA: The Sickle Cell Anemia Foundation, 1998 (http://globin.cse.psu.edu).

Gardner RJM, Sutherland GR. Chromosome Abnormalities and Genetic Counseling, 3rd ed. New York, **NY, USA**: Oxford University Press, 2004.

Contribution to a Book:

An International System for Human Cytogenetic Nomenclature (ISCN 2013). In: Schaffer LG, McGowan-Jordan, Schmid M, Editors. Basel, **Switzerland**: S. Karger, 2013

Strachan T, Read AP. **Genetic testing in individuals and populations (Chapter 17).** Human Molecular Genetics, 2nd ed. New York, **NY, USA**: Wiley-Liss; 1999 (http://www.ncbi.nlm.nih.gov/books/NBK7586/).

Web page:

Capodano AM. Nervous system: meningioma. Atlas Genet Cytogenet Oncol Haematol. July 2000 (http://atlasgeneticsoncology.org/Tumors/MeningiomaID5014.html)

Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution 30: 2725-2729

- Place all the tables at the end of your file. Start each table on a new page, complete with a title and footnotes (if any). Number the tables (Arabic numerals) in the order of appearance in the text. Always separate the individual columns using tabulators, not space bar.
- Figures should be prepared on separate pages. Use Arabic numerals to number the figures in the order pf afferanace in the text.

8. Plagiarism detection

Balkan Journal of Medical Genetics uses CrossCheck plagiarism software as part of the editorial process, in order to ensure that the content published is original and trustworthy. CrossCheck is a medium that allows for comprehensive manuscripts screening, aimed at eliminating plagiarism and providing a high standard and quality peer-review process. By submitting your manuscript to this journal, you accept the established procedure of screening your manuscript or plagiarism against previously published articles.