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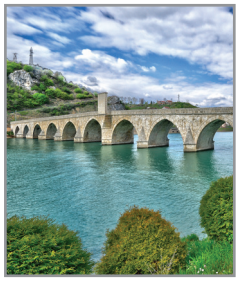

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ORIGINAL ARTICLE

PREIMPLANTATION GENETIC TESTING WITHIN THE PUBLIC HEALTHCARE SYSTEM IN SLOVENIA

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ABSTRACT

Preimplantation genetic testing (PGT) is the earliest form of prenatal diagnosis that has become an established procedure for couples at risk of passing a severe genetic disease to their offspring. At UMC Ljubljana, we conducted a retrospective register-based study to present 15 years of PGT service within the public healthcare system in Slovenia. We collected the data of the PGT cycles from 2004 to 2019 and compared clinical outcomes for chromosomal and monogenic diseases using different embryo biopsy and testing approaches. In addition, we assessed the extent to which PGT has become the preferred option compared to classic prenatal diagnostics. We treated 211 couples, 110 with single gene disorder, 88 with structural chromosome rearrangement and 13 for numerical chromosome aberration. There were 375 PGT cycles with oocyte retrieval, while embryo transfer was possible in 263 cases resulting in 78 deliveries and 84 children. Altogether, the clinical pregnancy rate per embryo transfer was 31% in 2004-2016 (blastomere biopsy) and 43% in 2017-19 (blastocyst biopsy), respectively. We assessed that approximately a third of couples would opt for PGT, while the rest preferred natural conception with prenatal diagnosis. Our results show that providing a PGT service within the public healthcare system has become a considerable option in pregnancy planning for couples at risk of transmitting a severe genetic disease to their offspring. In Slovenia, approximately a third of couples would opt for PGT. Although the number of cycles is small, our clinical results are comparable to larger centres.

Keywords: chromosome aberration, embryo biopsy, in vitro fertilization, monogenic disease, preimplantation genetic testing.

INTRODUCTION

Preimplantation genetic testing (PGT) is an established procedure for couples at risk of transmitting a genetic disease to their offspring. PGT involves in vitro fertilization using ICSI and genetic analysis of the embryo prior to transfer and implantation. Such practice allows the selection of an unaffected embryo for the specific pathogenic variant tested, thus avoiding the termination of pregnancy following classical prenatal diagnostic testing. The first PGT procedure was performed in 1990 for sex selection of X-linked disorder in the United Kingdom (1, 2). With advances in assisted reproductive technology (ART) and molecular genetic methods, PGT has become an essential reproductive option as it significantly reduces the risk of affected offspring. Another motive for PGT is the reduced psychological burden and uncertainty of future parents.

PGT can be performed for any severe monogenic disease (PGT-M) or chromosome rearrangement (PGT-SR). In addition, preimplantation aneuploidy screening (PGT-A, formerly preimplantation genetic screening - PGS) is applied worldwide in a subgroup of infertile patients with normal karyotypes undergoing in vitro fertilization (3). Although the technical procedures for PGT-M, PGT-SR and PGT-A are similar, the indications differ. In Slove-

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nia, prospective parents at risk of transmitting structural chromosome rearrangement or monogenic disease to their offspring may opt for PGT-SR or PGT-M, while PGT-A for infertile couples is not routinely practiced. However, PGT-A may be performed if a parent is a carrier of numerical chromosome aberration.

While modern PGT methodologies, performance, and outcomes of PGT services are similar between individual centres, the practices of how and to whom to offer PGT vary between countries depending on different jurisdictions and policy approaches (4). There is also limited data on what proportion of patients would opt for PGT as a first choice for testing instead of natural conception with classical prenatal diagnostics.

We conducted a retrospective register-based study to present 15 years of development and provision of PGT within the public healthcare system in Slovenia.

MATERIALS AND METHODS

Subjects

Two hundred and eleven (211) couples with a known genetic predisposition, 110 with monogenic disorder, 88 with chromosome structural rearrangement, and 13 with mosaic sex or numeric chromosome abnormality, were eligible for the PGT procedure. Ovarian stimulation and oocyte retrieval were performed according to standard protocol (5). All couples signed informed consent prior to the PGT procedure. Clinical operations have been conducted following the principles expressed in the Helsinki declaration.

Methods

Preimplantation genetic testing was implemented in 2004. Prior to enrolment, the couples underwent genetic counselling. Genetic counselling is organized stepwise to provide all the relevant education and information associated with the procedure. Couples attending PGT cycles are informed about the benefits, limitations, and risks of the PGT procedure and the expected delivery rate per embryo transfer. The multidisciplinary approach manages coordination between hormone stimulation, embryology part, and genetics. Since biopsy is performed only on good quality blastocysts, single embryo transfer is preferred. The confirmatory prenatal diagnostic testing is still recommended following a PGT-M, and to a lesser extent of PGT-SR, due to difficulties of testing the limited number of cells obtained by embryo biopsy as well as recognition of the biological and human factors that may lead to misdiagnosis in a PGT cycle (6). A follow-up of pregnancies, deliveries, and postnatal development of born children, along with the cycle data, is maintained.

For cycles from 2004 to the end of 2016, cleavage-stage embryo biopsy was performed on day three after fertilization, and two blastomeres, when possible, were withdrawn. Then according to the indication, either fluorescent in situ hybridization (FISH) analysis or polymerase chain reaction (PCR) based protocol were performed. The FISH based protocol consisted of set-up with probe selection and pre-cycle work-up on peripheral blood lymphocytes from both reproductive partners. FISH was carried out according to standard protocol using commercially available probes by Abbott Vysis, Cytocell, or Agilent SureFISH, and guidelines and recommendations by ESHRE (7). The turnaround time was 48 hours, which allowed for fresh embryo transfer on day five. PCR based protocol for single-gene disorders was performed according to guidelines and recommendations by ESHRE (8). PGT set-up included indirect analysis and direct genotyping, if appropriate (8).

In 2017, blastocyst biopsy (trophectoderm biopsy-TE) on days 5 to 7 was introduced. This allowed for whole genome amplification and next-generation sequencing (NGS) based 24-chromosome screening for chromosome and segmental abnormalities with a resolution of 10-20 Mb. Another advantage of this approach is that a pre-diagnostic set up is usually not required. The NGS-based protocol was carried out according to the manufacturer's recommendations (VeriSeq PGS, Illumina). In addition, genetic testing for single-gene disorders was carried out as mentioned above.

We have reviewed the medical records from 2004 to 2019 at our institute to determine the proportion of couples with genetic indications that opted for preimplantation genetic testing and signed informed consent. Based on this data, we defined the proportion of Slovenian couples who would opt for PGT as a first genetic testing.

Data analysis

By first reviewing medical records, we estimated the proportion of couples that would opt for PGT. We retrospectively collected the referrals for all performed PGT-M, PGT-SR, and PGT-A cycles from 2004-2019. In addition, we calculated the average and median age of female partners enrolled in PGT. Then we analysed data of the PGT cycles regarding the referrals (PGT-M, PGT-SR, PGT-A or X-linked disorder), the type of embryo biopsy (blastomere biopsy in 2004-2016, blastocyst biopsy in 2017-2019) and genetic testing approach for chromosome rearrangements (FISH for chromosome rearrangements in 2004-2016, next-generation sequencing-based 24-chromosome screening in 2017-2019). Then, we compared the clinical outcomes in 2004-16 and 2017-19 using Chi-square statistics to test the clinical effectiveness of different PGT approaches.

RESULTS

Following genetic counselling, approximately 32% of couples would opt for PGT, either because of chromosome rearrangements (88/284) or monogenic disorder (110/333). Referrals of performed PGT cycles are presented in tables 1 and 2. Of a total of 211 couples, there were 110 for PGT-M (Table 1), 88 carriers of either simple reciprocal chromosomal translocation or carriers of a complex or cryptic chromosome rearrangement for PGT-SR (table 2). In addition, there were 10 couples with sex chromosome mosaicism and 3 couples with repeated aneuploid conception for PGT-A. The age of female partners engaged in our PGT program in selected years were as follows: average age of the females in the couples was 32.6 (25-38 years, median 33 years) in 2004-2016 and 33 years (24-39 years, median 33 years) in 2017-2019.

Data collection of our PGT program throughout the years 2004-2019 are presented in the tables 3 and 4. A total of 211 couples underwent 375 PGT cycles. The most frequent indications were single gene disorder, followed by chromosome rearrangement, with X-linked disease being the least represented. There were 263 embryo transfers, which resulted in 94 clinical pregnancies, while 16 pregnancies (16/94, 17%) ended in spontaneous miscarriage. Eighty-four unaffected children were born, examined by the paediatrician and geneticist. Embryo diagnosis was possible in 94% in the years 2004-2016 but dropped to 83% in years 2017-19. The diagnostic drop in the later years was mainly due to amplification failure or poor-quality biopsies.

We present the data for years 2004-2016 and 2017-19 separately because different biopsy and genetic testing methods were used.

Data from 2004-2016 for FISH analysis for chromosome rearrangements and multiplex PCR for monogenic disorders performed on blastomeres are collected in table 3. Altogether, the clinical pregnancy rate was 31% per embryo transfer. There were, on average, 4 embryos suitable for biopsy per cycle. The miscarriage rate was 20% (10/51). There were 8 twin pregnancies and one triple pregnancy. In addition, two cases of hyperstimulation were reported. There were 11 cycles with no PGT either because oocytes were not fertilized, embryo arrest or poor-quality blastocysts.

In 2017-19 we implemented TE biopsy and NGS based 24-chromosome screening for chromosome rearrangements and aneuploidy screening. The data are represented in table 4. Altogether, the clinical pregnancy rate was 43% per embryo transfer. There were, on average, 3 embryos suitable for biopsy per cycle. The miscarriage rate was 9% (4/43). There were no twin or triple pregnancies nor any cases of hyperstimulation reported. No cases of misdiagnosis were reported. There were 20 cycles with no PGT either because oocytes were not fertilized, there was embryo arrest or poor-quality blastocysts.

Table 1. List of monogenic disorders.

Disorder	Number of couples
Duchenne muscular dystrophy	6
Huntington Disease	15
Facioscapulohumeral dystrophy	3
Spinal Muscular Atrophy	5
GJB1 X -linked Charcot Marie Tooth	4
Charcot Marie Tooth disease I	6
Von Hippel Lindau syndrome	4
Retinoblastoma	2
Myotonic dystrophy 1	10
Cystic Fibrosis	3
Sandhoff disease	2
Alport syndrome	4
Haemophilia A	6
IL1RAPL1 intellectual disability	2
Fragile X syndrome	3
Incontinentia pigmenti	2
ARPKD	2
Fabry disease	2
Other*	29

*Includes only one couple for each referral: Autosomal recessive deafness IA, Achondroplasia, WWOX encephalopathy, Glycine encephalopathy, Spondyloepiphyseal dysplasia congenita, Marfan syndrome, Neurofibromatosis I, Tuberous sclerosis I, Congenital adrenal hyperplasia, Alagille syndrome, Proliferative vasculopathy and hydranencephaly-hydrocephaly syndrome, Emery -Dreifuss Muscular dystrophy, Pachyonychia congenita, Metachromatic leukodystrophy, Fraser syndrome, Myofibrillar myopathy, Hypohydrotic ectodermal dysplasia, Schimke immunoosseous dysplasia, MED12 genopathy, Tavil Andersen syndrome, Norrie disease, Epidermolysis bulosa dystrophica, Adenomatous polyposis coli, MYH7-Hypertrophic cardiomyopathy, CDH1-cancer predisposition, FOXC1- Axenfeld-Rieger syndrome, RYR1 congenital neuromuscular disease.

Table 2. List of chromosomal rearrangements.

Translocation	Number of couples
45,XY,der13;14)(q10;q10)	8
45,XX,der(13;14)(q10;q10)	3
Simple reciprocal translocation male/ female carrier	71
47,XXY,t(12;22)(q12;q13.3) (5)/46,XY,t(12;22)(q12;q13.3)(45)	1
45,XX,der(15;20)(q10;q10), der(20;21)(p10;q10)	2
46,XX,t(11;18)(q23;q21).ish ins(11;18) (q21;q21.1q21.3)(WCP18+)	1
46,XX.ish t(X;17)(p22.1;p13.3)	1
46,XX.ish t(17;22)(q25.1;q13.33)	1

Table 3. Data collection 2004-2016.

Referral	XL disorder (sex selection)	PGT-A	PGT-SR	PGT-M	Total
Couples	6	10	48	55	119
Cycles (OR)	20	27	88	106	241
ET	19	30	56	58	163
Embryos for biopsy	87	122	453	364	1026
Diagnosis	81(93%)	109(89%)	442(97%)	332 (91%)	964 (94%)
Pregnancy	6	5	20	20	51
Miscarriage	0	2	2 (+2*)	6	10 (19.6%)
Children	7	3	19	16	45
	1x twins	N/A	1x twins, 1x triples	2x twins	4x twins, 1x triples
Deliveries	6	3	16	14	39
Pregnancy rate / ET (%)	32%	17%	36%	34%	31%
Delivery rate / ET (%)	32%	10%	29%	24%	24%
Cycles with no PGT	0	0	2	9	11

Legend: *- post amniocentesis.

Table 4. Data collection 2017-2019.

Referral	X-linked disorder (sex selection)	PGT-A	PGT-SR	PGT-M	Total
Couples	4	3	40	45	92
Cycles (OR)	5	6	49	74	134
ET	3	4	29	64	100
Embryos for biopsy	11	16	112	219	358
Diagnosis	10 (91%)	13 (81%)	91 (81%)	182 (83%)	296 (83%)
Pregnancy	2	2	13	26	43
Miscarriage	1	0	1	2	4 (9%)
Children	1	2	12	24	39
Deliveries	1	2	12	24	39
Pregnancy rate / ET (%)	N/A	N/A	45%	41%	43%
Delivery rate / ET (%)	33%	50%	38%	36%	37%
Cycles with no PGT	0	1	11	8	20

We compared the clinical outcome between both periods (2004-2016 versus 2017-19) using the Chi-square method with a p-value of less than 0.05 considered as significant. Implementation of blastocyst biopsy and chromosome-wide analysis significantly improved delivery rate per ET for chromosomal and monogenic indications in years 2017-19 (Chi-square 4.184, p= 0.03 and Chi-square 5.21, p= 0.02, respectively), while pregnancy rate per ET (Chi-square 3.08, p=0.07) was not statistically significant.

DISCUSSION

Our results of 15 years of experience show that PGT has become an established practice in addition to traditional prenatal diagnosis in Slovenia.

PGT is performed for requests associated with a high risk for a severe medical condition in offspring, either of chromosomal or monogenic origin. The most common referrals for PGT-SR were reciprocal translocation in female partners and Robertsonian translocation in male partners. By contrast, PGT-M was mainly requested for Huntington's disease, Duchenne muscular dystrophy, Haemophilia A, Myotonic dystrophy, Spinal muscular atrophy, and Charcot Marie Tooth disease. Furthermore, couples at high risk for adult-onset disorders or familial cancer predisposition presented 20% of all PGT-M referrals.

Our results are consistent with the published ESHRE PGT Consortium data collection (9, 10). Trophectoderm biopsy and genome-wide analysis increased the accuracy and reliability of the preimplantation genetic testing. When com-

paring the dataset from 2004-2016 to 2017-19, the delivery rates per embryo transfer significantly increased. The increase may be due to the substantial amount of starting material, whole genome amplification and genome-wide screening.

We observed that in 2004-2016, there were, on average, four embryos suitable for biopsy per cycle, while in 2017-2019, up to three embryos. This lower number was expected because, in 2004-2016, embryos were biopsied on day three at the cleavage stage and in 2017-2019 at the blastocyst stage, and not all cleavage stage embryos reached the blastocyst stage. Hormone stimulation may be associated with hyperstimulation syndrome, a life-threatening condition in the most severe form. Therefore, each patient's hormonal stimulation protocol in our clinic is adjusted to optimize follicle growth and avoid complications associated with hyperstimulation syndrome. Since 2017, only the freeze-all approach has been performed in PGT cycles, which is more convenient to prevent hyperstimulation than before, when fresh embryo transfers were performed. Putting the patients and their safety first is our priority as well as a critical indicator of the quality of a healthcare system, including IVF-PGT procedures.

Most PGT cycles in Slovenia were requested for PGT-M and PGT-SR (375 cycles, 91%). In addition, PGT-A cycles (33 cycles, 9%) were performed because of genetic indications, i.e., parental sex chromosome mosaicism, X-linked monogenic disorder or repeated aneuploid conception. In many IVF centres, PGT-A cycles predominate and are used to shorten the time to pregnancy in the treatment of infertile couples without genetic indication. In the recent ESHRE data collection (9), PGT-A comprised more than 60% of all reported procedures. PGT-A, as an extension of IVF, is not performed in our country nor in Denmark, France, Germany, Hungary, Lithuania, Norway, and the Netherlands (10, 11). However, embryo sex selection by PGT-A is allowed in some European countries to screen for X-linked diseases.

The monogenic referrals account for more than 50% of cycles and are increasing yearly. An increase in PGT-M is mainly due to improved genetic diagnostics by next-generation sequencing and preconception carrier screening.

The availability of PGT for couples with severe genetic indications represents a considerable reproductive option in Slovenia. The costs of PGT cycles are covered by the National Health Insurance, which allows equal access to health care services for eligible couples. Our national public healthcare system provides PGT services in accordance with the needs of the patients to ensure fair and accessible patient-centred medicine. Furthermore, following genetic counselling, about a third of couples at high risk of transmitting a genetic disease to their offspring would opt for the PGT procedure.

The practices of PGT vary across different jurisdictions and policy approaches, ranging from restrictive to permissive policy models (4). Countries may regulate PGT through state funding (Austria, Belgium, Germany, France, Italy, the Netherlands, Switzerland, United Kingdom, Sweden, Denmark, Finland, Canada), private (Australia, Israel, United States, Singapore, Brazil, Japan), or a mixture of the two models (Denmark, Finland, United Kingdom) (4). However, PGT practices change with time, according to technological development, diagnostic improvements, ethical considerations, and patient needs and demands.

There are certain limitations of our retrospective register-based study. First, we know that a small sample size (n=163 ET in 2004-2016 and n=100 ET in 2017-2019) represents a study limitation. Nevertheless, the clinical outcomes are comparable to larger centres and reflect the actual needs of our patients. It was expected that blastocyst biopsies would result in increased implantation and live-birth rates compared to blastomere biopsy (12); however, a large retrospective cohort study showed that a freeze-all strategy is beneficial in high responders but not in intermediate or low responders, thus refuting the idea that freeze-all cycles are preferable for all patients (13). Lastly, we neither addressed the clinical characteristics of the patients in terms of hormone levels, stimulation protocol, endometrium preparation, or the number of retrieved and matured oocytes nor whether socioeconomic status influences the decision regarding PGT. The study was focused on the development and provision of PGT services in our country rather than assessing the routine protocol for IVF-PGT procedure.

In conclusion, we report on our 15 years of experience in PGT, provided by the Slovenian healthcare system, where about a third of couples at risk for transmitting a severe genetic disorder to their offspring would opt for PGT. The results of our study show that the clinical outcomes of PGT cycles are comparable to other larger centres. Furthermore, our study demonstrates that PGT, when provided by the public healthcare system offering accessibility and equity, has become a considerable option in addition to traditional prenatal diagnosis.

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The authors received no specific funding for this work. All data are fully available without restriction. All relevant data are within the manuscript. The author's contributions: MV, BPeterlin – wrote the main manuscript text. MV, AV, MŠ – prepared analyses of the PGT cycles. KW, AV, MV, HJ, and NT – performed genetic analyses. MŠ, IVK – performed ICSI and embryo biopsy. EBV, HBF – performed gynaecological management. BPros-

enc – coordinated PGT procedures. BPeterlin designed and supervised the study and revised the manuscript. All authors reviewed and approved the final manuscript.

DECLARATION OF INTEREST

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

ETHICAL APPROVAL

This was register-based study where all the participants signed individual personal approval and permission before starting the treatment and did not have to be notified to the Ethics Committee according to Slovene law, (Personal Data Protection Act, Official Gazette of the Republic of Slovenia No 94/07, 2004). Additionally, by our law, we are obligated to collect data about assisted reproduction procedures and monitor the success rates (Healthcare Databases Act, Official Gazette of the Republic of Slovenia No 65/00, 2000; No 47/15, 2015; 31/18, 2018).

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ORIGINAL ARTICLE

CHROMOSOMAL ABNORMALITIES IN EARLY PREGNANCY LOSSES: A STUDY OF 900 SAMPLES

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ABSTRACT

Chromosomal abnormalities are the most common causes of early pregnancy losses (EPLs). In this study, we aimed to evaluate the incidence and spectrum of chromosomal abnormalities in EPLs and correlate them with different clinical characteristics. We performed Quantitative Fluorescent PCR (QF-PCR), followed by subtelomeric Multiplex Ligation Probe Amplification (MLPA) analysis to detect chromosomal abnormalities in 900 products of conceptions (POCs) from EPLs collected over a period of 10 years.

Chromosomal abnormalities were present in 56.25% of uncontaminated EPLs, with significantly higher incidence in women ≥36 years (71.37%, p<0.0001) in comparison to women ≤30 years of age (43.40%). Trisomies were also more common in women ≥36 years (79.68%, p<0.0001) than in those ≤30 years of age (48.70%). In contrast, triploidy and monosomies were more prevalent in women ≤30 years of age (26.09%, p<0.0001 and 16.52%, p=0.0066 respectively) than in women ≥36 years of age (6.42% and 6.42% respectively). Trisomy 16 was more common in women ≤30 (39.29%, p=0.0009) than in those ≥36 years of age (16.78%), while trisomy 22 was predominant among women ≥36 (23.49%, p=0.013), and was not present in the group of women ≤30 years of age. The frequency of chromosomal abnormalities in POCs from women with sporadic (61.19%) was higher than in those with recurrent EPLs (55.21%). This difference, however, was not statistically significant (p=0.164). Although some differences in the chromosomal aneuploidy rates among women with different ABO blood groups, as well as among 6-8 and 9-11 gestational week EPLs were observed, further larger studies are required to confirm these findings.

In conclusion, our study enriches the knowledge about chromosomal abnormalities as a cause of EPLs and confirms the higher incidence of foetal chromosomal abnormalities in EPLs in women of older reproductive age. Furthermore, it shows that using QF-PCR and MLPA methodologies, a high detection rate of chromosomal abnormalities in EPLs can be reached.

Key words: Chromosomal abnormality, early pregnancy loss (EPL), QF-PCR, MLPA

INTRODUCTION

Human reproduction is characterized by a high rate of abnormal conceptions, most of which are spontaneously eliminated before the pregnancy is clinically recognized. Pregnancy loss (PL), spontaneous abortion or miscarriage refers to the spontaneous (unintended) loss of pregnancy before the foetus reaches viability, i.e. before twenty weeks of gestation [1]. Early pregnancy loss (EPL) represents a spontaneous loss of pregnancy before the 12th week of gestation (first trimester). In the United States, recurrent pregnancy loss (RPL) is defined as having two or more consecutive failed clinical pregnancies, documented by ultrasound or histopathology, while in the United Kingdom it is defined as having three or more consecutive early pregnancy losses [2,3]. Up to 15% of all clinically

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recognized pregnancies are miscarried, and nearly 2% of the couples that are trying to conceive experience RPL.

The aetiology of EPL can include various factors, such as maternal endocrine dysregulation, anatomical abnormalities of the uterus, implantation factors, various infections during the pregnancy and foetal chromosomal abnormalities. About 40-65% of the miscarried foetuses are associated with various chromosomal abnormalities, the most common of which are chromosomal trisomies, followed by polyploidies and monosomy X [4-6].

Some studies have detected nearly equal frequencies in sporadic and recurrent EPLs, while others show a lower rate of chromosomal abnormalities in RPLs [7, 8]. Due to age-related oogenesis errors, advanced maternal age represents a considerable risk factor for EPL [9]. Indeed, many studies have confirmed a higher incidence of POCs with chromosomal abnormalities in women with advanced age. Published data indicate that foetal triploidies and monosomies are more common in younger women, while trisomies are more prevalent in older women [10, 11]. A limited number of data provide insight into the distribution of chromosomal abnormalities in POCs in reference to the week of gestation. Up to now, there is no published study that correlates the maternal ABO blood groups and Rhesus factor with foetal chromosomal abnormality, even though the available data indicate that incompatible mating and adverse pregnancy outcomes may correlate with ABO blood groups [12].

Chromosomal karyotyping has been the gold standard for studying the chromosomes for many decades, but this method is hampered by a high culture failure or maternal cell contamination. Currently, chromosomal microarrays represent a first-tier method for chromosomal abnormality investigations, however due to the high price, it is rarely used for EPLs. On the other side, quantitative fluorescent PCR (QF-PCR) and multiplex ligation probe amplification (MLPA) methods have emerged to determine any chromosomal abnormalities in POCs. This is due to their lower cost, faster reporting times, and accurate results [13, 14].

Here we present the results of our 10 year study of EPLs using QF-PCR, followed by subtlomeric MLPA, including the distribution of foetal chromosomal abnormalities in relation to the clinical characteristics of women experiencing EPLs.

MATERIALS AND METHODS

Study subjects

Our study included 900 POC samples from women who experienced EPLs (gestational age ≤12 weeks). POC samples, previously selected by a gynaecologist/pathologist [15] and accompanied with maternal whole blood sample, were referred for analysis of chromosomal abnormalities to the Research Centre for Genetic Engineering and Biotechnology "Georgi D. Efremov", at the Macedonian Academy of Sciences and Arts, Skopje. Signed informed consent was obtained from all participants in this study. The study has been approved by the ethical committee of the Macedonian Academy of Sciences and Arts (09-1047/6 from 04.05.2016).

Table 1 displays the clinical characteristics of the women with EPLs, including maternal age, ethnic origin, history of previous EPL, previous live birth, maternal ABO blood group, Rhesus (Rh) factor, and the gestational week (gw) of the EPLs studied. The samples were categorized into three groups according to the maternal age: ≤30, 31-35, and ≥36 years. The majority of patients were of Macedonian (n=528) ethnic origin as well as Albanian (n=208). Gestational ages of the POCs consisted of samples from gw=6 to gw=11 (mean gestational age 8.5 weeks).

Methods

DNA extraction from the POC samples, as well as from maternal blood was performed using the standard phenol/chloroform method or the automated magnetic bead-based protocol using the MagCore Super instrument (RBC Bioscience). The study primarily used the quantitative fluorescent (QF)-PCR method with STR markers on chromosomes 13, 18, 21 and sex chromosomes. Three markers were located on chromosome 13, four on chromosomes 18 and 21 each and six on the sex chromosomes. Except aneuploidies on the given chromosomes, this method also allowed for the determination of triploid samples, and exclusion of maternal DNA contamination in the analysed samples. This method is described in detail by Noveski et al. [16]. All results for chromosomal aneuploidies obtained by the QF-PCR analyses were confirmed by the subsequent subtelomere MLPA analyses.

Chromosomal gains and losses were detected with the Multiplex Ligation Probe Amplification (MLPA) method, using the SALSA MLPA P036 Subtelomeres mix 1 and SALSA MLPA P070 Subtelomeres mix 2B (MRC-Holland). Each MLPA kit contains two probes for each chromosome. For metacentric chromosomes the two probes were located subtelomerically, while for the acrocentric chromosomes, one probe was located subcentromeric, while the other was subtelomeric. The detailed MLPA protocol as well as the chromosomal location of each probe contained in the kits is available on the MRC-Holland site. Capillary electrophoresis was performed on the AB3500 Genetic Analyser (Life Technologies), and the obtained results were analysed and interpreted using the Coffalyzer software (MRC-Holland). Mean values, standard deviations, percentages, odds ratios, and p-values were

Table 1. Characteristics of the study groups

Characteristic	Study groups	All cases		Macedonian		Albanian		Other	
		n (%)	MA±SD	n (%)	MA±SD	n (%)	MA±SD	n (%)	MA±SD
		n=768	32.9±5.35	n=528	34.14±4.90	n=208	33.16±5.46	n=32	31.90±5.68
Maternal age	≤30	265 (34.50)	27.19±2.56	131 (24.81)	27.82±2.18	121 (58.17)	26.53±2.72	13 (40.62)	26.92±3.22
	31-35	241 (31.37)	32.87±1.39	178 (33.71)	32.89±1.40	52 (25.00)	32.82±1.41	11 (34.37)	32.27±0.90
	≥36	262 (34.11)	38.96±2.39	219 (41.47)	38.94±2.31	35 (16.82)	39.02±2.61	8 (25.00)	39.5±3.46
	ND	/	/	/	/	/	/	/	/
History of PL	Sporadic	268 (34.89)	33.16±5.26	202 (38,25)	33.95±4.78	48 (23,07)	29.60±5.55	18 (56,25)	33.61±5.81
	RPL	259 (33,72)	33.1±5.24	164 (31,06)	34.67±4.71	86 (41,34)	30.56±5.11	9 (28,12)	28.77±4.14
	ND	241 (31,38)	/	162 (30,68)	/	74 (35,57)	/	5 (15,62)	/
Previous live birth	Yes	142 (18,48)	34.66±4.75	95 (17,99)	35.76±4.48	41 (19,71)	32.82±4.64	6 (18,75)	29.66±2.25
	No	383 (49,86)	32.57±5.32	269 (50,94)	33.77±4.76	93 (44,71)	29.07±5.15	21 (65,62)	32.66±6.26
	ND	243 (31,64)	/	164 (31,06)	/	74 (35,57)	/	5 (15,62)	/
Maternal ABO group	0	183 (23,821)	33.09±5.52	123 (23,29)	34.73±4.98	55 (26,44)	29.38±4.98	5 (15,62)	33.60±4.72
	A	183 (23,82)	33.61±4.95	138 (26,13)	34.41±4.60	40 (19,23)	31.32±4.99	5 (15,62)	30.00±7.68
	B	67 (8,72)	32.82±5.03	46 (8,71)	33.91±4.63	15 (7,21)	30.46±5.99	6 (18,75)	30.33±2.42
	AB	36 (4,68)	31.63±5.57	21 (3,97)	33.33±5.38	9 (4,32)	28.11±5.46	6 (18,75)	31.00±4.28
	ND	299 (38,93)	/	200 (37,87)	/	89 (42,78)	/	10 (31,25)	/
Maternal RhD status	RhD+	410 (53,38)	33.19±5.26	288 (54,54)	34.4±4.81	102 (49,03)	30.14±5.23	20 (62,5)	31.30±5.00
	RhD-	59 (7,68)	32.91±5.21	40 (7,57)	34.3±4.75	17 (8,17)	29.64±5.08	2 (6,25)	30.00±2.82
	ND	299 (38,93)	/	200 (37,87)	/	89 (42,78)	/	10 (31,25)	/
Gestational week	6	36 (4,68)	33.02±4.64	24 (4,54)	34.66±3.71	11 (5,28)	29.90±4.90	1 (3,12)	28.00±0.00
	7	80 (10,41)	34.27±5.29	58 (10,98)	34.75±4.98	16 (7,69)	31.93±5.37	6 (18,75)	35.83±7.02
	8	102 (13,28)	32.89±5.43	71 (13,44)	34.45±5.05	26 (12,5)	29.53±4.53	5 (15,62)	28.20±5.21
	9	65 (8,46)	32.21±5.28	46 (8,71)	32.91±4.83	17 (8,17)	30.23±5.99	2 (6,25)	33.00±8.48
	10	33 (4,29)	33.72±5.51	24 (4,54)	34.91±4.97	7 (3,36)	31.14±6.54	2 (6,25)	28.5±3.53
	11	39 (5,07)	30.74±4.98	26 (4,92)	31.65±4.31	11 (5,28)	29.27±6.35	2 (6,25)	27.00±1.41
	ND	413 (53,77)	/	279 (52,84)	/	120 (57,69)	/	14 (43,75)	/
Fetal sex	Male	379 (49.34)	33.03±5.26	264 (50.00)	34.09±4.74	101 (48.55)	30.21±5.46	14 (43.75)	33.42±6.01
	Female	389 (50.65)	32.93±5.44	264 (50.00)	34.19±5.06	107 (51.44)	30.20±5.27	18 (56.25)	30.72±5.28
	Total	768 (100.00)	/	528 (100.00)	/	208 (100.00)	/	32 (100.00)	/

MA, mean age; SD, standard deviation; ND, no data

determined where appropriate, using the MedCalc software (*MedCalc Software Ltd. https://www.medcalc.org/ Version 22.016; accessed November 17, 2023*). A p-value below 0.05 was considered statistically significant.

RESULTS

The QF-PCR analysis performed on 900 POC samples showed maternal DNA contamination in 14.66%. These samples were excluded from further investigation and MLPA analyses were performed on a total of 768 POC samples.

Chromosomal abnormalities - overall incidence

Chromosomal abnormalities were present in 56.25% of uncontaminated POC samples. Chromosomal trisomies were detected in 66.20%, triploidy in 15.28%, and monosomies were present in 10.88% of the positive cases. The group of monosomies consisted of monosomy X which was predominant (91.49%) and monosomy 21 was present in 8.51%.

All chromosomes, apart from chromosomes 1 and 19, were affected by trisomy; the most common was trisomy 16, present in 26.57% of all trisomy samples, followed by trisomy 22 (15.73%), 21 (10.13%) and 15 (9.09%) (Figure 1).

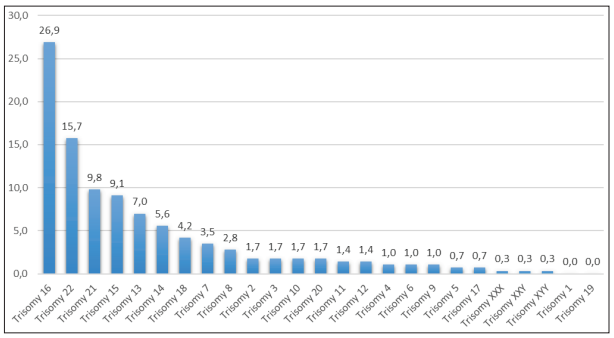

Figure 1. Distribution of chromosomal trisomies in EPLs as a percentage of all detected trisomies (n=286).

Chromosomal abnormalities and maternal age

As shown in Table 2 the overall frequency of chromosomally abnormal POCs increased with maternal age (43.40% in the women ≤30 years of age, 53.94% in women 31-35 years and 71.37% in woman ≥36 years). The odds for chromosomally abnormal EPLs were 1.52 higher in the group of women 31-35 years of age (95% CI: 1.07-2.16; **p=0.018**) and 3.25 higher in women ≥36 years (95% CI: 2.26-4.66; **p<0.0001**), when compared to the group of women ≤30 years of age (Table 3).

The spectrum of the chromosomal abnormalities differed in the three groups of women according to their age.

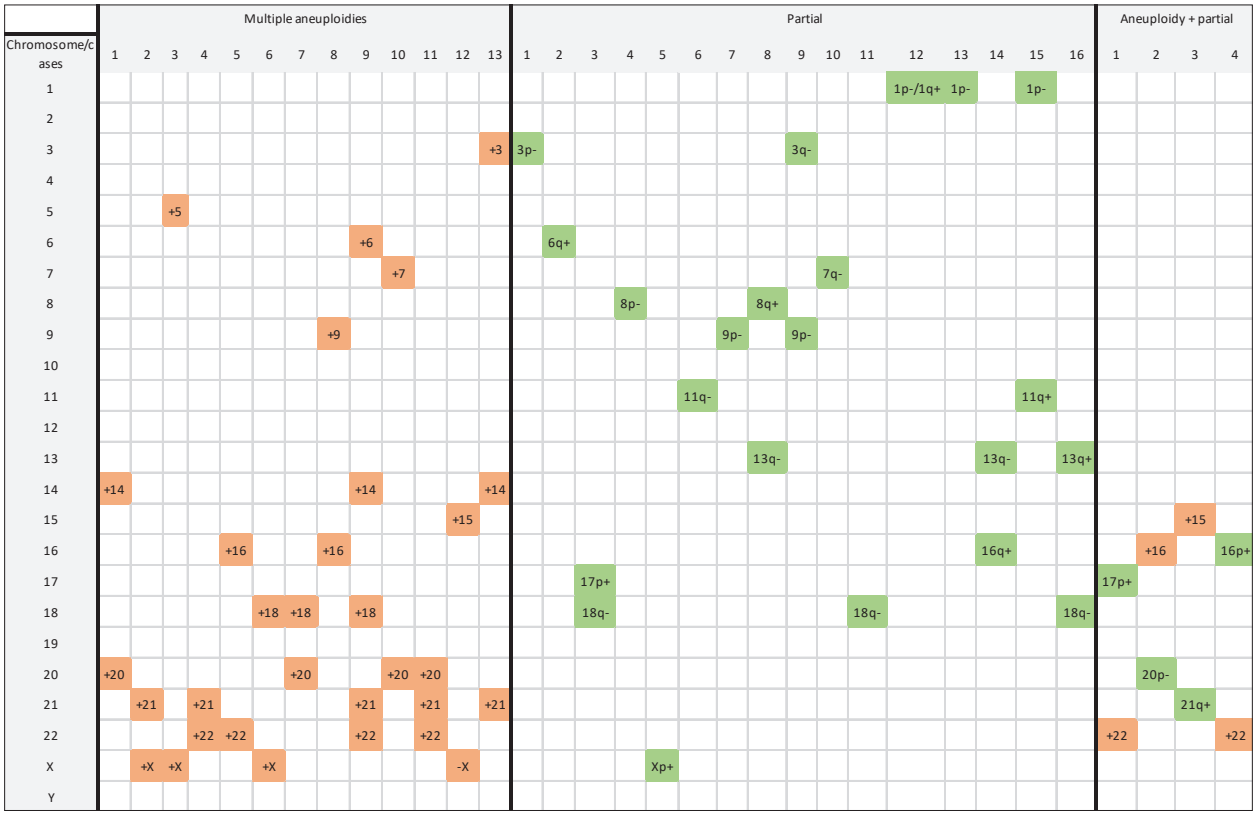

Figure 2. Multiple aneuploidies, partial chromosomal abnormalities, aneuploidies accompanied by partial abnormality detected among the studied EPLs.

Partial chromosomal abnormalities (terminal deletions and duplications), multiple chromosomal aneuploidies, and chromosomal aneuploidies accompanied by partial chromosomal abnormality were found in 3.70%, 3.01%, and 0.93% of all positive samples (Table 2). A detailed description of the later three groups of chromosomal abnormalities is provided in Figure 2. Chromosomes 20, 21, 22 and X were most involved in multiple aneuploidies, chromosomes 1, 13 and 18 in partial aneuploidies, while chromosomes 15, 16 and 22 were found in combination with a partial aneuploidy. In cases of partial chromosomal abnormalities, parental karyotyping could not be performed at the time.

In the group ≥36 years, the trisomies were predominant (79.68%), while triploidy and monosomies were present in small portions (6.42% each). In the group of women ≤30 years of age, a more even distribution of the chromosomal abnormalities was observed, with trisomies present in 48.70%, triploidy in 26.09% and monosomies in 16.52%. The odds for EPLs with trisomy were 1.88 times higher in the group of women 31-35 years of age (95% CI: 1.26-2.81; **p=0.0017**) and 4.92 higher in women ≥36 years (95% CI: 3.35-7.21; **p<0.0001**), when compared to the group of women ≤30 years of age (Table 3).

Table 2. Frequency of chromosomal abnormalities in EPLs according to different clinical characteristics.

Clinical characteristics	Study groups	Total	Chromosomal abnormalities	Trisomy n(%)	Triploidy n(%)	Monosomy n(%)	Multiple aneuploidies n (%)	Partial n(%)	Aneuploidy + partial n(%)	Total n(%)
		n	n(%)							
	Total	768	432 (56.25)	286 (66.20)	66 (15.28)	47 (10.88)	13 (3.01)	16 (3.70)	4 (0.93)	432 (100)
Age group	≤30	265	115 (43.40)	56 (48.70)	30 (26.09)	19 (16.52)	2 (1.74)	7 (6.09)	1 (0.87)	115 (100)
	31-35	241	130 (53.94)	81 (62.31)	24 (18.46)	16 (12.31)	0 (0.00)	7 (5.38)	2 (1.54)	130 (100)
	≥36	262	187 (71.37)	149 (79.68)	12 (6.42)	12 (6.42)	11 (5.88)	2 (1.07)	1 (0.53)	187 (100)
Ethnic origin	Macedonian	528	309 (58.52)	216 (69.90)	38 (12.30)	29 (9.39)	13 (4.21)	10 (3.24)	3 (0.97)	309 (100)
	Albanian	208	106 (50.96)	58 (54.72)	27 (25.47)	16 (15.09)	0 (0.00)	3 (2.83)	2 (1.89)	106 (100)
	Other	32	17 (53.13)	12 (70.59)	1 (5.88)	2 (11.76)	0 (0.00)	2 (11.76)	0 (0.00)	17 (100)
History of PL	Sporadic	268	164 (61.19)	109 (66.46)	22 (13.41)	19 (11.59)	6 (3.66)	7 (4.27)	1 (0.61)	164 (100)
	RPL (≥2)	259	143 (55.21)	94 (65.73)	20 (13.99)	17 (11.89)	4 (2.80)	5 (3.50)	3 (2.10)	143 (100)
Previous live birth	Yes	142	88 (61.97)	60 (68.18)	11 (12.50)	14 (15.91)	2 (2.27)	1 (1.14)	0 (0.00)	88 (100)
	No	383	218 (56.92)	143 (65.60)	31 (14.22)	21 (9.63)	8 (3.67)	11 (5.05)	4 (1.83)	218 (100)
Maternal Blood Group	0	183	103 (56.28)	63 (61.17)	14 (13.59)	15 (14.56)	5 (4.85)	4 (3.88)	2 (1.94)	103 (100)
	A	183	115 (62.84)	77 (66.96)	16 (13.91)	12 (10.43)	4 (3.48)	4 (3.48)	2 (1.74)	115 (100)
	B	67	34 (50.75)	26 (76.47)	4 (11.76)	3 (8.82)	0 (0.00)	1 (2.94)	0 (0.00)	34 (100)
	AB	36	23 (63.89)	16 (69.57)	3 (13.04)	3 (13.04)	0 (0.00)	1 (4.35)	0 (0.00)	23 (100)
Maternal RhD status	RhD +	410	244 (59.51)	163 (66.80)	33 (13.52)	31 (12.70)	6 (2.46)	7 (2.87)	4 (1.64)	244 (100)
	RhD -	59	31 (52.54)	20 (64.52)	4 (12.90)	3 (9.68)	1 (3.23)	3 (9.68)	0 (0.00)	31 (100)
Gestational week	6	36	19 (52.78)	14 (73.68)	2 (10.53)	1 (5.26)	0 (0.00)	1 (5.26)	0 (0.00)	19 (100)
	7	80	45 (56.25)	38 (84.44)	5 (11.11)	0 (0.00)	0 (0.00)	2 (4.44)	0 (0.00)	45 (100)
	8	102	56 (54.90)	38 (67.86)	8 (14.29)	5 (8.93)	1 (1.79)	3 (5.36)	1 (1.79)	56 (100)
	9	65	41 (63.08)	24 (58.54)	9 (21.95)	4 (9.76)	3 (7.32)	1 (2.44)	0 (0.00)	41 (100)
	10	33	22 (66.67)	10 (45.45)	3 (13.64)	8 (36.36)	0 (0.00)	0 (0.00)	1 (4.55)	22 (100)
	11	39	21 (53.85)	12 (57.14)	5 (23.81)	4 (19.05)	0 (0.00)	0 (0.00)	0 (0.00)	21 (100)
Fetal sex	Male	379	203 (53.56)	141 (69.45)	45 (22.16)	3 (1.47)	3 (1.47)	8 (3.94)	3 (1.47)	203 (100)
	Female	389	229 (58.86)	146 (63.75)	22 (9.60)	45 (19.65)	8 (3.49)	7 (3.05)	1 (0.43)	229 (100)

Table 3. Comparisons of the incidence of chromosomal abnormalities between the three groups according to the maternal age (Odds ratios and p-values).

	≤30 (n)	31-35 (n)	OR (95% CI)	p	>36 (n)	OR (95% CI)	p
Abnormal	115	130	1.52 (1.07-2.16)	**0.018**	187	3,25 (2.26-4.66)	**< 0.0001**
Trisomy	56	81	1.88 (1.26-2.81)	**0.0017**	149	4.92 (3.35-7.21)	**< 0.0001**
Triploidy	30	24	0.64 (0.24-1,17)	0.152	12	0.19 (0.09-0.39)	**< 0.0001**
Monosomy	19	16	0.89 (0.43-1.85)	0.771	12	0.34 (0.16-0.74)	**0.0066**
Trisomy 16	22	25	0.86 (0.43-1.74)	0.678	29	0.31 (0.15-0.62)	**0.0009**
Trisomy 22	0	10	16.59 (0.95-289.31)	**0.054**	35	35.03 (2.11-581.6)	**0.013**
Trisomy 21	7	9	0.87 (0.30-2.50)	0.803	13	0.66 (0.25-1.77)	0.419
Trisomy 15	0	4	6.56 (0.34-124.34)	0.210	22	19.94 (1.18-334.53)	**0.037**
Trisomy 13	7	7	0.66 (0.21-2.00)	0.465	6	0.29 (0.09-0.91)	**0.034**
Trisomy 14	7	4	0.36 (0.10-1.30)	0.121	5	0.24 (0.07-0.80)	**0.020**
Other abnormalities	10	10	1.10 (0.45-2.70)	0.828	14	1.43 (0.62-3.30)	0.389

Multiple chromosomal trisomies were more common among the group ≥36 years (5.88%), while partial chromosomal aneuploidies were more frequent in women ≤35 (Table 2).

Considering the individual trisomies, trisomy 16 had the highest incidence in the group ≤30 years (39.29%), and lowest in the group ≥36 years of age (16.78%). Furthermore, trisomies 13 and 14 were also more common

among the group ≤30 years (12.50% and 12.50% each), than in the other two groups. By contrast, trisomy 22 was more common in the group ≥36 years (23.49%) than in the group 31-35 years (12.35%). It was entirely absent in the youngest group of women (≤30 years of age). Similarly, the trisomy 15 was most common in the group of women ≥36 (14.77%) than in other groups (0.00% and 4.94% in groups of women ≤30 and 31-35 years, respectively) (Table 4). The odds of EPLs with trisomy 16 were 0.31 lower in the group of women ≥36 years (95% CI: 0.15-0.62; **p=0.0009**) while for trisomy 22, the odds were 16.59 higher in the group of women 31-35 years of age (95% CI: 0.95-289.31; **p=0.054**) and 35.03 higher in women ≥36 years (95% CI: 2.11-581.6; **p=0.013**) when compared to the group of women ≤30 years of age. The odds for trisomy 15 were higher (OR=19.94, 95% CI: 1.18-334.53; **p=0.037**), while for trisomies 13 and 14 were lower in women ≥36 years (OR=0.29, 95% CI: 0.09-0.91; **p=0.034** and OR=0.24, 95% CI: 0.07-0.80; **p=0.02**, respectively) when compared to the group of women ≤30 years of age (Table 3).

Although not statistically significant, a higher incidence of chromosomally abnormal POCs were detected in the Macedonian ethnic group (58.52%) in comparison to the Albanian group (50.96%; p=0.06) (Table 2). Triploidy and chromosomal monosomies were significantly more common among the Albanian ethnic group (25.47% and 15.09% respectively; p=0.0001) than in the Macedonian group (12.30% and 9.39% respectively). In contrast, chromosomal trisomies were more prevalent in POCs of Macedonian ethnic origin (69.90%) than in the Albanian group (54.72), (p=0.0044).

Regarding the individual trisomies, the most evident difference was observed in trisomy 16, being more common among Albanians (32.76%, p=0.207) than among Macedonians (24.54%). Trisomy 22 was found to be more frequent in the Macedonian group compared to the Albanian (18.52% vs. 6.90%, respectively, **p=0.035**) (Table 4). However, these differences can be explained by the age distribution of samples with different ethnicity. Namely, most Macedonians (41.47%) belonged to the ≥36 years

Table 4. The most common chromosomal trisomies in the different studied groups shown in table with number of cases as well as percentage.

Clinical characteristics	Study groups	Trisomy 16		Trisomy 22		Trisomy 21		Trisomy 15		Trisomy 13		Trisomy 14		Other		Total	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Age group	≤30	22	39.29	0	0.00	7	12.50	0	0.00	7	12.50	7	12.50	13	23.21	56	100.00
	31-35	29	35.80	10	12.35	9	11.11	4	4.94	7	8.64	4	4.94	18	22.22	81	100.00
	≥36	25	16.78	35	23.49	13	8.72	22	14.77	6	4.03	5	3.36	43	28.86	149	100.00
Ethnic origin	MK	53	24.54	40	18.52	23	10.65	18	8.33	11	5.09	12	5.56	59	27.31	216	100.00
	AL	19	32.76	4	6.90	5	8.62	7	12.07	8	13.79	3	5.17	12	20.69	58	100.00
History of miscarriage	Sporadic	25	22.94	21	19.27	9	8.26	12	11.01	7	6.42	5	4.59	30	27.52	109	100.00
	RPL	24	25.53	17	18.09	5	5.32	11	11.70	7	7.45	6	6.38	24	25.53	94	100.00
Previous live birth	Yes	9	15.00	11	18.33	2	3.33	11	18.33	3	5.00	3	5.00	21	35.00	60	100.00
	No	40	27.97	27	18.88	12	8.39	12	8.39	10	6.99	8	5.59	34	23.78	143	100.00
Maternal Blood Group	0	20	31.75	10	15.87	6	9.52	11	17.46	4	6.35	3	4.76	9	14.29	63	100.00
	A	19	24.68	14	18.18	3	3.90	9	11.69	4	5.19	3	3.90	25	32.47	77	100.00
	B	6	23.08	5	19.23	0	0.00	4	15.38	2	7.69	1	3.85	8	30.77	26	100.00
	AB	4	25.00	7	43.75	0	0.00	1	6.25	0	0.00	1	6.25	3	18.75	16	100.00
Maternal RhD status	RhD+	43	26.38	29	17.79	7	4.29	22	13.50	8	4.91	8	4.91	46	28.22	163	100.00
	RhD-	6	30.00	3	15.00	2	10.00	3	15.00	2	10.00	0	0.00	4	20.00	20	100.00
Gestational week	6	5	35.71	2	14.29	1	7.14	0	0.00	0	0.00	1	7.14	5	35.71	14	100.00
	7	13	34.21	7	18.42	1	2.63	0	0.00	3	7.89	4	10.53	10	26.32	38	100.00
	8	8	21.05	7	18.42	1	2.63	4	10.53	2	5.26	3	7.89	13	34.21	38	100.00
	9	3	12.50	4	16.67	4	16.67	5	20.83	3	12.50	1	4.17	4	16.67	24	100.00
	10	3	30.00	1	10.00	0	0.00	3	30.00	0	0.00	0	0.00	3	30.00	10	100.00
	11	3	25.00	2	16.67	3	25.00	1	8.33	1	8.33	0	0.00	2	16.67	12	100.00
Foetal sex	Male	40	28.36	19	13.47	13	9.21	10	7.09	10	7.09	10	7.09	19	13.47	141	100.00
	Female	37	25.34	26	17.80	15	10.27	16	10.95	9	6.16	6	4.10	37	25.34	146	100.00

group and only 24.81% were in the group ≤30, while the majority of Albanians (58.17%) belonged to the group ≤30, and only 16.82% were in the group ≥36. This is in accordance with the tendency of earlier childbearing among Albanian women.

Chromosomal abnormalities and history of EPLs

The frequency of chromosomal abnormalities in POCs from women with sporadic EPLs was higher than in those with RPLs (61.19% vs. 55.21%, respectively, p=0.164), this difference, however, was not statistically significant (Table 2). No evident difference was observed between these two groups regarding the presence of different classes of chromosomal abnormalities and chromosomal trisomies (Tables 2 and 4).

Chromosomal abnormalities and previous live birth

In the group of women with a previous live birth, a slightly higher, but not significantly different, incidence of chromosomally abnormal POCs was found compared to the group of women without a live birth (61.97% vs. 56.92%, respectively, p=0.297) (Table 2). However, this is most probably due to the higher mean age of the women with a live birth (34.66 years) in comparison to that of women with no live birth (32.57 years). Triploidy and trisomies did not differ much among these groups, while monosomies were more common in the group with (15.91%, p=0.119) compared to the group without live births (9.63%), but without statistical significance.

Trisomy 16 and 21 were more prevalent in the group without live births (27.97% and 8.39%, respectively) than in the group with live births (15.00%, **p=0.049** and 3.33%, p=0.195; respectively), while trisomy 15 was more common in the group with live births (18.33%, **p=0,041**) vs those without (8.39%) (Table 4). These findings were statistically significant for trisomies 16 and 15, but not for trisomy 21.

Chromosomal abnormalities and maternal blood groups/RhD status

The highest incidence of chromosomally abnormal POCs was detected in women with the AB blood group (63.89%), followed by A (62.84%), O (56.28%) and B (50.75%) (Table 2). The O blood group had the highest incidence of monosomies (14.56%) in contrast to the B with the lowest rate of monosomies in our study (8.82%) (p=0.312). The opposite was observed concerning the trisomies, where B had the highest, and O the lowest incidence (76.47% and 61.17%, respectively) (p=0.176).

Higher presence of trisomy 22 was observed among women of the AB blood group (43.75%), in comparison to women of other (combined B, A and O) blood groups (17.46%) (**p=0.012**). Furthermore, trisomy 21 was more frequently observed among women in the O blood group (9.52%; 6/63), compared to other groups (2.52%) (**p=0.034**) (Table 4).

Women with positive RhD positive (RhD+) had slightly higher incidence of abnormal POCs (59.51%) than the RhD negative (RhD-) women (52.54) (p=0.310).

Chromosomal abnormalities according to the gestational age

Overall, increased incidence of chromosomal abnormalities was present in advanced gestational age. The POCs eliminated in gestational week (gw) 10 showed the highest incidence of chromosomal abnormalities (66.67%), followed by gw 9 (63.08%), while the lowest rate was observed in the POCs eliminated in the gw 6 (52.78%).

Higher incidences of triploidy and monosomies were observed in POCs eliminated between gw 9 and gw 11 (p=0.135 and **p=0.0015**, respectively), while trisomies were more common among studied POCs from gw 6 to 8 (**p=0.0026**) (Table 2).

Chromosomal abnormalities and POCs sex

Among the 768 POCs, 379 were male sex (49.34%) and 389 were of female sex (50.65%). A slightly lower number of chromosomally abnormal male POCs was observed (53.56%) in comparison to the female POCs (58.86%). Triploidies were more common among male POCs in contrast to the females (25.56% vs. 13.75%, respectively; p=0.026). Chromosomal monosomies were more prevalent in female than in male POCs (28.12% vs. 1.7%; p<0.0001), but this was expected since monosomy X was predominant (95.55%) in this group, and there were only 2 cases with monosomy 21 (4.44%). Regarding the chromosomal trisomies, no significant difference was observed between males and females (Tables 2 and 4). In the group of RPL, a higher percentage of male POCs were euploid in comparison to the female POCs (50% vs. 39.85%), but the difference was not significant (p=0.1).

DISCUSSION

Foetal chromosomal abnormalities have been recognized as a major cause of EPLs [4]. Several methods have been used for the detection of chromosomal abnormalities with different detection rates [17-21]. Using a combination of QF-PCR and MLPA analyses, the chromosomal abnormality rate in our study was 56.25%, which agrees with the majority of previously published data. Chromosomal trisomies were the most frequent abnormalities (66.20%), followed by triploidy (15.28%) and monoso-

mies (10.88%). Multiple chromosomal aneuploidies and partial chromosomal abnormalities were also identified. Thus, our approach could detect most of the chromosomal abnormalities known to cause EPLs.

In comparison to the conventional cytogenetic methods, our approach does not require viable tissue material, lowering the rate of unsuccessful analyses and has a potential to detect maternal cell contamination. Indeed, in our study 14.67% of the POC samples showed maternal contamination and were excluded from further analysis. Furthermore, the use of QF-PCR enables not only detection of maternal cell contamination, but also detection of foetal triploidy, which could not be possible by using only MLPA analysis. Although QF-PCR/MLPA approach could not accurately detect other polyploidies, such as tetraploidy, these abnormalities rarely occur and represent only less than 3% of all chromosomal abnormalities in EPLs [19]. Some disadvantages of the QF-PCR and MLPA methods used in present study include the inability to detect balanced structural chromosomal rearrangements, as well as interstitial deletions and duplications, ring chromosomes, and inversions. Some of these variations could be detected with other molecular approaches such as aCGH and NGS-based methodologies, but because of their higher price, they are not widely used in the investigation of EPLs samples [5, 6].

In our study, a significantly higher frequency of chromosomally abnormal POCs was observed in the group of women ≥36 years (71.37%) compared to the groups ≤30 (43.4%; OR=3.25; **p<0.0001**) and 31-35 years (53.9%; OR=1.52, **p=0.018**). Thus, our study confirms previous observations of increased EPL aneuploidy rate in women with advanced age [13, 22]. Extensive research has been performed with the aim to explain the reason behind the increased rate of aneuploidy with advanced maternal age. Some of the leading causes identified include recombination failure, cohesion and spindle deregulation, abnormalities in post-translational modification of histones and tubulin, and mitochondrial dysfunction [23, 24].

The high trisomy rate in our study (66%), with trisomy 16 being the most frequent, followed by trisomies 22, 21, 13 and 15 is in accordance with the data from previous published studies [21, 25-27]. The trisomy rate increased with maternal age, being the highest (79.68%) in women ≥36 years of age and lowest in women ≤30 years (48.70%). On the other hand, our study showed higher rates of monosomies and triploidy in younger women, which has been also shown by other authors [26, 28].

The distribution of individual trisomies differed among the three age groups. The frequency of trisomy 16 (calculated on all studied samples) was similar in the three groups, while trisomies 22, 21, 15 as well as other rare trisomies showed the highest frequencies in the group ≥36 years (Table 5). Many previous studies have also shown that trisomies 21 and 22 are more common in women with advanced age and have shown that they occur mostly because of maternal non-disjunction in meiosis I. Both chromosomes 21 and 22 are acrocentric and, on average, each is held by a single chiasma, which can be commonly lost during meiosis [29]. Thus, our study confirms the findings that trisomies 22, 21 and 15 occur due to age-related factors, but also imply that other, non-age-related factors might be involved in the occurrence of trisomy 16.

Table 5. Distribution of the chromosomal trisomies among all studied EPLs

Trisomy	≤30 n (%)	31-35 n (%)	≥36 n (%)
16	22 (8.30)	29 (12.03)	25 (9.54)
22	0 (0.00)	10 (4.15)	35 (13.36)
21	7 (7.64)	9 (3.73)	13 (4.96)
15	0 (0.00)	4 (1.66)	22 (8.40)
13	7 (2.64)	7 (2.90)	6 (2.29)
14	7 (2.64)	4 (1.66)	5 (1.91)
Other	13 (4.91)	18 (7.47)	43 (16.41)
Total trisomy	56 (48.71)	81 (62.3)	149 (79.68)
Total POC	265	241	262

Our results favour the findings of lower chromosomal aneuploidy rate in recurrent compared to the sporadic EPLs [7], however with a similar distribution of the chromosomal abnormalities and trisomies 16 and 22 being the most common in both groups [30]. The differences observed among the women with different ethnic origins as well as those with and without live births could be related to the maternal age. Although, we have observed some differences in the chromosomal aneuploidy rates among women with different ABO blood groups and RhD status (with higher rates in groups A and AB, as well as in RhD positive women) further larger studies are required to confirm our findings.

Our results show that triploidy and monosomies occur more frequently in POCs eliminated in gw 9-11, while trisomies are more common in gw 6-8 POCs, findings observed also by other studies [31]. Thus, our study suggests that chromosomal trisomies have a more negative effect on POCs, causing earlier elimination during the pregnancy in contrast to the triploidies and monosomies.

We have observed a slight predominance of chromosomally abnormal POC in females than in males (58.86% vs. 53.56%), although without statistical significance. The female predominance was also observed by other authors indicating relative weakness in female embryo formation and development, supported by an animal model where, male embryos development was favoured in comparison to female development [32, 33].

CONCLUSION

Our study furthers the understanding regarding chromosomal abnormalities as a cause of EPLs and confirms the higher incidence of foetal chromosomal abnormalities in EPLs in women of older reproductive age. Furthermore, it shows that by using QF-PCR and MLPA methodologies, a high detection rate of chromosomal abnormalities in EPLs can be found.

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CONFLICT OF INTEREST

None declared.

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ORIGINAL ARTICLE

DROPLET DIGITAL PCR AS A MOLECULAR TOOL FOR THE DETECTION OF THE *EGFR* T790M MUTATION IN NSCLC PATIENTS WITH THE *EGFR* ACTIVATING MUTATIONS

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ABSTRACT

Background

Almost 50% of NSCLC patients who initially show a successful response to tyrosine kinase inhibitors targeted therapy (TKI therapy) eventually develop acquired *EGFR* T790M mutation. The T790M secondary mutation can cause resistance to the targeted therapy and disease relapse. Since this mutation can be present at very low frequencies in liquid biopsy samples, droplet digital PCR (ddPCR), due to its high sensitivity, has opened the possibility for minimally invasive monitoring of the disease during TKI targeted therapy.

Materials and methods

For this study, a total of 45 plasma samples from NSCLC patients with previously detected *EGFR*-activating mutations were analyzed. Extracted circulating free DNA was amplified and examined for the presence of T790M mutation using ddPCR technology. For the data analysis, QuantaSoft Software was used.

Results

Of 45 tested plasma samples, a total of 14 samples were identified as positive for the T790M mutation. The same samples eventually showed the presence of T790M mutation in FFPE. Droplet digital PCR showed its great advantage in high sensitivity detection of rare allele variants. Our ddPCR assay detected T790M mutant allele in frequencies from 0.1%. The average number of droplets generated by ddPCR was 9571.

Conclusion

Monitoring of the T790M mutation has an important role in the examination of the effects of the prescribed TKI therapy. Since monitoring of potential changes during TKI therapy requires repeated sampling, our results showed that ddPCR technology has made it possible to use liquid biopsy as an adequate minimally invasive alternative for single nucleotide polymorphisms (SNP) detection.

Keywords: ddPCR, liquid biopsy, NSCLC patients, T790M

INTRODUCTION

Lung cancer is one of the most commonly diagnosed cancers, while non-small cell lung cancers (NSCLC) account for 80-85% of all lung cancer cases[1,2]. Determining the presence of *EGFR* mutations in this type of lung cancer is an essential step, as it helps in selecting tyrosine kinase inhibitor therapy (TKI therapy). TKIs target the EGFR receptor in NSCLC patients and thus harbor activating *EGFR* mutations[3,4]. The presence of mutations in NSCLC is crucial for a patient's diagnosis, therapy assessment, and prognosis[5].

However, a significant number of patients who initially respond successfully to TKI therapy, eventually develop acquired resistance to EGFR-TKIs[6,7]. It has been found that specific secondary mutations occur and cause resistance to the applied therapy and lead to disease relapse[8,9]. The most frequent secondary mutation in NSCLC patients is a secondary mutation in exon 20, called the T790M mutation, which occurs in nearly 50% of cases where

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the disease recurs in patients who have undergone TKI therapy[8]. As a result, third-generation EGFR-TKIs have been developed to target resistance mutation by blocking T790M mutant *EGFR* irreversibly[10].

Since secondary *EGFR* mutations, such as T790M, cause resistance to the prescribed TKI therapy, monitoring potential changes after the administration of therapy is crucial. This approach implies multiple sampling, and therefore the standard method of tissue biopsy, which is invasive, can make it difficult to resample regularly. To overcome this, liquid biopsy, which is minimally invasive, is becoming a more frequent method in clinical diagnostics[11]. It is also a suitable sampling method for patients in whom tumor formations have developed in inaccessible locations in the body or for those who may be at risk of some health complications in the event of a tissue biopsy[12].

Droplet digital PCR has several unique advantages, especially regarding rare mutation detection and precise DNA quantification[5]. Droplet digital PCR utilizes water-oil emulsion technology to partition each sample into 20 000 nanoliter-sized droplets, where target and background DNA are distributed randomly into the droplets. When properly designed, ddPCR can detect 1 mutant in 10 000 wild-type alleles[13]. Using ddPCR to test for mutation T790M from plasma samples, could be a promising approach for treating NSCLC patients.

MATERIALS AND METHODS

For this study, a total of 45 blood samples were collected from NSCLC patients who had previously tested positive for *EGFR*-activating mutations. The samples were collected at the Alea Genetic Center in Sarajevo. The selection of blood samples was based on identified *EGFR* mutations from lung adenocarcinoma tissue samples, and during this study they were tested for the presence of T790M mutation. The Alea Genetic Center did not perform the analysis for *EGFR*-activating mutations, and patients were selected according to their pathohistological and molecular testing results from different institutions. In this study, we concentrated solely on T790M mutation, activating *EGFR* mutations were determined by laboratories that tested the tissue samples.

Blood plasma was separated from the blood, with 1-4 ml taken from each sample and stored at a temperature of -20˚C. The circulating free DNA (cfDNA) was extracted from these plasma samples using QIAamp® Circulating Nucleic Acid Kit, following the manufacturer's instructions[14]. To process plasma samples faster and more efficiently, the QIAvac 24 Plus with the QIAvac Connecting System and QIAGEN Vacuum Pump was used according to the manufacturer's instructions[15]. Extracted cfDNA was quantified using Qubit 3 Fluorometer® and Qubit® dsDNA HS assay kit[16]. Starting DNA concentration for ddPCR reaction was 15 ng for every sample. Using Bio-Rad QX200 Droplet Digital PCR technology, all 45 samples were tested for a specific *EGFR* T790M mutation and its corresponding wild-type amplicons. The commercial assay used for T790M mutation and wild-type amplicon detection was PrimePCR™ ddPCR™ Mutation Detection Assay Kit: *EGFR* WT for p.T790M, and *EGFR* p.T790M. The multiplex assay was 20X concentrated and ddPCR supermix for probes was 2X concentrated. In order to portion each sample into droplets, they were placed into a QX200 droplet generator. Droplets were then transferred to a 96-well plate and placed into Bio-Rad T100 thermal cycler. The amplification was performed according to the manufacturer's instructions[17]. After DNA amplification, the droplet-containing plate was positioned in the QX200 droplet reader, allowing for the analysis of each droplet individually through a two-color system (FAM and HEX/VIC)[13]. For the analysis of the results, QuantaSoft Software was used.

When processing the results, wild-type and mutant T790M alleles were expressed as a number of FAM (mutant) and HEX (wild-type) droplets. The frequency of mutant alleles was determined by calculating the ratio of mutant droplets to the sum of mutant and wild-type droplets. Since ddPCR enables absolute quantification, mutant cfDNA templates were expressed as a number of copies per µl of tested DNA. The ddPCR threshold for positive mutant alleles was set at two or more positive droplets considering the added amount of DNA and generated number of droplets[18,19].

RESULTS

A total of 45 plasma samples were enrolled in this study. All selected plasma samples from NSCLC patients had previously shown the presence of *EGFR*-activating mutations on FFPE samples, in the following order: del19 (66.66%), L858R (17.77%), G719X (8.88%) and ins20 (4.44%). Out of 45 FFPE samples, one sample was invalidated for molecular testing for *EGFR*-activating mutations and only the patient's plasma sample was enrolled in the study.

Out of 45 samples, 14 samples were identified as positive for the T790M mutation, while 31 samples did not show the presence of the T790M mutant allele variant. The same 14 samples eventually showed the presence of T790M mutation in FFPE, while 31 tissue samples were identified as negative. In comparison to tumor tissue results, the sensitivity and specificity for *EGFR* T790M mutation in plasma samples were 100%. Our ddPCR as-

Table 1. Plasma samples that showed the presence of the T790M mutant allele variant

SAMPLE NUMBER	NUMBER OF DROPLETS	WILD TYPE/MUTANT T790M	T790M MUTANT ALLELE FREQUENCY (%)	MUTANT CONC (COPIES/ML)
3	6538	61/3	5	0.9
4	10038	7671/1634	12.3	209
6	8924	1534/8	0.5	0.8
8	12111	725/5	0.7	0.49
15	10891	480/4	0.8	0.43
18	8670	77/3	3.8	3
19	8015	210/7	3.3	1
20	12711	1428/2	0.1	0.28
34	7284	494/6	1.2	1
35	5775	577/25	4.1	5.1
36	14326	770/7	0.9	0.57
43	12788	632/16	2.5	1.5
44	12668	612/7	1.1	0.65
45	12789	536/18	3.3	1.7

say detected T790M mutant allele in frequencies ranging from 0.1%. The number of mutant molecules per µL of tested cfDNA ranged from 0.28 to 209 as depicted in Table 1. The average number of droplets generated by ddPCR was 9571.

As can be noticed from Table 1, plasma sample number 4 had an excessive amount of T790M mutant alleles. It is worth noting that the amount of tissue material from this patient was insufficient for valid results. Therefore, the result obtained from this tissue sample was reported as an invalid result and was not considered in the comparison between plasma and tissue samples. In the patient's plasma sample, 10 038 droplets were generated and the ratio of the number of droplets containing mutated gene variants to the total number of generated droplets was 12.3% (Figure 1).

However, in plasma sample 38, which was reported negative for the T790M mutation, one highly differentiated positive droplet was observed. For this sample, a small number of amplified copies were detected. To confirm the result, this plasma sample was run and analyzed twice, obtaining identical results, which indicates the accuracy of the procedure and the validity of the results. Although insufficient to be declared as positive, this result should not be ignored, and it indicates the need for liquid rebiopsy to determine the exact mutational status of the patient. This patient should be monitored for early detection of the T790M to adjust therapy if needed. Out of 14 plasma *EGFR* T790M positive samples, detected *EGFR*-activating mutations on FFPE samples were as follows: del19 (76.92%), L858R (15.38%), and G719X (7.69%).

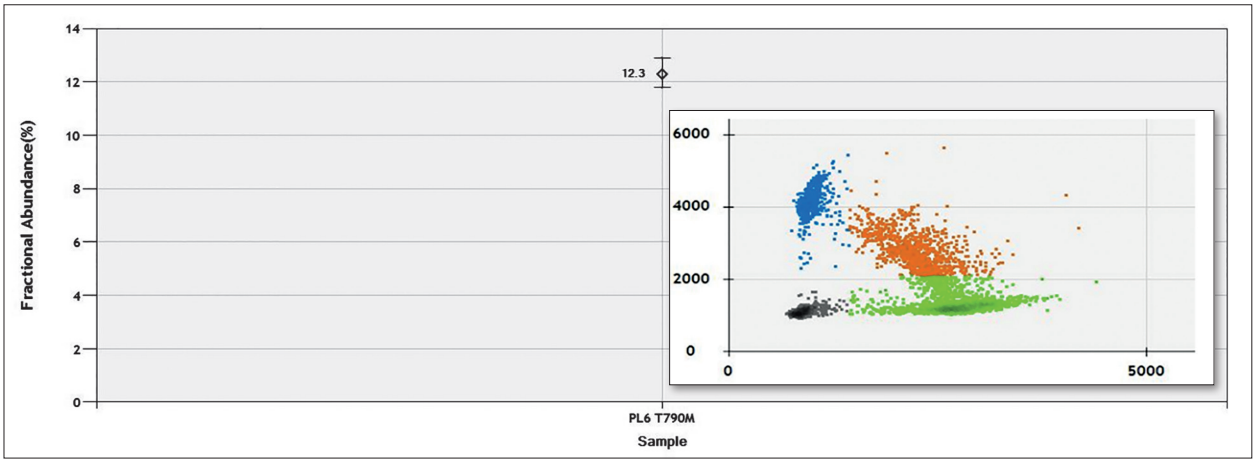

Figure 1. Sample number 4 with 12.3% mutant allele frequency

DISCUSSION

The main objective of this type of study is to improve the approach to treating oncology patients by providing individualized and accessible therapy. When one considers that around 50% of NSCLC patients experience disease relapse during TKI-targeted therapy and eventually acquire the T790M mutation, it becomes crucial to quickly discover molecular resistance mechanisms[20]. In current practice, the preferred method for monitoring resistance mechanisms is to perform a tissue re-biopsy. However, with the development of highly sensitive and reliable molecular technologies, such as ddPCR, analysis can be performed from other, minimally invasive types of samples[21].

Our study indicates that the sampling of patients can be significantly facilitated in order to monitor T790M status. Some studies estimate that up to 40% of relapsed NSCLC cases, cannot undergo molecular analysis due to issues with tumor tissue biopsies[22,23]. Two main factors contribute to this problem. Firstly, it is often challenging to obtain sufficient tissue material. As a result, clinicians use a well-known phrase "Tissue is an issue", since the patient's diagnosis is practically based on small amounts of tissue material. Secondly, tissue samples from NSCLC patients can be difficult to obtain since the lesions frequently develop in inaccessible locations[22]. For these patients, a more acceptable type of sample would be liquid biopsy, as an alternative to tissue re-biopsy. Although liquid biopsy generally contains a low concentration of circulating tumor biomarkers, highly sensitive and precise technologies such as ddPCR technology can overcome this drawback[25].

With its great sensitivity, ddPCR can accurately identify a mutant allele present at low frequency in a wild-type background. Since this technology has an advantage in mutation detection at low frequency, ddPCR has enabled more significant implementation of liquid biopsy in molecular diagnostics with the aim of early diagnosis of tumors, minimally invasive monitoring of disease, and therapy response assessment[25]. In this way, the sampling of patients is significantly facilitated and enables regular monitoring of the patient's healthcare condition.

In 2019, Salihefendić et al.[5] studied whether ddPCR technology could be used as a confirmatory method for the detection and quantification of somatic mutations, previously detected by NGS. A total of 35 samples from CRC and NSCLC patients were analyzed, and the results showed that there were no statistically significant differences between the results obtained by NGS and ddPCR methods. Therefore, high sensitivity and resolution of ddPCR, make it an adequate method for validating low-frequency somatic mutations[5].

In research published in 2016, Zheng et al.[27] reported that monitoring the status of cfDNA from plasma in NSCLC patients treated with TKI therapy can enable the detection of the acquired T790M mutation up to 6.8 months before the clinical progression of the patient's condition. In their research, almost half of the detected T790M positive patients were detected from plasma samples before disease progression (45.7%), and the time of detection varied from 0.8 to 6.8 months before clinical progression[27]. For patients with detected *EGFR* mutation who receive TKI therapy, regular analysis of the *EGFR* gene during treatment is significant in order to detect the T790M or other secondary mutations and to change the therapy.

CONCLUSION

Monitoring the T790M mutation has an important role in the examination of the effects of the prescribed TKI therapy. This approach implies multiple tissue biopsy sampling, which, as an invasive sampling method, has its limitations and risks. Our results showed that ddPCR technology has made it possible to use liquid biopsy as an adequate minimally invasive alternative for the detection of rare allele variants. Plasma ddPCR-based genotyping can be significant in the detection of specific mutations and patient monitoring. Using plasma as a sample for the detection of *EGFR* mutations by highly sensitive methods such as ddPCR can enable early detection of the T790M mutation, but also other inhibiting and activating mutations.

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CONFLICTS OF INTEREST:

There are no conflicts of interest.

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ORIGINAL ARTICLE

ASSOCIATION BETWEEN THE POLYMORPHISM OF ANGIOTENSIN-CONVERTING ENZYME GENE AND INTERLEUKIN-1 BETA GENE AND THE RESPONSE TO ERYTHROPOIETIN THERAPY IN DIALYSIS PATIENTS WITH ANEMIA

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ABSTRACT

Introduction

The polymorphism of the angiotensin-converting enzyme (ACE) gene and interleukin-1 beta (IL-1b) gene could be associated with resistance in the treatment of anemia in dialysis patients with recombinant human erythropoietin (rHuEPO). The aim of the study was to evaluate the association between the polymorphism of the ACE and IL-1b genes and the response to rHuEPO therapy in dialysis patients with anemia.

Material and methods

The study investigated 69 patients on dialysis with anemia treated with recombinant human erythropoietin for 12 months. Genotyping of ACE and IL-1b polymorphism was done in all study patients at the initiation of the study. The patient's demographic characteristics, dialysis vintage, and laboratory parameters were also evaluated as factors associated with rHuEPO resistance. The erythropoietin resistance index (ERI) was calculated as the weekly rHuEPO dose per kg of body weight, divided by the hemoglobin (Hb) concentration in g/dl.

Results

The Hb ≥ 110 g/l was registered in 37 (53.6%) patients. Patients with Hb ≥ 110 g/l were characterized by significantly higher serum levels of albumin, cholesterol, and iron than those with Hb < 110 g/l. The serum level of the CRP, the weekly dose of rHuEPO, and ERI were significantly higher in patients with Hb < 110 g/l compared to patients with Hb ≥ 110 g/l. The ERI value of ≥ 10 IUkg/weekly/g/dl was present in 27 (39.1%) patients. The serum levels of ferritin and CRP, and weekly dose of rHuEPO were significantly higher in patients with ERI value ≥ 10 IU kg/weekly/g/dl compared with the patients with ERI value < 10 IUkg/weekly/g/dl. There was no significant association between the ERI and polymorphism of the ACE and IL-1b genes in study patients.

Conclusion

The polymorphism of the ACE and IL-1b genes was not significantly associated with the response to erythropoietin therapy in dialysis patients with anemia. Iron deficiency, malnutrition, and inflammation were factors associated with anemia and resistance to erythropoietin therapy in dialysis patients.

***Keywords*:** anemia, dialysis, erythropoietin, gene, polymorphism, therapy

INTRODUCTION

Recombinant human erythropoietin (rHuEPO) has been used as a treatment for anemia in patients with chronic kidney disease for more than 30 years. Chronic kidney disease is characterized by decreased secretion of endogenous erythropoietin from kidneys (1-3). The treatment of anemia with rHuEPO in dialysis patients lowers the blood transfusions, increases the patient's quality of life, and reduces the risk of cardiovascular morbidity and mortality (4-7). Based on current recommendations, the treatment of anemia with rHuEPO in patients on hemodialysis begins when the value of hemoglobin is lower than 100 g/l (6). On average, 85% of dialysis patients receive rHuEPO for

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correction of anemia to achieve a target value of hemoglobin up to 110-120 g/l (8, 9). The initial dose of rHuEPO is 50-100 IU/kg body weight/three times per week, with subcutaneous or intravenous administration.

Resistance or reduced response to treatment with rHuEPO in dialysis patients might be caused by several factors, such as iron deficiency, vitamin B12/folic acid deficiency, hypothyroidism, infection-inflammation, inadequate dialysis, hyperparathyroidism, malnutrition, bleeding, and malignancy. Irreversible factors are hemoglobinopathies and bone marrow diseases (8). The erythropoiesis resistance index (ERI) evaluates the rHuEPO responsiveness and is calculated as the weekly rHuEPO dose per kg of body weight, divided by the hemoglobin (Hb) concentration in g/dl (10). Resistance to human erythropoietin is encountered in 5-10% of patients on hemodialysis (11).

The polymorphism of certain genes could be associated with erythropoietin resistance during the treatment of anemia in patients on dialysis (12, 13). Every polymorphism has a rsID number ("rs" followed by a number), a unique label used by researchers and databases to identify a specific polymorphism. Polymorphism (rs1799752) of the angiotensin-converting enzyme (ACE) gene is characterized by the presence (insertion, I) or absence (deletion, D) of a 287-bp sequence of DNA (ACE I/D) in intron 16 of the ACE gene located on the chromosome 17 (17q23) (14). The angiotensin-converting enzyme is a key enzyme in the creation of angiotensin II. Additionally, angiotensin II stimulates the proliferation of erythroid precursors, which is proven by in vitro models, i.e. it affects erythropoiesis (15, 16). The genetic cluster for interleukin 1 (IL-1), located on chromosome 2 (2q14.1), is presented with IL-1a, IL-1b, and IL-1RN genes that provide genetic information on the synthesis of cytokines IL-1alpha, IL-1beta, and endogenous receptor antagonist IL-1 (17). IL-1beta suppresses the endogenous secretion of erythropoietin (18). The polymorphism of the IL-1b gene is IL-1B-511 C/T (rs 1143627) (19).

AIM OF THE STUDY

Our study aimed to evaluate the association between the polymorphism of the ACE and IL-1b genes and the response to erythropoietin therapy in dialysis patients with anemia.

PATIENTS AND METHODS

The study included 69 patients with stage 5 chronic kidney disease on maintenance hemodialysis or peritoneal dialysis. All patients signed an informed consent for participation in the study. The study was approved by the ethical commission of the Faculty of Medicine, Ss. Cyril and Methodius University in Skopje, Skopje, RN Macedonia. The design of the study was a prospective, longitudinal study, with a duration of 12 months.

Inclusive criteria for study patients:
- older than 18 years,
- treatment with dialysis for at least 3 months,
- treatment of anemia with recombinant human erythropoietin.

Exclusion criteria for study patients:
- bleeding diagnosed before involvement in the study,
- the persistence of malignant disease,
- hemoglobinopathies and diseases of the bone marrow.

The patients were recruited from the hemodialysis unit and peritoneal dialysis unit at the University Hospital of Nephrology in Skopje and the Department of Nephrology and Dialysis at the General City Hospital "8^{mi} Septemvri" in Skopje. The medical histories of the patients were used to determine demographic characteristics, etiology of kidney disease, dialysis vintage, and total weekly dose of erythropoietin. The laboratory data were obtained from the routine laboratory analyses of dialysis patients during the study period of 12 months. The total red blood cell count, hematocrit, hemoglobin (Hb), total protein, albumin, alkaline phosphatase, calcium, phosphorus, C-reactive protein (CRP), iron, and total iron binding capacity (TIBC) were analyzed monthly. The transferrin saturation index (TSAI) was calculated using the following equation: (serum Fe/TIBC) x 100% (20). The serum concentration of ferritin was determined once in three months, with a target value of more than 500 ng/ml, but not exceeding 800 ng/ml (20). The serum concentration of intact parathyroid hormone (iPTH) and cholesterol was determined once in six months. The erythropoietin resistance index (ERI) was calculated monthly as the weekly rHuEPO dose per kg of body weight, divided by the hemoglobin concentration in g/dl (10).

Genotyping of ACE and IL-1b polymorphism was done in all study patients at the initiation of the study in the Center for Biomolecular Pharmaceutical Analysis at the Institute of Pharmaceutical Chemistry at the Faculty of Pharmacy in Skopje. Genomic DNA from all study participants was isolated from peripheral blood using the MagCore Genomic DNA Whole Blood Kit (RBC Bioscience), following the manufacturer's instructions. The ACE polymorphism (rs1799752) was genotyped by

fluorescent PCR followed by fragment analysis on 3500 Automated Genetic Analyzer (Thermo Fisher Scientific), using the following primers: ACE_I/D_F:5'-CTGGAGACCACTCCCATCCTTTCT-3' and ACE_I/D_R: 6FAM-5'-GATGTGGCCATCACATTCGTCAGAT-3'. A total of 100 ng of DNA was amplified in 25μL final volume including 2 mM Mg^{2+}, 0.2 mM of each dNTPs, 0.5μM of each primer and 1U HOT FIREPol® DNA Polymerase (Solis Bio-Dyne), using the following program: initial denaturation at 95°C for 10 minutes; 35 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C; and final elongation at 72°C for 10 minutes. The IL-1b gene (rs1143627) polymorphism was genotyped by allele discrimination PCR on a Stratagene Mx3005P (Agilent Technologies) real-time PCR system using TaqMan® SNP genotyping assay (reference ID: C___1839944_10; Thermo Fisher Scientific). The genotypes were determined in a reaction mix containing 20 ng DNA in a total volume of 25 μL, according to the manufacturer's recommended protocol. Positive and negative controls were included on each plate and reproducibility was checked by re-genotyping 10% of the cases.

STATISTICAL ANALYSIS

The statistical analysis of the data was performed by using SPSS 17. The parametric variables were presented as an average value ± standard deviation (X ± SD). The parametric variables between two groups were compared with Student's t-test for independent samples, and between three different groups with the ANOVA test. Fisher's exact test was used to compare proportions. The obtained P value less than or equal to 0.05 was considered statistically significant.

RESULTS

The study included 69 patients, 63 patients on maintenance hemodialysis, and 6 patients on peritoneal dialysis. Among the patients, 48 (69.5%) were male, and 21 (30.5%) were female. The etiology of kidney disease in most of the patients was chronic glomerulopathy (n = 21, 30.5%). The other etiological causes for kidney disease were: obstructive nephropathy (n = 16, 23.2%), nephroarteriolosclerosis (n = 14, 20.3%), autosomal dominant polycystic kidney disease (n = 7, 10.1%), and unknown cause (n = 11, 15.9%). Demographic characteristics, dialysis vintage, laboratory parameters, and erythropoietin resistance index (ERI) of all study patients are presented in Table 1.

The mean hemoglobin value in all patients was 109.4 ± 11.1 g/l, achieved by the application of an average of 6909 IU of erythropoietin per week. The mean value of TSAI (%) was 29.4 ± 7.6%, and the mean value of the ferritin was 468.8 ± 343.1 μg/l. The mean value of calculated erythropoietin resistance index (ERI) was 9.6 ± 6.2 IU kg/week/dl, Table 1.

Table 1. Demographic characteristics, dialysis vintage, laboratory parameters, and erythropoietin resistance index (ERI) of all study patients.

	X	SD
Age (years)	62.1	16.3
Weight (kg)	72.3	16.0
Dialysis vintage (months)	94.8	88.7
Hemoglobin (g/l)	109.4	11.1
Red blood cell count (10^{12}/l)	3.6	0.4
Hematocrit (rv)	0.34	0.04
Alkaline phosphatase (U/L)	102.3	58.3
Total protein (g/l)	69.5	5.2
Albumin (g/l)	40.2	4.2
Calcium (mmol/l)	2.3	0.2
Phosphates (mmol/l)	1.6	0.5
Iron (μmol/l)	10.9	2.9
TIBC (μmol/l) *	37.6	8.2
TSAI (%)**	29.4	7.6
CRP (mg/l) ***	12.1	14.2
Ferritin (ng/ml)	468.8	343.1
iPTH (pg/ml)****	499.3	490.3
Erythropoietin (IU/per week)	6909.6	3465.7
ERI (IU kg/week/g/dl)*****	9.6	6.2

*Total iron binding capacity (TIBC),
**Transferrin Saturation Index (TSAI),
***C reactive protein (CRP),
**** Intact parathyroid hormone (iPTH),
***** Erythropoietin resistance index (ERI)

The concentration of hemoglobin ≥ 110 g/l was registered in 37 (53.6%) patients, Table 2. Patients with hemoglobin ≥ 110 g/l were characterized with significantly higher serum levels of albumin, cholesterol, and iron compared to the patients with hemoglobin < 110 g/l. The serum levels of the CRP, the weekly dose of rHuEPO, and ERI were significantly higher in patients with hemoglobin < 110 g/l compared to patients with hemoglobin ≥ 110 g/l, Table 2.

The ERI ≥ 10 IU/kg/week/g/dl was detected in 27 (39.1%) patients. Patients with ERI ≥10 IU/kg/week/g/dl had significantly lower levels of hemoglobin, cholesterol, iron, and TSAI% compared to patients with ERI< 10 IU/kg/week/g/dl. The serum levels of ferritin and CRP, and a weekly dose of erythropoietin were significantly higher in patients with ERI ≥ 10 IU/kg/week/g/dl compared to patients with ERI <10 IU/kg/week/g/dl, Table 3.

Table 2. Comparison of demographic characteristics, dialysis vintage, laboratory parameters, and erythropoietin resistance index (ERI) between patients with hemoglobin value <110 g/l and ≥ 110 g/l.

	Hb < 110 g/l, (N=32)	**Hb ≥ 110 g/l, (N=37)**	**P**
	X ± SD	**X ± SD**	
Age (years)	58.6 ± 18.3	65.1 ± 138	0.099
Weight (kg)	70.8 ± 16.9	73.5 ± 15.3	0.486
Dialysis vintage (months)	84.0 ± 79.7	104.1 ± 95.9	0.351
Hemoglobin (g/l)	99.9 ± 9.2	116.9 ± 4.8	0.000
Red blood cell count (10^{12}/l)	3.3 ± 0.4	38. ± 0.2	0.000
Hematocrit (rv)	0.31 ± 0.03	0.36 ± 0.02	0.000
Alkaline phosphatase (U/L)	114.4 ± 96.5	103.1 ± 53.1	0.539
Total protein (g/l)	68.1 ± 6.5	70.3 ± 3.8	0.086
Albumin (g/l)	38.6 ± 5.9	40.8 ± 2.7	0.044
Calcium (mmol/l)	2.3 ± 0.2	2.4 ± 0.3	0.282
Phosphates (mmol/l)	1.6 ± 0.5	1.6 ± 0.5	0.494
Cholesterol (mg/dl)	3.5 ± 0.8	4.1 ± 0.8	0.005
Iron (µmol/l)	9.7 ± 3.0	11.7 ± 2.6	0.005
TIBC (µmol/l) *	35.6 ± 9.4	38.1± 7.6	0.229
TSAI (%)**	27.8 ± 7.9	31.4 ± 7.2	0.051
Ferritin (ng/ml)	566.6 ± 529.5	437.0 ± 299.7	0.210
iPTH (pg/ml) ***	560.7 ± 550.5	442.9 ± 410.8	0.317
CRP(mg/l) ****	21.8 ± 34.3	8.3 ± 9.9	0.025
Erythropoietin (IU/per week)	8714.6 ± 2982.4	5234.6 ± 2836.1	0.000
ERI (IU kg/week/g/dl)*****	13.5 ± 6.1	6.2 ± 3.4	0.000

*Total iron binding capacity (TIBC), **Transferrin Saturation Index (TSAI), ***Intact parathyroid hormone (iPTH),
**** C reactive protein (CRP), ***** Erythropoietin resistance index (ERI).

Table 3. Comparison of demographic characteristics, dialysis vintage, and laboratory parameters between patients with ERI ≥ 10 and ERI < 10 IU/kg/week/g/dl.

	ERI ≥ 10, (N =27)	**ERI < 10, (N = 42)**	**P**
	X ± SD	**X ± SD**	
Age (years)	59.1 ± 19.7	63.9 ± 13.5	0.234
Weight (kg)	68.6 ± 14.1	74.6 ± 16.9	0.131
Dialysis vintage (months)	70.6 ± 56.4	110.3 ± 102.0	0.070
Hemoglobin (g/l)	100.6 ± 11.4	114.4 ± 6.9	0.000
Red blood cell count (10^{12}/l)	3.4 ± 0.4	3.7 ± 0.3	0.000
Hematocrit (rv)	0.32 ± 0.04	0.35 ± 0.03	0.000
Alkaline phosphatase (U/L)	106.6 ± 66.2	109.5 ± 82.4	0.879
Total protein (g/l)	69.1 ± 5.8	69.5 ± 4.9	0.762
Albumin (g/l)	39.5 ± 4.9	40.0 ±4.37	0.639
Calcium (mmol/l)	2.3 ± 0.3	2.3 ± 0.2	0.672
Phosphates (mmol/l)	1.6 ± 0.5	1.6 ± 0.5	0.940
Cholesterol (mg/dl)	3.5 ± 0.7	3.9 ± 0.9	0.045
Iron (µmol/l)	9.2 ± 2.7	11.8 ± 2.6	0.000
TIBC (µmol/l) *	36.3 ± 9.3	37.4 ± 8.0	0.609
TSAI (%)**	26.4 ± 7.5	31.8 ± 7.1	0.003
Ferritin (ng/ml)	630.4 ± 555.4	412.3 ± 290.4	0.038
CRP(mg/l)***	24.2 ± 36.8	8.5 ± 9.9	0.010
iPTH (pg/ml)****	518.5 ± 511.6	483.1 ± 464.3	0.770
Erythropoietin (IU/per week)	9392.1 ± 2496.0	5213.4 ± 2811.9	0.000

* Total iron binding capacity (TIBC), **Transferrin Saturation Index (TSAI), ***C reactive protein (CRP),
**** Intact parathyroid hormone (iPTH), ***** Erythropoietin resistance index (ERI).

Table 4. The frequency of the ACE I/D genotypes (rs1799752) in all patients, and the mean value of ERI in the patients with different genotypes of ACE I/D.

ACE I/D* rs1799752			**ERI**
	Number of patients	%	X ± SD
II	10	14.5	11.87 ± 6.56 [a, b]
ID	41	59.4	8.42 ± 5.84 [a, c]
DD	18	26.1	10.92 ± 6.56 [b, c]

*Angiotensin-converting enzyme gene with insertion (I) or deletion (D) of a 287-bp sequence of DNA,
[a] P=0.237, [b] P=0.914, [c] P=0.307

Table 5. The frequency of genotypes of the IL-1B 511 C/T (rs1143627) in all patients and the mean value of ERI in patients with different genotypes of IL-1B 511 C/T.

IL-1B 511 C/T* rs1143627			**ERI**
	Number of patients	%	X ± SD
CC	7	10.1	8.34 ± 3.64 [a, b]
CT	35	50.7	9.83 ± 6.75 [a, c]
TT	27	39.1	9.56 ± 5.68 [b, c]

*Polymorphism of IL-1b gene is IL-1B 511 C/T with bases cytosine (**C**)/ thymine (**T**)
[a] P=0.826, [b] P=0.886, [c] P=0.983

The polymorphism of the ACE gene (ACE I/D, rs1799752) is presented by three genotypes: ACE II, ACE ID, and ACE DD. The ACE ID genotype was with the highest frequency, at 59.4%, detected in 41 patients. There was no significant difference in ERI between the three genotypes of the ACE I/D, Table 4.

The polymorphism of the IL-1b gene (IL-1B-511 C/T, rs1143627) is presented with three different genotypes: IL-1B CC, IL-1B CT, and IL-1B TT. The IL-1B CT genotype was with the highest frequency of 50.7%, detected in 35 patients. There was no significant difference in ERI between the three genotypes of the IL-1B-511 C/T, Table 5.

DISCUSSION

The study included patients with stage 5 chronic kidney disease on dialysis with anemia treated with recombinant human erythropoietin. The mean hemoglobin level of 109.4 ± 11.1 g/l was achieved in study patients with an average dose of erythropoietin of 6909 IU per week. The target value of hemoglobin in dialysis patients is 110-120 g/l (8,9). The mean level of TSAI (%) in the study patients was 29.4 ± 7.6%, and the mean level of ferritin was 468.8 ± 343.1 μg/l. KDIGO (Kidney Disease: Improving Global Outcomes) guidelines for anemia in chronic kidney disease recommend not exceeding a TSAI of 30% and a serum ferritin level of 800 ng/ml (20). The mean value of iPTH in study patients was 499.3 ± 490.3 pg/ml. KDIGO guidelines recommended maintaining iPTH levels 2- to 9-fold the upper normal limit, corresponding to a range of 130–600 pg/mL (21). The mean value of the ERI was 9.6 ± 6.2 IU/kg/week/g/dl. In the study of Santos EJF et al. with a total number of 99 patients with anemia treated with erythropoietin, the mean value of ERI was 15.3 ± 9.0 IU/kg/week/g/dl (23). The mean ERI value of 7.0 ± 4.4 IU/kg/week/g/dl was noted in 60 patients on peritoneal dialysis in the study performed by Kaneko S et al. (24).

A hemoglobin level above 110 g/l with erythropoietin therapy was achieved in 53.6% of the study patients. Patients with hemoglobin ≥ 110 g/l were characterized by significantly higher serum values of albumin, cholesterol, and iron compared to patients with hemoglobin <110 g/l. The serum values of CRP, weekly dose of rHuEPO, and ERI were significantly higher in patients with hemoglobin < 110g/l, compared to patients with hemoglobin ≥ 110g/l. Malnutrition and inflammation in dialysis patients were associated with anemia and resistance to erythropoietin therapy (25, 26). Radić J et al. studied 101 patients on peritoneal dialysis, divided into two groups, a group of 60 patients (59.4%) with hemoglobin ≥ 110 g/l and a group of 41 patients (40.6%) with hemoglobin<110 g/l. The serum value of the albumin was significantly higher in the group of patients with the level of hemoglobin ≥ 110 g/l compared to the group of patients with the level of hemoglobin <110 g/l, (44.2 ± 8.5 *vs.* 39.9 ± 8.5, P = 0.003). Also, the serum value of CRP was significantly higher in the group with hemoglobin <110 g/l compared to the group with hemoglobin ≥ 110 g/l, (7.8 ± 7.9 *vs.* 3.5 ± 6.3, P = 0.005) (26).

Patients involved in the study with an ERI ≥10 IU/kg/week/g/dl had significantly lower serum values of iron and TSAI% and significantly higher serum values of ferritin and CRP compared to patients with an ERI <10 IU/kg/g/dl. Iron deficiency and inflammation were the most common causes of reduced erythropoietin response during the treatment of anemia in dialysis patients (27). Ferritin and CRP are well-known acute-phase proteins of inflammation (28,29).

The frequency of polymorphism of the ACE I/D gene in the study patients was: 14,5% with ACE II, 59,4% with ACE I/D, and 26,1% with ACE DD. A study by Jeong KH et al. included 167 patients on hemodialysis, with a similar frequency of polymorphism of the ACE I/D gene: 25.1% with ACE II, 54.5% with ACE I/D, and 20.4% with ACE DD (19). The frequency of polymorphism of the IL-1B C/T gene in the study patients was 10.1% with IL-1B CC, 50.7% with IL-1B CT, and 39.1% with IL-1B TT. The distributions of IL-1B C/T polymorphism in the study of Jeong KH and al. with 167 hemodialysis patients was 21.6% with IL-1B CC, 43.1% with IL-1B CT, and 35.3% with IL-1B TT (19). In the same study, the ACE DD and IL-1B CC genotypes were associated with significantly lower values of ERI compared to other genotypes (ACE II: 13.2 ± 5.5 *vs.* ACE I/D: 13.9 ± 7.6 *vs.* ACE DD: 10.0 ± 5.1, P = 0.038, and IL-1B CC: 9.6 ± 5.1 *vs*. IL-1B CT: 15.2 ± 7.5 vs. IL-1B TT: 12.2 ± 5.7, P = 0.004) (19). The association of ACE DD with lower ERI value was also confirmed in a group of 50 patients with chronic kidney disease and anemia in a study by Nand N et al. (13). The same study did not confirm the association of polymorphism of the IL-1b gene with ERI (13). A study by Varagunam M et al. included 46 patients on peritoneal dialysis with anemia, treated with erythropoietin, and showed that genotype ACE DD was associated with lower total weekly doses of erythropoietin compared to genotypes ACE II and ACE I/D (22). Kiss Z et al. evaluated 660 hemodialysis patients with anemia treated with erythropoietin and the patients with ACE DD genotype had significantly higher ERI compared to the patients with ACE II (P = 0.046) (30). In our study, there was no significant association of polymorphism of the ACE and IL-1b genes with rHuEPO responsiveness in dialysis patients. Several published studies did not find a significant association between genetic polymorphism and erythropoietin treatment response in dialysis patients. The pro-inflammatory cytokine polymorphism was not associated with rHuEPO responsiveness in a study with 112 patients on peritoneal dialysis (12). Hatano M et al. did not find a significant association between the ACE polymorphism and the rHuEPO dose in 91 hemodialysis patients (31). The polymorphism of the ACE was genotyped in 70 Iraqi patients on hemodialysis and there was no significant effect of polymorphism on hemoglobin levels (32).

Our study has several limitations: the small sample size, no measurement of serum angiotensin II levels, and erythropoietin levels in studied patients, which might be a possible explanation for rHuEPO responsiveness. Many other ACE gene polymorphism could affect the response to rHuEPO as rs4343, rs429, and rs4341, which may be in linkage disequilibrium with studied rs1799752.

In conclusion: iron deficiency, inflammation, malnutrition, and hyperparathyroidism are factors associated with anemia and resistance to erythropoietin therapy in dialysis patients. The genetic polymorphism have been identified as possible causes of resistance to erythropoietin in dialysis patients. Studies with a larger sample size should be performed to confirm the association of polymorphism with erythropoietin responsiveness.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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ORIGINAL ARTICLE

CO-EXISTENCE OF *CYP2C19**1/*2 AND *ABCB1c*.3435 CT GENOTYPE HAS A POTENTIAL IMPACT ON CLINICAL OUTCOME IN CAD PATIENTS TREATED WITH CLOPIDOGREL

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ABSTRACT

Clopidogrel, is a standard treatment in the prevention of major adverse cardiovascular events (MACE) in patients with coronary artery disease (CAD). Clopidogrel response is highly variable, mainly due to the presence of polymorphisms in the genes involved in drug metabolism. The aim of this study was to evaluate the association between the presence of the *ABCB1* C3435T and *CYP2C19**2 polymorphism and the clinical outcome in patients with CAD treated with clopidogrel. A total of 96 patients with CAD were included in the study. Genomic DNA from peripheral blood was extracted from all patients with standard phenol/chloroform protocol. The genotyping was performed by Real-Time PCR using TagMan assays. The frequency of the reduced-function allele, in both genes, was higher in patients with negative outcome (36.36% vs 21.15%). A negative clinical outcome and an increased risk for MACE was observed in patients with concomitant inheritance of the *CYP2C19* *1/*2 and *ABCB1* CT genotype vs patients with other genotypes (22.73% vs 9.62%; OR 3.455; 95% CI= [0.936-12.743], p=0.05722. A trend towards higher risk of MACE was also noted in carriers of the *CYP2C19*1/*1* and *ABCB1* CC/CT genotype. Our results support the data on the association of the *CYP2C19 *2* alone, or in combination with the *ABCB1* C polymorphism with the increased risk of MACE. The results also indicate that the presence of *ABCB1* C343T polymorphism might be potentially considered as independent predictor of MACE in patients on clopidogrel. However, these results are preliminary and should be confirmed on a larger number of patients.

***Key words*:** *ABCB1,* clopidogrel, *CYP2C19,* coronary artery disease, *P*-glycoprotein, pharmacogenetics

INTRODUCTION

Cardiovascular diseases are a leading cause of death worldwide. Coronary artery disease (CAD) is the most common type of cardiovascular disease, and for those patients, Percutaneous Coronary Intervention (PCI) with stenting is the standard of care. However, in many cases post-operative patients develop major cardiovascular events (MACE) as cardiac death, myocardial infarction, stroke, and stent thrombosis which are serious concerns (1). According to guidelines, antiplatelet therapy is the first-line option in primary and secondary prevention of most cardiovascular diseases, followed by a thrombotic event, but despite successful treatment, the possibilities for recurrent ischemic events still exist (2, 3). Therefore, the use of drug-eluting stents in combination with dual antiplatelet therapy of aspirin and clopidogrel significantly reduces the incidence of ischemic events and stent thrombosis in patients with CAD (4).

Clopidogrel (adenosine diphosphate receptor $P2Y_{12}$ blocker) is a prodrug that needs to be converted into an active drug by several hepatic cytochrome P450 (CYP) enzymes. For this reason, the activity of these enzymes is assumed as the primary determinant for therapeutic response to this drug. *CYP2C19* plays a key role in the metabolic transformation. The *CYP2C19* gene is highly polymorphic, and therefore the association between *CYP2C19* gene variants and clopidogrel efficacy appears to be clinically actionable. The presence of certain *CYP2C19* polymorphisms can lead to variations in the level of functional proteins that influence the degree of clopidogrel metabolites and promote different inter-individual clopidogrel responses (5, 6). Specifically, the presence of any dysfunctional *CYP2C19* allele (*2,*3,*4,*5) is associated with adverse cardiovascular events, whereas the presence of *CYP2C19* allele (*17) is associated with increased risk of bleeding (7, 8). Guidelines recommend the testing of *CYP2C19*2 *3* and **17* in order to avoid adverse outcomes in patients with CAD treated with clopidogrel (9). According to the presence of reduced-

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function alleles, patients are classified into two clinically significant categories: intermediate metabolizers and poor metabolizers. Due to the insufficient activity of the enzyme these patients are suggested to consider alternative P2Y12 inhibitors (ticagrelor and ticlopidine) (10).

The intestinal absorption of clopidogrel is mediated by ATP-dependent drug efflux pump, and P-glycoprotein, transporting a high variety of molecules across the extra- and intra-cellular membranes. Although it is expressed mostly on the intestinal epithelial cells, an increased expression can alter the bioavailability of clopidogrel. The P-glycoprotein is encoded by the *ABCB1* gene located on chromosome 7 (11). Among several single nucleotide polymorphisms (SNPs) that were examined within this gene, the *ABCB1* C3435T has been shown to have an effect on absorption of clopidogrel (12). Namely, individuals carrying the loss of function allele variant were associated with lower levels of the active drug metabolite and were considered to have a high rate of adverse clinical outcomes (13, 14). However, recent studies have presented conflicting results on the association of the *ABCB1* C3435T and adverse events in patients treated with clopidogrel.

It is known that the pharmacodynamic response of clopidogrel can vary among individuals. Nearly 25% of patients treated with standard doses of clopidogrel experience low ex vivo inhibition of ADP-induced thrombocyte aggregation. The precise mechanism of resistance of clopidogrel is still unclear, although additional factors including epigenetics, demographics, complications and drug-drug interactions may also be involved in the response heterogeneity (15).

The aim of this study was to evaluate the association between the presence of the *ABCB1* C3435T and *CYP2C19**2 polymorphisms and the clinical cardiovascular outcome in post-operative patients with coronary artery disease treated with clopidogrel.

MATERIALS AND METHODS

Study population

A total of 96 patients were included the study. Samples from all patients were derived from the Special Hospital for Surgical Diseases "Filip II" in Skopje, R.N. Macedonia. The demographic and clinical characteristics of the patients enrolled in the study are presented in Table 1.

Table 1. Demographic and clinical characteristics of the patients with coronary artery disease treated with clopidogrel.

Parameter	Total number of patients (N=96)	Patients with positive outcome (N=52)	Patients with negative outcome (N=44)
Demographic characteristics			
Age	60.42±9.05	60.64±9.19	60.16±9.22
Male	59 (61.46%)	33 (63.46%)	26 (59.09%)
Clinical characteristics			
History of myocardial infarction	27 (28.13%)	10 (19.23%)	17 (38.64%)
History of diabetes	48 (50%)	24 (46.15)	24 (54.54%)
NYHA classification*			
Class I	22 (22.92%)	15 (28.85%)	7 (15.91%)
Class II	31 (32.29%)	14 (26.92%)	17 (38.64%)
Class III	41 (42.71%)	22 (42.31%)	19 (43.18%)
Class IV	2 (2.08)	1 (1.92%)	1 (2.27%)
Diagnosis**			
105-06	4 (4.17%)	3 (5.77%)	1 (2.27%)
120-23	54 (56.25%)	32 (61.54%)	22 (50%)
125	17 (17.71%)	8 (15.38%)	9 (20.45%)
135	3 (3.13%)	1 (1.92%)	3 (6.82%)
165-66, 170-74	17 (17.71%)	8 (15.38%)	9 (20.45%)

NYHA classification*
Class I No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation or dyspnea.
Class II Slight limitation of physical activity. Comfortable at rest but ordinary physical activity results in fatigue, palpitation or dyspnea.
Class III Marked limitation of physical activity. Comfortable at rest but less than ordinary activity results in fatigue, palpitation or dyspnea.
Class IV Unable to carry out any physical activity without discomfort. Symptoms at rest. If any physical activity is undertaken, discomfort is increased.

Diagnosis**
105-06 mitral and aortal stenosis (rheumatic etiology)
120-23 angina, acute myocardial infarction, complications after myocardial infarction
125 – atherosclerotic cardiovascular disease
135 – aortal stenosis (nonrheumatic etiology)
165-66, 170-74 atherosclerosis, aortal aneurism, other peripheral vascular diseases, arterial embolism and thrombosis

Table 2. Genotype frequencies of *ABCB1* and *CYP2C19* in patients treated with clopidogrel

CYP2C19 / **Genotype**	***ABCB1***	**Positive outcome N=52**	**Observed Frequency (%)**	**HWE Frequency (%)**	**Negative outcome N=44**	**Frequencies (%)**	**Expected Frequencies (%)**	**OR**	**95%**	**p value**
***ABCB1* CC homozygotes**										
*1/*1	CC	9	17.31	17.7	11	4.84	5.4	1.00		
*1/*2	CC	3	5.77	5.0	2	4.55	3.4	0.545	0.074-4.008	0.54819
*2/*2	CC	0	0	0.4	0	0	0.6	0.826	0.015-45.693	1.0000
ABCB1 CT heterozygotes										
*1/*1	CT	19	36.54	32.9	11	25	11.1	1.00		
*1/*2	CT	5	9.62	16.8	10	22.73	9.7	3.455	0.936-12.743	0.05722
*2/*2	CT	3	5.77	2.2	2	4.55	2.1	1.152	0.166-7.990	0.88644
ABCB1 TT homozygotes										
*1/*1	TT	10	19.23	19.6	4	9.09	8.6	1.00		
*1/*2	TT	3	5.77	5.1	3	6.82	7.8	2.500	0.346-18.039	0.35720
*2/*2	TT	0	0	0.3	1	2.27	1.8	7.00	0.237-206.784	0.14323

Clopidogrel was administrated to all patients by the following regimen: a loading dose of 600 mg, on the first day of the treatment, and maintenance dose of 75 mg daily for up to 15 months. The follow up period was > 12 months. The primary endpoint of this study was the occurrence of the first clinical sign of the following MACE. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics committee of the Faculty of Pharmacy- Skopje.

DNA isolation

Genomic DNA was isolated from leucocytes obtained (PBMCs) from 3ml peripheral blood collected by venepunction in vacutainers containing EDTA (Ethylenediaminetetraacetic acid) as an anticoagulant. DNA isolation was performed using the standard phenol/chloroform extraction protocol. Subsequently, the concentration of the obtained DNA was measured with the NanoDrop 2000c UV – Vis spectrophotometer (ThermoFisher Scientific, Wyman Street Waltham, MA USA). DNA purity was verified by UV absorption at 260/280 nm, while DNA integrity was assessed using 1% agarose gel containing ethidium bromide. DNA samples were stored at 4°C.

Genotyping

The genotyping analysis for both polymorphisms *(CYP2C19*2* and *ABCB1* C3435T) was done by Real-Time PCR using the allelic discrimination method on the MxPro 3005P instrument (Agilent technologies, Santa Clara, CA, USA). The amplification and the detection of the specific SNPs alleles were performed using specific TaqMan Drug Metabolism Genotyping Assays according to manufacture recommendations (ThermoFisher Scientific, Foster City, CA, USA). The presence of the SNPs was determined using the MxPro Software v.5.1.

Statistical analysis

The obtained data was analyzed using SPSS software. Genotype distribution for the studied polymorphisms was in correlation with the Hardy-Weinberg equilibrium, according to the X^2 test. X^2 and Fischer exact probability test were used to compare the genotype distributions and allelic frequencies between the patient population and positive/negative outcome. Odds ratios (OR) were calculated with 95% confidence interval limit (95% CI). P value ≤0.05 was considered as statistically significant.

RESULTS

By comparison of the results obtained from the genotyping and clinical presentation of the disease we observed that a negative clinical outcome is more frequent in the subgroup of patients treated with clopidogrel that carry the *CYP2C19* *1/*2 genotype together with the *ABCB1* CT genotype. These results indicate that those patients are at an increased risk of adverse cardiovascular events vs patients who are homozygotes for the normal allele (22.73% vs 9.62%; OR 3.455; 95% CI= [0.936-12.743], p=0.05722 (Table 2). Consequently, allelic distribution of the reduced-function allele, in the *CYP2C19* and *ABCB1* genes, was higher in patients with worse cardiovascular outcome (36.36% vs 21.15%) (Table 3, Figure1). Additionally, in the subgroup of patients presented with the *CYP2C19*1/*1* genotype and co-existence of the *ABCB1* CC or CT genotype, we noted a trend towards higher risk of MACE occurrence compared to patients with the TT genotype (OR=1.316 TT→CT; OR=3.056 TT→CC).

Based on the results from the CYP2C19/ABCB1 genotyping, patents were divided into three groups according to combined genotype/phenotype: extensive metabolizers (EM), intermediate metabolizers (IM) and poor metabo-

Table 3. Allelic distribution of the *ABCB1* and *CYP2C19* polymorphisms in CAD patients treated with clopidogrel

Allele	Patients with positive outcome (N=52)	Patients with negative outcome (N=44)
	number of patients	
***CYP2C19**1/ *ABCB1* C**	36	34
***CYP2C19**1/ *ABCB1* T**	37	28
***CYP2C19**2/ *ABCB1* C**	11	14
***CYP2C19**2/ *ABCB1* T**	11	16

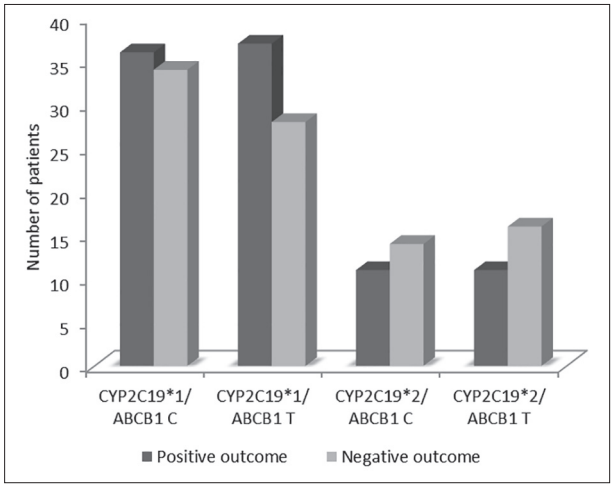

Figure 1. Allelic distribution of *ABCB1* and *CYP2C19* in patients with CAD treated with clopidogrel

lizers (PM) (Table 4). Patients carrying normal function alleles were classified as extensive metabolizers, patients carrying at least one or two loss-of-function allele were classified as intermediate metabolizers, whereas the patients carrying two loss-of-function alleles were classified as poor metabolizers. In the subgroup of patients with negative outcome, the presence of intermediate metabolizers was more frequent compared to the subgroup of patients with positive outcome (0.7272 vs 0.5192; p=0.02805). The frequency of patients referred to as poor metabolizers was higher in the subgroup with a positive outcome (0.2272 vs 0.3077; p=0.22842), but without statistical significance (Table 4). Therefore, the patients classified as intermediate metabolizers, carrying at least one loss-of-function allele were assumed to have higher risk of adverse cardiovascular events or MACE.

DISSCUSION

The main goal of optimal antiplatelet therapy for patients with acute coronary disease and/or undergoing percutaneous coronary intervention is to reduce the incidence of cardiovascular events. The clinical outcome of clopidogrel, as a well-established antiplatelet therapy, has high inter-individual variability within patients

Although recent studies present contradictory data, our results support the association between the presence of the *CYP2C19 *2,* alone or in combination with the *ABCB1* C and an increased risk for adverse cardiovascular events compared to the carriers of the alternative alleles. The concomitant inheritance of the genotype *CYP2C19*1/*2* and *ABCB1 CT* have shown a significant trend toward increased risk for MACE. These finding are line with data presented in the studies conducted on a larger sample size. (16-18)**.**

According to another study *ABCB1* C3435T and *CYP2C19* *2 polymorphisms are recognized as significant, independent predictors for the primary endpoint of cardiovascular death, myocardial infarction or stroke. The presented results indicate that the risk for MACE is higher in patients, either as carriers of a *CYP2C19* or *ABCB1* reduced-function allele, or both (19). In our study it was also demonstrated that patients with normal *CYP2C19* genetic status and presence of the *ABCB1* CT/CC genotype have a higher incidence of MACE, indicating that the presence of the *ABCB1* C allele could be a potential negative predictor for disease outcome in patients treated with clopidogrel.

In a large cohort, conducted on 2208 patients with an acute myocardial infarction receiving clopidogrel therapy,

Table 4. Phenotype frequencies of *CYP2C19* and *ABCB1* in clopidogrel treated patients

Phenotype/ Metabolizer	Patients with positive outcome (N=52)			Patients with negative outcome (N=44)			OR	p
	N	Observed Frequency (%)	HWE Frequency (%)	N	Observed Frequency (%)	HWE Frequency (%)		
Normal*	9	0.1731	0.1872	2	0.0454	0.1673	1.000	
Intermediate**	27	0.5192	0.4909	32	0.7272	0.4834	5.333	0.02805
Poor***	16	0.3077	0.3218	10	0.2272	0.3491	2.812	0.22842

*Carrier of CYP2C19*1/*1/ ABCB1 CC genetic polymorphism
**Carrier of CYP2C19*1/*1/ ABCB1 CT genetic polymorphism
CYP2C19*1/*1/ ABCB1 CT genetic polymorphism
CYP2C19*1/*2/ ABCB1 CT genetic polymorphism
CYP2C19*1/*2/ ABCB1 CC genetic polymorphism
***Carrier of CYP2C19*2/*2/ ABCB1 CC genetic polymorphism
CYP2C19*2/*2/ ABCB1 CT genetic polymorphism
CYP2C19*1/*1/ ABCB1 TT genetic polymorphism
CYP2C19*1/*2/ ABCB1 TT genetic polymorphism
CYP2C19*2/*2/ ABCB1 TT genetic polymorphism

Simon et al., presented no significant association between the *ABCB1* and *CYP2C19* polymorphisms and the clinical outcome, but the presence of two *CYP2C19*-deficient alleles and either one or two *ABCB1* variant alleles was associated with rate of events five times higher when compared to patients with the wild-type (20). Another study also supports the evidence that patients who are poor metabolizers are at greater risk of thrombotic events when treated with clopidogrel (17, 21).

Several studies including the TRITON TIMI study evaluated the contribution of *ABCB1* variants in patient carrying the risk allele *CYP2C19*2* and confirmed that clopidogrel response depends on the complex mechanisms of action, including hepatic activation and also that variable response can occur due to other factors such as polymorphisms in other genes involved in clopidogrel pharmacokinetics and pharmacodynamics.

Since this study was conducted on a small sample size, limitations should be considered when interpreting the results. This was the first conducted study in Macedonia to evaluate the concomitant influence of the *ABCB1*3435 CT and *CYP2C19*1/**2 genotypes on clinical cardiovascular outcomes in coronary artery disease patients on clopidogrel treatment. The evaluation was based on occurred major adverse cardiovascular events and plasma concentrations of clopidogrel, and its active metabolites were not monitored during the follow up. Moreover, we cannot completely exclude the probability that other risk factors, such as BMI, smoking status, lifestyle, diet etc., influence the clinical cardiovascular outcome. However, the main limitation of this study was the small number of patients included, leading to restricted statistical significance for some of the results. Additional, larger studies will be of great importance to further evaluate the relation between concomitant inheritance of the *ABCB1* C3435T and *CYP2C19**2 polymorphisms and clopidogrel treatment outcomes.

CONCLUSION

In summary, our results support the previous data on the association between the presence of the *CYP2C19 *2* alone, or in combination with the *ABCB1* C, and the increased risk of MACE compared to the carriers of the wild type alleles. Namely, a higher incidence of major adverse cardiovascular events was detected in patients with concomitant inheritance of the CYP2C19*1/*2 and ABCB1 CT genotype and in patients with normal CYP2C19 genetic status and presence of the *ABCB1 3435*C allele. The results also indicate that the presence of *ABCB1* C343T polymorphism might be potentially considered as an independent predictor of adverse cardiovascular events in patients treated with clopidogrel. However, this was an initial study conducted on a subset of the Macedonian population, and the results should be confirmed with a larger cohort.

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ORIGINAL ARTICLE

DETERMINATION OF THE RELATIONSHIP BETWEEN DNA METHYLATION STATUS OF *KLOTHO* AND *ARNTL* GENES WITH HYPERTENSION

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ABSTRACT

Hypertension is a multifactorial chronic disease due to the interaction of environmental factors with genetic alteration. *KLOTHO* and *ARNTL* genes play an important role in the development of hypertension. Therefore, we analyzed the methylation status of *KLOTHO* and *ARNTL* genes by using methylation-sensitive high-resolution melting (MS-HRM) in a total of 78 hypertensive and 49 control subjects. In this study, we could not identify a significant association between *KLOTHO* and *ARNTL* methylation and the hypertensive phenotype. Moreover, we could not find a direct association between *KLOTHO* and *ARNTL* methylation and the fasting blood sugar, triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, sodium (Na), creatinine (Cr), potassium (K), and urea levels in hypertensive patients. However, we found a significant difference between the methylated *KLOTHO* hypertensive patients and the unmethylated *KLOTHO* control subjects for potassium (K).

Keywords: Methylation, Hypertension, *KLOTHO*, *ARNTL*

INTRODUCTION

Hypertension (HTN) is a common chronic disease in advanced age and is considered to influence 29% of adult individuals worldwide by 2025 [1]. HTN is one of the leading causes of kidney disease and cardiovascular complications, including stroke, coronary artery disease, heart failure, and peripheral vascular disease [2]. Generally, HTN can be categorized into two groups, as primary (essential or idiopathic) and secondary (non-essential) hypertension. Approximately 90-95% of hypertensive cases have primary hypertension, 5-10% of hypertensive cases have secondary hypertension. Primary hypertension is not due to other diseases and is affected by genetic and lifestyle factors. However, secondary hypertension is developed from kidney diseases, endocrine disorders, or side effects of drugs [3]. Mechanisms involved in the development of primary hypertension have been reported to be still unclear [1]. Numerous factors, including genetic, epigenetic, advanced age, smoking, overweight, diabetes, arterial aging, endothelial dysfunction, and arteriosclerosis contribute to HTN [4,5]. The heritability for HTN was indicated to be 30-60% [6].

KLOTHO (*KL*) was discovered as an aging suppressor gene encoding α-KL protein due to the determination of short lifespan and various aging-related phenotypes resembling human aging. These phenotypes include vascular calcification, atherosclerosis, cardiovascular disease in *KL*-deficient mice [7]. The *KL* gene is highly conserved in mice, rats and humans [8,9], and mainly expressed in the distal convoluted and proximal tubules of the kidney, choroid plexus of the brain and also other tissues such as the parathyroid glands, sinoatrial node, vascular tissue, cartilage and bones [10]. More than 10 SNPs in the human *KL* gene have been reported [11,12]. *KL* gene polymorphisms such as G-395A [5,13], C1818T [2], and F352V [14] were associated with hypertension. The *KL* gene expression was shown to be lower in essential hypertensive patients in the Indian population [3]. Furthermore, a decrease in *KL* levels has been found in renovascular hypertensive

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patients. Thus, these results shed light on how the *KL* gene plays a crucial role in the pathophysiology of primary and secondary hypertension. Additionally, according to these results, it was suggested that *KL* could be utilized as a biomarker for the determination of kidney injury in hypertensive patients [1,15].

The circadian clock is a highly conserved system and provides adaptations of organisms to the environment cues along the 24-hour light/dark cycle [16]. It regulates most physiological processes, such as sleep-wake cycles, immune response, metabolism, renal function, and blood pressure [17]. The circadian clock is involved in the maintenance of vascular function. It has been suggested that impaired clock function can lead to health complications such as cardiovascular disease and hypertension by contributing to vascular dysfunction [16, 18]. Aryl hydrocarbon receptor nuclear translocator-like protein 1 (*ARNTL* or brain and muscle ARNT-like 1 (*BMAL1*)) is a core clock component and regulates the rhythmic expression of many genes as a transcription factor [19]. The *ARNTL* rs9633835 and rs6486121 polymorphisms were associated with susceptibility to hypertension [20,21].

There have been studies investigating the connection between the *KL* and *ARNTL* genes and hypertension through polymorphism and expression analyses, there is no published study that explores the correlation between the methylation status of these genes and hypertension, as well as the associated biochemical variables in both hypertensive and control subjects. Methylation is an epigenetic modification process that alters gene expression without changing the nucleotide sequence, thus contributing to the regulation of gene expression and maintenance of genomic stability[22]. In this study, therefore, the methylation status of the *KL* and *ARNTL* genes was analyzed in both hypertensive and control subjects. Furthermore, the possible effect of *KL* and *ARNTL* gene methylation on biochemical variables was analyzed in hypertensive and control subjects.

MATERIAL AND METHODS

Study subjects

78 hypertensive patients (31 female and 47 male) and 49 control subjects (36 female and 13 male) were included in this study, and peripheral blood specimens of the participants were collected. Participants were recruited from Burhan Nalbantoğlu State Hospital, Nicosia (from November 2022 to April 2023). The diagnostic criteria of hypertension were defined as systolic blood pressure (SBP)/diastolic blood pressure (DBP) >140/90 mmHg or antihypertensive medication use for decreasing high blood pressure [23]. The participants with cancer, respiratory diseases, cerebral infarction, congenital heart disease, diabetes mellitus or chronic kidney diseases, and autoimmune diseases were excluded, moreover, subjects who were discontent to participate in the study were excluded as well. The medical history of all participants was questioned. All clinical investigations performed for this study were conducted in accordance with the principles of the Declaration of Helsinki. The study was approved by the Scientific Research Ethics Committee of the Near East University (YDU/2020/80-1066). All subjects signed the written consent form before participating in the study.

Measurements of the Biochemical Parameters

Peripheral blood samples were collected from participants after overnight fasting. All participants' serum was obtained by centrifugation at 2000 rpm for 20 min at 4 °C. The fasting blood sugar (FBS), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), sodium (Na), potassium (K), creatinine (Cr), and urea levels were determined with an automated clinical biochemistry analyzer (Abbott Architect C8000).

Methylation Analysis

Peripheral blood samples of hypertensive patients and normal controls were transferred to 2 ml vacuum tubes with K2EDTA. Genomic DNA was isolated from whole blood samples by using the AllPrep DNA/RNA/Protein isolation kit (Qiagen in Manchester, United Kingdom). A NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA) was used to analyze the amount and purification of DNA samples. Sodium bisulfite modification of DNA samples was done by using the EpiTect Bisulfite Modification Kit (Qiagen, Manchester, UK). According to the EpiTect® MS-HRM PCR Handbook (Qiagen, Manchester, UK), primers were designed. The MS-HRM analysis was performed to analyze the promotor methylation status of *KLOTHO* and *ARNTL* genes according to the EpiTect® HRM™ PCR Handbook protocol (Rotor-Gene Q, Qiagen). As methylated and unmethylated controls, universal methylated and unmethylated DNA (EpiTect Control DNA Set, Cat No./ID: 59568) were preferred [24].

Statistical Analysis

Descriptive statistics for qualitative (frequency and percentage) and quantitative varaibles (arithmetic mean, standard deviation) were calculated. Pearson's Chi-Square test or Fisher's exact test was used to investigate the associations between gene methylation status and several patient characteristics, where appropriate. The McNemar

test was used to investigate the association of methylation in both genes. A two-way Analysis of Variance test was performed to analyze the effects of gene methylation and hypertension on several biochemical parameters. Sidak's posthoc test was applied to investigate the pairwise differences, in the case of statistical significance. The Statistical Package for Social Science (SPSS) (Demo Version 26.0 for Mac) and GraphPad Prism (Demo Version 9.51 for Mac) software were used for all statistical calculations. The level of significance was accepted to be 0.05.

RESULTS

The mean age of 76 patients who suffered from hypertension is 60.04 ± 13.57 years old, while the control subjects were at 44.45 ± 13.94 years old (n=49).

Promoter methylation status of *KLOTHO* and *ARNTL* in hypertensive and control subjects

A comparison of the methylation status of *KLOTHO* and *ARNTL* in hypertensive and control subjects was shown in Table 1.

A total of 46 of the 67 methylated *ARNTL* subjects were hypertensive (68.7%) while 27 of the 48 unmethylated *ARNTL* subjects were hypertensive individuals (56.2%). The association between the methylation status of the *ARNTL* gene and hypertension was not significant ($p > 0.05$) (Table 1 and Figure 1-2).

A total of 62 of the 100 methylated *KLOTHO* subjects were hypertensive (62.0%). On the other hand, 16 of the 27 unmethylated *KLOTHO* subjects were hypertensive (59.3%). Likewise, for the *ARNTL* gene, the association between the methylation status of the *KLOTHO* gene and hypertension was not significant ($p > 0.05$) (Table 1 and Figure 3).

The difference in metabolic characteristics between methylation levels of the *KLOTHO* and *ARNTL* genes

The mean values of biochemical parameters for *KLOTHO* and *ARNTL* genes regarding the methylation status were shown in Table 2 and Table 3, respectively.

The difference between the methylation status categories of *KLOTHO* and *ARNTL* genes for age, glucose, triglyceride, total cholesterol, HDL-C, and LDL-C levels in blood circulation, Na, K, Cr, Urea were investigated in this study. The results indicate no statistically significant difference for age, glucose, triglyceride, total cholesterol, HDL-C, and LDL-C levels in blood circulation, Na, K, Cr, Urea for both genes ($p > 0.05$).

Table 1. *KLOTHO* and *ARNTL* gene methylation in hypertensive and control subjects

Subjects	*KLOTHO* gene				*ARNTL* gene			
	Methylation		Unmethylation		Methylation		Unmethylation	
	Count	$\%_A$	Count	$\%_B$	Count	$\%_A$	Count	$\%_B$
Hypertension	62	62.0%	16	59.3%	46	68.7%	27	56.2%
Control	38	38.0%	11	40.7%	21	31.3%	21	43.8%
Total	100	100%	27	100%	67	100%	48	100%
p Value	p>0.05				p>0.05			

(% in total according to methylation status in subjects for each gene)
($\%_A$ in total methylated subjects; $\%_B$ in total unmethylated subjects)

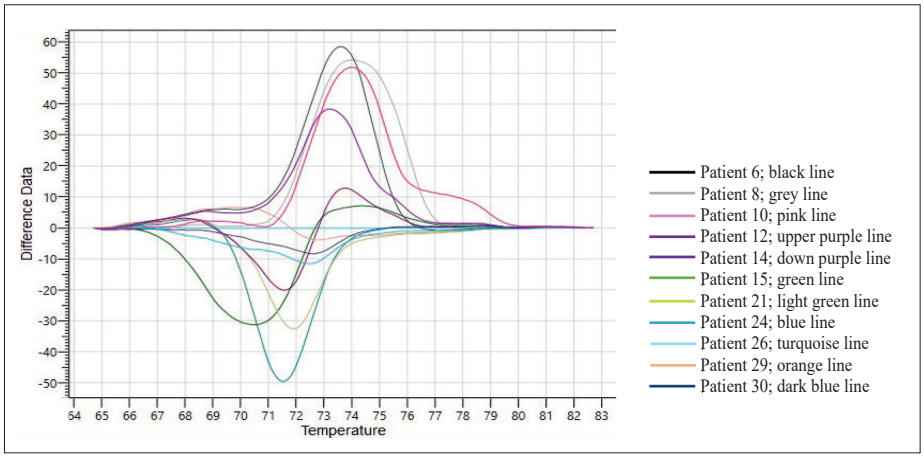

Figure 1. Methylated and unmethylated peaks of *ARNTL* gene for the patient groups. (The peaks obtained in different Tm degrees for the unmethylated and methylated cases were shown. Methylated *ARNTL* patient 6, 8, 10, and 12 are shown in black, grey, pink, and upper purple, respectively. Unmethylated *ARNTL* patient 14, 15, 21, 24, 26, 29, and 30 are shown in down purple, green, light green, blue, turquoise, orange, and dark blue, respectively)

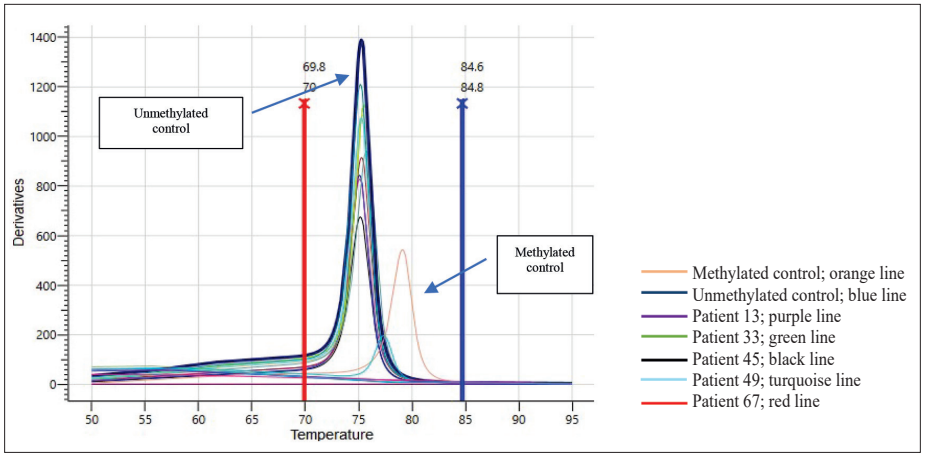

Figure 2. Unmethylated *ARNTL* patients. *ARNTL* unmethylated control is shown in blue, and methylated control is shown in orange. Unmethylated *ARNTL* patient 33, 49, 67, 13 and 45 are shown in green, turquoise, red, purple, and black, respectively.

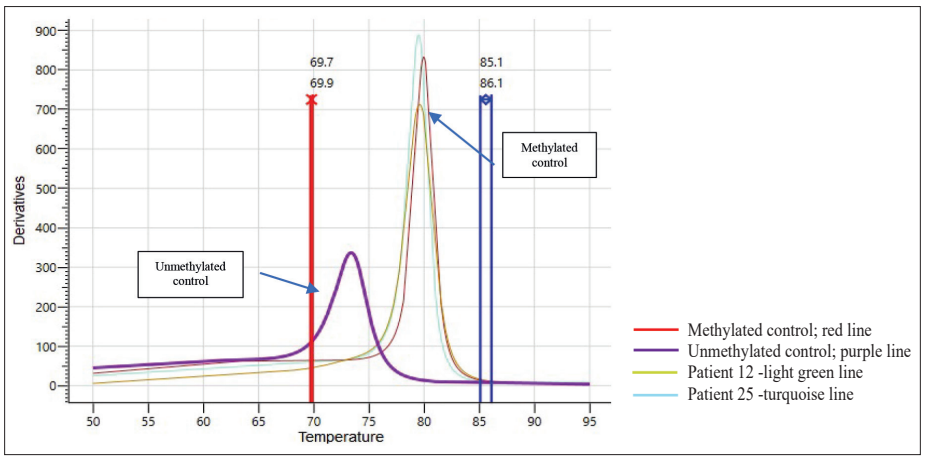

Figure 3. Methylated *KLOTHO* patients. *KLOTHO* unmethylated control is shown in purple, and methylated control is shown in red. Methylated *KLOTHO* patient 25 and 12 are shown in turquoise and light green, respectively.

Table 2. Mean Values of Biochemical Parameters for *KLOTHO* Gene

Parameter	Methylated *KLOTHO*		Unmethylated *KLOTHO*	
	Hypertensive subjects	Control Subjects	Hypertensive subjects	Control subjects
Fasting glucose (mg/dL)	129.96 ± 83.08 (54)	97.97 ± 12.43 (38)	117.20 ± 34.03 (15)	98.18 ± 5.23 (11)
Triglyceride (mg/dL)	166.82 ±91.28 (38)	122.22 ± 31.10 (37)	183.93 ±104.06 (14)	132.00±28.43 (11)
Total cholesterol (mg/dL)	195.97 ± 61.63 (36)	170.59 ±43.12 (37)	197.07 ± 49.94 (14)	191.09 ± 42.03 (11)
HDL-C (mg/dL)	45.84 ± 14.31 (37)	52.19 ± 12.72 (37)	46.50 ± 14.34 (14)	49.64 ± 6.83 (11)
LDL-C (mg/dL)	131.92 ± 65.55 (37)	129.62 ± 28.28 (37)	125.71 ± 60.77 (14)	130.27 ± 33.02 (11)
Sodium (Na) (mmol/L)	137.98 ± 4.29 (53)	138.26 ± 7.50 (38)	136.60 ± 12.98 (15)	143.27 ± 8.60 (11)
Potassium (K) (mmol/L)	4.45 ± 0.60 (53)	4.88 ± 1.11 (38)	4.71± 0.75 (15)	5.55 ± 1.20 (11)
Creatinine (Cr) (mg/dL)	1.11± 0.59 (54)	0.66 ± 0.14 (38)	1.06 ± 0.95 (15)	0.65 ± 0.11 (11)
Urea (mg/dL)	28.66 ± 23.10 (53)	15.18 ± 3.40 (38)	21.14 ± 17.82 (14)	20.91 ± 13.40 (11)

A statistically significant difference was detected between the methylated *KLOTHO* hypertensive patients and unmethylated *KLOTHO* control subjects for potassium (K) (p= 0,0014) (Figure 4).

The Association Between *ARNTL* and *KLOTHO* Methylation Status

ARNTL and *KLOTHO* were both methylated in 53 out of 115 subjects (46.1%) and unmethylated in 10 out of the 115 subjects (8.8%) (Table 4). The association between methylation status of *KLOTHO* and *ARNTL* genes was not statistically significant (p > 0.05).

Table 3. Mean Values of Biochemical Parameters for *ARNTL* Gene

Parameter	Methylated *ARNTL*		Unmethylated *ARNTL*	
	Hypertensive subjects	Control Subjects	Hypertensive subjects	Control subjects
Fasting glucose (mg/dL)	120.00 ± 70.48 (42)	96.67 ± 15.54 (21)	142.65 ± 87.50 (23)	98.14 ± 6.02 (21)
Triglyceride (mg/dL)	177.58 ±98.15 (33)	132.50 ± 31.30 (20)	172.06±91.38 (16)	120.57±28.13 (21)
Total cholesterol (mg/dL)	197.58 ± 59.89 (33)	179.60 ±59.04 (20)	195.07 ± 52.06 (14)	168.14 ± 28.81 (21)
HDL-C (mg/dL)	47.55 ± 15.91 (33)	48.55 ± 8.75 (20)	41.87 ± 10.33 (15)	53.48 ± 12.15 (21)
LDL-C (mg/dL)	128.30 ± 60.90 (33)	134.65 ± 35.32 (20)	136.93 ± 71.65 (15)	128.24 ± 26.19 (21)
Sodium (Na) (mmol/L)	137.38 ± 8.36 (42)	138.67 ± 6.63 (21)	138.00 ±4.57 (22)	141.00 ± 9.11 (21)
Potassium (K) (mmol/L)	4.45 ± 0.70 (42)	5.02 ± 1.05 (21)	4.59± 0.57 (22)	5.11 ± 1.30 (21)
Creatinine (Cr) (mg/dL)	1.00± 0.51 (42)	0.64 ± 0.15 (21)	1.07 ± 0.52 (23)	0.68 ± 0.12 (21)
Urea (mg/dL)	26.78 ± 23.29 (40)	17.95 ± 10.51 (21)	23.22 ± 13.49 (23)	15.76 ± 2.95 (21)

Table 4. The Interaction Between *ARNTL* and *KLOTHO* Methylation Status

	Subjects (p + c)		
Methylation Status of *ARNTL*+ *KLOTHO* Genes	**Count**	***%***	**p value**
***ARNTL* Methylated+ *KLOTHO* Methylated**	53	46.1%	p>0.05
***ARNTL* Methylated+ *KLOTHO* Unmethylated**	14	12.1%	
***ARNTL* Unmethylated+ *KLOTHO* Methylated**	38	33.0%	
***ARNTL* Unmethylated+ *KLOTHO* Unmethylated**	10	8.8%	
Total	115	100%	

(% in total subjects containing patients (p) and controls (c))

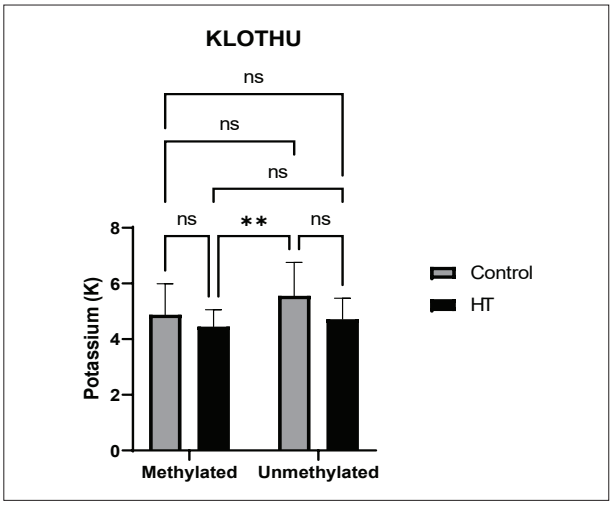

Figure 4. The association between the methylation status of *KLOTHO* and hypertension for potassium (K) (ns: not significant, **: p-value <0,05)

DISCUSSION

Hypertension is an important risk factor for kidney disease and cardiovascular diseases such as stroke, heart failure (HF) and arrhythmias [1,2]. The risk of hypertension significantly increases with age in both men and women [25]. However, the risk of hypertension exhibits variation based on gender. Particularly, the incidence of hypertension was found to be higher in postmenopausal women than those age-matched men and premenopausal women. It was suggested that obesity-mediated sympathetic activity, low estrogen level, and high renin-angiotensin system (RAS) activity might contribute to postmenopausal hypertension [26].

Human KL protein exists as a full-length single-pass transmembrane protein and soluble KL The soluble KL protein can be found in blood, urine, and cerebrospinal fluid [8,9]. A large proportion of soluble KL is provided from the kidney [7]. KL suppresses oxidative stress and aldosterone secretion, inhibits insulin/IGF-1 (insulin-like growth factor 1) and Wnt (wingless-related integration site) pathways, apoptosis, fibrosis and cell senescence, and regulates calcium-phosphate homeostasis [1].

KL-deficient mice were indicated to display aging-related phenotypes, including hypertension, arterial stiffness, and chronic kidney disease (CKD) [27]. The silencing of *KL* gene was found to be related to hypertension and high level of aldosterone in human adrenocortical cells [28]. It was indicated that membranous and soluble KL can inhibit Wnt/β-mediated activation of RAS by blocking the binding of Wnt ligands, and thus preserving kidney function and normalizing BP. Therefore, it was suggested that KL has an ameliatory effect on hypertension in CKD patients [29]. The soluble KL levels were found to be positively correlated with high-density lipoprotein-cholesterol (HDL-

C) and negatively correlated with serum triglycerides in controls, and inversely correlated with body mass index (BMI) in hypertensive patients [3]. It was suggested that low plasma KL levels could increase total body sodium in patients, leading to chronic inflammation and high blood pressure. In addition, it was indicated that this process could be related to CKD patients with low serum KL levels [7].

It was reported that women with low serum KL concentration have a high risk of postmenopausal hypertension. It was suggested that serum KL concentration may be a significant biomarker to evaluate the risk of hypertension in postmenopausal women [26]. Several studies reported that the lower KL concentration was related to higher blood pressure, increasing the risk of hypertension [30,31]. In contrast, serum KL levels were detected not to be significantly different between subjects with and without hypertension in the general Chinese population [27]. Furthermore, a larger population-based study indicated no association between serum KL concentration and blood pressure [32,33]. In our study, we found no statistically significant link between *KL* methylation and hypertension (P>0.05). The difference between the studies' findings could be the result of a limited sample size or the age of the population. Since changes in *KL* expression have been reported during aging, focusing on large-scale studies that evaluate the association between KL concentration, *KL* methylation, and hypertension in age-matched patients with different disease conditions and control subjects would be important. The soluble KL protein is produced by the kidney. Therefore, it is claimed that, in cases where the kidneys are normally functional and soluble KL protein is at normal levels, *KL* expression may vary in other tissues. Thus, it is suggested that *KL* expression in other tissues may decline due to different factors, including genetic variants and epigenetic alteration [34]. Therefore, this information highlights how this situation should be taken into consideration in further studies.

Particularly, it is defended that the effect of genetic polymorphisms in the *KL* gene on blood pressure should not be ignored [27]. Individuals with the GA+AA genotype of *KL* G-395A polymorphism were found to have a lower SBP, relative to the individuals with the GG genotype [35]. Thus, it was suggested that the A variant of *KL* G-395A might prevent the development of essential hypertension by increasing the *KL* levels [5, 13]. The uncertain mechanism affecting the human *KL* promoter region was indicated to inhibit *KL* gene expression in HEK293 cells [36]. The *KL* promoter region has been reported to be sensitive to DNA methylation [37, 38]. The *KL-VS* variant contains six variants, and two variants thereof are located in exon 2 cause amino acid substitutions, F352V and C370S. The *KL-VS* variant has been associated with enhanced SBP, serum cholesterol and cardiovascular disease in Baltimore Caucasian and African American subjects [39-41]. A meta-analysis has indicated that the G-395A, C1818T SNPs of the *KL* gene might be associated with a hypertension risk [2]. The F352V (rs9536314 T> G) polymorphism of the *KL* gene was correlated with salt-sensitive hypertension in an Italian study [14]. The *KL* rs9536314 and rs564481 polymorphisms were associated with increasing levels of HDL-C in females [42]. High HDL-C levels were suggested to be protective against *KL* dysfunction [40]. Particularly, HDL and KL have been indicated to participate in the regulation of similar signaling pathways, including both molecules that promote nitric oxide (NO) synthesis, angiogenesis, and inhibit apoptosis, and insulin signaling in cell culture models [42, 43]. The fasting glucose was found to be predominantly higher in women with the T allele of *KL* rs564481 relative to non-carriers in Japanese [12] and Korean women [11]. It was reported that KL reduces the levels of serum creatinine. The decrease in *KL* expression has been related to aging-related kidney damage. Particularly, enhance in serum creatinine was indicated to be a diagnostic factor of acute kidney injury (AKI) [29]. Although several studies have reported a relationship between some biochemical parameters and KL protein, in our study, the relationship between the methylation status of *KL* and fasting glucose, triglyceride, total cholesterol, HDL-C, LDL-C, Na, K, Cr and Urea levels, were not significant ($p > 0.05$). It is considered that these contradictory results may be due to different genetic backgrounds in different populations. However, levels of K were significantly different between the methylated *KL* hypertensive patients and unmethylated *KL* control subjects (p= 0,0014).

The circadian clock regulates the circadian oscillation of human blood flow by enhancing it during the day and decreasing it during the night [44]. Circadian clock genes were implicated in modulating many processes involved in the regulation of the blood pressure in the kidney, heart, vasculature, and metabolic organs [17]. It has been reported that the circadian oscillation of blood pressure decreases with age [45].

The global *ARNTL* knockout (KO) mice were found to have lower BP with impaired BP rhythm. It was shown that decreased BP can be associated with changes in the vasculature in *ARNTL* KO mice [18]. Furthermore, the global *ARNTL* KO mice were indicated to lose diurnal sodium excretion [46]. Smooth muscle components of the blood vessel wall provide sufficient blood flow to organs and blood pressure homeostasis by regulating its contractile state. Smooth muscle-specific *ARNTL* KO male mice were shown to exhibit decreased BP and less impaired BP rhythm [16]. *ARNTL* rs3816358 polymorphism has been related to non-dipper hypertension in young hypertensive

patients [47]. In rats, the *ARNTL* gene was stated to be situated in hypertension susceptibility loci. The 18477-T/G variant in the *ARNTL* promotor was reported to significantly decrease the Gata-4-mediated transcriptional activation of the *ARNTL* promotor. Moreover, in respect of this polymorphism, *ARNTL* promoter activity was indicated to be consistently 2-fold higher in normotensive Wistar-Kyoto (WKY) rats than in spontaneously hypertensive rats (SHR) [21]. It is suggested that hypertensive patients with the GG genotype of *ARNTL* A1420G may exhibit high nighttime SBP [48]. The essential arterial hypertension (EAH) patients with GG genotype of the circadian locomotor output cycles protein kaput (*CLOCK)* 257TG polymorphism were found to exhibit lower *ARNTL* expression than EAH patients with other genotypes at 9.00, 13:00, and 17:00 time points [49]. The downregulation of *ARNTL* levels was detected in hypertensive female patients [4]. Therefore, all these findings support that *ARNTL* is an essential factor during the development of hypertension. However, the mechanisms underlying ''circadian genes'' in the regulation of blood pressure have not been comprehended yet. According to our study, the *ARNTL* gene was methylated in 68.7% of the hypertensive subjects and the relationship between methylation status and hypertension was not significant ($p > 0.05$). Furthermore, the relationship between the methylation status of *ARNTL* and fasting glucose, triglyceride, total cholesterol, HDL-C, LDL-C, Na, K, Cr and Urea levels, were found to not be significant ($p>0.05$).

In summary, we did not identify a statistically significant association between *KLOTHO* and *ARNTL* methylation status and hypertension and their association with fasting blood sugar, triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, Na, K, Cr, Urea levels in hypertensive patients. The limitations of our study are the age mismatch between patients and controls, and the small number of participants. Since several factors such as age, gender and obesity and diabetes are risk factors for hypertension, patients and controls matched by age, gender, and BMI should be included in future studies to minimize the impact of these factors on the results [3]. Therefore, as mentioned in this study, these conditions should not be ignored in future large population-based studies.

CONCLUSION

Several studies have revealed the influence of *KL* and *ARNTL* polymorphisms and altered gene expression on hypertensive patients. Considering these results we focused in this study on understanding the importance of the methylation status of these genes in hypertension and their interaction with biochemical parameters. Although we could not detect a significant difference in *KL* and *ARNTL* methylation status in hypertension patients and controls, this study will shed light on the analysis and determination of new epigenetic pathways involving *KL* and *ARNTL* genes. Furthermore, except for *ARNTL*, considering that other core circadian clock genes may participate in the onset and progression of hypertension, their epigenetic analysis may provide further information on the determination of the mechanisms involved in the hypertension progress.

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ORIGINAL ARTICLE

DO GENE POLYMORPHISMS PLAY A ROLE IN NEWBORN HYPERBILIRUBINEMIA?

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ABSTRACT

Objectives

Polymorphisms of the uridine-diphospho-glucuronosyltransferase 1A1 (*UGT1A1*) gene, hepatic solute carrier organic anion transporter 1B1/B3 (*SLCO1B1/3*) gene, and glutathione S-transferase (*GST*) gene have been associated with significant hyperbilirubinemia in some populations. This study aims to determine whether the variation of *UGT1A1*, *SLCO1B1/3* and *GST* genes play an important role in neonatal hyperbilirubinemia in Turkish newborn infants.

Methods

The study included 61 idiopathic hyperbilirubinemia cases, 28 prolonged jaundice cases, and 41 controls. Ten common polymorphisms in four genes involved in bilirubin metabolism were examined. Polymerase chain reaction-restriction fragment length polymorphism method was used to detect variants of those genes.

Results

No association was found between the variants of *UGT1A1* at nt 211, the *SLCO1B1* gene at nt 388, 463, 521, 1463, the *SLCO1B3* gene at nt 334, 727+118, 1865+19721, and the *GST* gene at nt 313, 341, and neonatal hyperbilirubinemia. There was no difference between the case and control groups in terms of allele frequencies of these genes (except *SLCO1B3* at nt 334) ($p>0.05$ in all comparisons). The presence of the G allele of the *SLCO1B3* at nt 334 variant gene seemed to protect from jaundice in infants with idiopathic hyperbilirubinemia.

Conclusion

These gene polymorphisms currently studied do not seem to modulate the risk of hyperbilirubinemia in Turkish newborn infants.

Key words: Neonatal hyperbilirubinemia, gene polymorphisms, *UGT1A1*, *SLCO1B1/B3*, *GST*

INTRODUCTION

Hyperbilirubinemia is a common finding in the neonatal period and can sometimes lead to serious consequences such as kernicterus. Most cases of neonatal hyperbilirubinemia (NH) are physiological, and approximately 13.4% of the cases are non-physiological [1, 2]. In approximately half of cases of pathological jaundice, there is no identifiable factor [3]. Studies have shown that African newborns have lower serum bilirubin levels and Asian babies develop higher values than their Caucasian counterparts [4]. In a study, the incidence of non-physiological significant hyperbilirubinemia in Turkish newborns was found to be between approximately 10.5% and 25.3% [5]. This may be due to differences in the genetic backgrounds of the populations, suggesting the existence of genetic risk factors for the development of NH [6].

Unconjugated bilirubin is rapidly and selectively taken up across the basolateral membrane of the hepatocyte as a carrier-mediated process involving the partially dissolved carrier organic anion-carrying polypeptide-1B1/B3 (*SLCO1B1* and *SLCO1B3* genes). *SLCO1B1* and *SLCO1B3*

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are sinusoidal transporters that facilitate hepatic uptake of a wide variety of endogenous substrates [1, 2]. In hepatocytes, glutathione S-transferases (*GSTs*) are involved in binding to nonsubstrate ligands, such as unconjugated bilirubin [3]. Unconjugated bilirubin is then conjugated with glucuronate through the enzyme activity of uridine diphosphate glucuronyl transferase (*UGT1A1* gene) [1]. In particular, polymorphisms of four genes specifically related to bilirubin production and metabolism (*UGT1A1*, *SLCO1B1*, *SLCO1B3*, and / or *GST*) may interact with environmental contributors to produce significant hyperbilirubinemia [3, 7].

The aim of this study is to investigate whether polymorphisms in the *UGT1A1*, *SLCO1B1*, *SLCO1B3* and *GST* genes are a contributing factor to idiopathic hyperbilirubinemia or prolonged jaundice with unexplained etiology in Turkish newborns.

MATERIALS AND METHODS

Patients

This study was conducted at Dr. Sami Ulus Maternity and Children's Research and Training Hospital between April 2011 and May 2012. Of the 130 newborn babies included in the study, 89 jaundiced babies consisted of the study group (61 babies with idiopathic hyperbilirubinemia, 28 babies with prolonged jaundice of unexplained etiology), and 41 healthy babies without jaundice consisted of the control group. NH refers to newborn babies with serum total bilirubin levels above 17 mg/dl in the first 7 days of life. Prolonged jaundice is a condition in which serum total serum bilirubin (TSB) is above 10 mg/dl, which persists after the 14th day of life in newborns. The control group consisted of healthy newborns with peak serum TSB level ≤12.9 mg/dl in the first week of life. All newborns were born at 38-42 weeks of gestation and weighed over 2500 grams. Neonates with known risk factors such as major congenital malformations, sepsis, perinatal asphyxia, maternal diabetes, polycythemia, glucose-6-phosphate dehydrogenase (G6PD) activity deficiency, cephalic hematoma, dehydration, hypothyroidism, liver disease, Rh/subgroup and/or direct Coombs (DC) positive ABO incompatibility, or hemolysis for any reason were excluded from the study. Newborns in the control group were followed up for the development of jaundice, and in addition to determining the STB concentration, complete blood count, peripheral blood smear, blood group typing, DC and thyroid function tests were examined. In hyperbilirubinemia and prolonged jaundice groups, in addition to the above parameters, serum direct and indirect bilirubin levels, reticulocyte count, G6PD, liver function tests, thyroid function tests, urine culture and, if necessary, C-reactive protein (CRP) were examined. The study was approved by the Hacettepe University Faculty of Medicine Ethics Committee with Decision number 08/II dated 02 May 2011.

Genotyping procedure

Blood samples were taken from all cases and then placed in EDTA vacuum containers. Genomic DNA was isolated from peripheral leukocytes using the standard phenol-chloroform procedure. DNA was isolated using the QIAamp DNA blood kit (Qiagen, Hilden, Germany).

- **Variants of the genes *UGT1A1* (nucleotides 211), *SLCO1B1* (nucleotides 388, 463, 521, 1463) and *SLCO1B3* (nucleotides 334, 727+118, 1865+19721)**

  The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was applied to detect variants of *UGT1A1* at nucleotide 211, *SLCO1B1* at nucleotides 388, 463, 521, 1463 and *SLCO1B3* at nucleotides 334, 727+118, 1865+19721. PCR mixture (25 µL) consisted of 200 ng DNA, 0.2 mM of each dNTP, 120 nM of primer, 2.5 µL of 10 x buffer, and 1 µL of 50 mM MgCl2 solution. The final concentration of the PCR mixture was 1.5 mM MgCl2 in a volume of 100 µl of working solution. PCR amplification was performed in a DNA thermal cycler for 35 cycles of initial denaturation for 5 min, denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, primer extension for 1.5 min at 72 °C, and final extension for 7 min at 72 °C. The PCR product was digested with appropriate restriction enzymes and analyzed on 3% agarose gel (variants of the *UGT1A1*53 gene were examined for 53 subjects with idiopathic hyperbilirubinemia, 27 subjects with prolonged hyperbilirubinemia and 37 healthy subjects. Variants of the *SLCO1B1* / *SLCO1B3* genes were investigated in 61 subjects with idiopathic hyperbilirubinemia, 28 subjects with prolonged hyperbilirubinemia and 41 healthy subjects).

- **Variant of *GST* (*GSTP1*) gene**

  PCR-RFLP method was applied to detect the *GST* variant (*GSTP1*). PCR mixture (25 µL) consisted of 200 ng DNA, 0.5 µL of 10 mM dNTP, 0.6 µL of 10 mM *GSTP* primer, 0.12 µL of 10 mM albumin, 2.5 µL of 10 x buffer, 1.25 µL of 50 mM MgCl2 solution. The final concentration of the PCR mixture was 1.5 mM MgCl2 in a volume of 100 µl of working solution. PCR amplification was performed in a DNA thermal cycler for 35 cycles of initial denaturation for 5 minutes, denaturation for 1 minute at 94°C, annealing for 1 minute at 64°C, primer extension for 1 minute at 72°C and final extension for 7 minutes at 72°C. The PCR product was digested with appropriate restriction enzymes and analyzed on a 3% agarose gel as previously described (variants of

Table 1. Demographic characteristics of the study population

	Idiopathic Hyperbilirubinemia (n:61)	Prolonged jaundice (n:28)	Control (n:41)	*P*
Gestational age (weeks, mean± SD)	38.1 ± 1.0	38.5 ± 1.0	38.8 ±1.0	>0.05
Birth weight (g, mean ± SD)	3145 ±397	3154 ±390	3322 ±416	>0.05
Gender (Male/Female)	30/31	13/15	20/21	>0.05
Peak STB* level (mg/dl)	19.3 ± 1.9	-	7.1 ±2.4	0.0001
Peak time of STB (days)	5.2 ±1.9	-	5.1 ± 1.5	>0.05
Feeding pattern (breast milk/breast milk+formula)	56/5	26/2	37/4	>0.05

*, Serum total bilirubin

the *GST* gene were examined for 55 subjects with idiopathic hyperbilirubinemia, 28 subjects with prolonged hyperbilirubinemia and 40 healthy subjects.

Statistical analysis

All means are presented as means ± standard errors of the means. The weights and gestational ages of the groups were compared with one-way ANOVA. The Chi-square test was used to determine whether there were differences in gender distribution and genotype distribution between groups. Whether the quantitative data conformed to normal distribution was tested with the Shapiro-Wilk test or the Kolmogorov Smirnov test. Since the STB values of the patients showed a normal distribution, the data are given as mean ± standard deviation values. Differences were considered statistically significant when the *P* value was less than 0.05.

RESULTS

Demographic characteristics of the three groups are shown in Table 1. There was no difference between the groups in terms of mean gestational age and birth weight. There was no difference between the groups in terms of feeding patterns, gender and mode of delivery. There is a significant difference between the groups in terms of bilirubin levels.

The genotype distributions on the basis of nucleotides in these three groups are shown in Table 2. There was no significant difference in genotype distribution between the case and control groups (*P*>0.05 in all comparisons). There were no statistically significant differences in the prevalence of the variant of *UGT1A1* gene at nt 211, variants of *SLCO1B1* gene at nt 388, 463, 521 and 1463 variants of

Table 2. Frequency of allelic and genotypic polymorphisms in *UGT1A1*, *SLCO1B1*, *SLCO1B3* and *GSTP1* genes in case and control groups

	Genotype frequency n (%)				Allele frequency n (%)		
UGT1A1 rs4148323 G71R c.211A>G	**A/A**	**A/G**	**G/G**	**Total**	**A**	**G**	**Total**
IH	49 (92.4)	2(3.8)	2(3.8)	53 (100)	100 (94)	6(6)	106(100)
Prolonged jaundice	21 (77.8)	6 (22.2)	0	27(100)	48 (89)	6(11)	54(100)
Control	35 (92.1)	3 (7.9)	0	38(100)	73 (96)	3(4)	76(100)
P				0.14			0.26
SLCOIBI rs2306283 c.388A>G	**A/A**	**A/G**	**G/G**	**Total**	**A**	**G**	**Total**
IH	17(27.8)	35 (57.3)	9(14.9)	61(100)	69(57)	53 (43)	122(100)
Prolonged jaundice	7(25)	13 (46.4)	8 (28.6)	28(100)	27(48)	29 (52)	56(100)
Control	19 (46.3)	16 (39)	6(14.7)	41(100)	54(66)	28 (34)	82(100)
P				0.09			0.11
rsl1045819 c.463C>A	**C/C**	**C/A**	**A/A**	**Total**	**C**	**A**	**Total**
IH	47 (77)	13 (21.3)	1 (1.7)	61(100)	107 (88)	15 (12)	122(100)
Prolonged jaundice	20 (71.4)	7(25)	1 (3.6)	28(100)	47 (84)	9(16)	56(100)
Control	32 (82)	7(18)	0	39(100)	71(91)	7(9)	78 (100)
P				0.59			0.46

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rsl1045819 c.521T>C	T n	**T/C**	**C/C**	**Total**	**T**	**C**	**Total**
IH	38 (62.3)	21 (34.4)	2 (3.3)	61(100)	97(80)	25 (20)	122(100)
Prolonged jaundice	21 (75)	6(21.4)	1 (3.6)	28(100)	48 (86)	8 (14)	56(100)
Control	31(75.6)	9(21.9)	1 (2.5)	**41**(100)	71(87)	11(13)	82(100)
P				0.27			0.35
rs59502379 c. 1463G>C	**G/G**	**G/C**	**C/C**	**Total**	**G**	**C**	**Total**
IH	61 (100)	0	0	61(100)	122(100)	0	122(100)
Prolonged jaundice	28(100)	0	0	28(100)	56(100)	0	56(100)
Control *P*	40 (100)	0	0	40 (100)	80 (100)	0	80(100)
SLC01B3 rs4149117 c.334T>G	**T/T**	**T/G**	**G/G**	**Total**	**T**	**G**	**Total**
IH	2(3.4)	13 (22.4)	43 (74.2)	58(100)	17(15)	99 (85)	116(100)
Prolonged jaundice	0	6(21.4)	22 (78.6)	28(100)	6(11)	50 (89)	56(100)
Control	0	3 (7.5)	37(92.5)	40(100)	3(4)	77 (96)	80(100)
P				0.20			**0.03**
rsl7680137 c.727+118C>G	c/c	**C/G**	**G/G**	**Total**	**C**	**G**	**Total**
IH	46 (75.4)	11(18)	4 (6.6)	61(100)	103 (94)	19(16)	122(100)
Prolonged jaundice	19(67.8)	7(25)	2 (7.2)	28 (100)	45(80)	11(20)	56(100)
Control	33 (80.5)	7(17)	1 (2.5)	41 (100)	73 (89)	9(11)	82(100)
P				0.49			0.36
rs2117032 c. 1865+19721C>T	**C/C**	**C/T**	**T/T**	**Total**	**C**	**T**	**Total**
IH	17 (27.8)	29 (47.5)	15 (24.7)	61(100)	63 (52)	59 (48)	122(100)
Prolonged jaundice	6 (21.4)	13 (46.4)	9 (32.2)	28 (100)	25 (45)	31(55)	56(100)
Control	4 (9.7)	21(51.2)	16(39.1)	41 (100)	29 (35)	53 (65)	82(100)
P				0.07			0.07
GSTP1							
rsl695 c.313A>G	**A/A**	**A/G**	**G/G**	**Total**	**A**	**G**	**Total**
IH	28 (50.9)	18 (32.7)	9(16.4)	55 (100)	74(67)	36 (33)	110(100)
Prolonged jaundice	12(42.8)	13 (46.4)	3 (10.8)	28(100)	37(66)	19 (34)	56(100)
Control	18(45)	19(47.5)	3 (7.5)	40 (100)	55(69)	25 (31)	80 (100)
c				0.74			0.94
rsl138272 c.341C>T	**C/C**	**C/T**	**T/T**	**Total**	**C**	**T**	**Total**
IH	48 (87.3)	7(12.7)	0	55 (100)	103 (94)	7(6)	110(100)
Prolonged jaundice	21 (75)	7(25)	0	28(100)	49(88)	7(12)	56(100)
Control	29 (72.5)	11(27.5)	0	40 (100)	69(86)	11(14)	80(100)
P				0.15			0.19

IH, Idiopathic hyperbilirubinemia

SLCO1B3 gene at nt 334, 727+118, 1865+19721 variants of *GSTP1* gene at nt 313 and nt 341, and allele frequency of these genes (except *SLCO1B3* at nt 334) among the three groups ($P>0.05$ in all comparisons, Table 2). The G allele frequency of the variant of *SLCO1B3* gene at nt 334 of the infants in the idiopathic hyperbilirubinemia group was 85%, which was significantly lower than that in the control group (96%, $P=0.03$; Table 2). There was no statistically significant difference in the frequency of the G allele of the *SLCO1B3* variant at nt 334 between infants in the prolonged jaundice and control groups ($P=0.16$). No variant of the *SLCO1B1* gene was found at nt 1463 (rs59502379).

Clinical features among genotypes based on nucleotides

There was no significant difference in the mean peak bilirubin levels and the onset time of hyperbilirubinemia

Table 3. Peak bilirubin levels and peak time of hyperbilirubinemia in the idiopathic hyperbilirubinemia group according to genotypic distributions

	Genotype distribution			*P*
UGT1A1 rs4148323 G71R C.211A>G	**G/G**	***AJG***	**G/G**	
Mean peak bilirubin levels (mg/dL)	19.4 ±2.1	20 ±2.8	19.7 ±1.3	0.87
Peak time of hyperbilirubinemia (day)	5.1 ± 1.9	6 ± 1.4	8.5 ±3.5	0.06
SLCOIBI rs2306283 c.388A>G	**A/A**	***AJG***	**G/G**	
Mean peak bilirubin levels (mg/dL)	18.8 ± 1.2	19.5 ±2.3	19.2 ±1.5	0.42
Peak time of hyperbilirubinemia (day)	5.0 ±2.0	5.2 ±2.2	5.4 ± **1.1**	0.89
rsl 1045819 c.463C>A	**C/C**	**C/A**	**A/A**	
Mean peak bilirubin levels (mg/dL)	19.2 ± 1.6	19.9 ± 2.9	17 ±0	0.28
Peak time of hyperbilirubinemia (day)	5.1 ± 1.9	5.6 ± **2.4**	5.0 ±0.0	0.74
rsl 1045819 c.521T>C	**T/T**	**T/C**	**C/C**	
Mean peak bilirubin levels (mg/dL)	19.3 ± 2.2	19.3 ± 1.5	18.1 ±0.9	0.67
Peak time of hyperbilirubinemia (day)	5.5 ±2.1	4.7 ± 1.8	4.5 ±2**.1**	0.27
rs59502379 c.I463G>C	**G/G**	**G/C**	**C/C**	
Mean peak bilirubin levels (mg/dL)	19.4 ± 1.5	-	-	-
Peak time of hyperbilirubinemia (day)	5.2 ± 1.8	-	-	-
SLC01B3 rs4149117 c.334T>G	**T/T**	**G/T**	**G/G**	
Mean peak bilirubin levels (mg/dL)	20.3 ±2.1	18.8 ± 1**.1**	19.4 ±2.2	0.49
Peak time of hyperbilirubinemia (day)	6.0 ± 1.4	4.8 ±2.1	5.2 ±2.0	0.69
rsl7680137 e.727+118C>G	**C/C**	**C/G**	**G/G**	
Mean peak bilirubin levels (mg/dL)	19.3 ± 2.0	18.9 ± 1.4	19 ±2.4	0.77
Peak time of hyperbilirubinemia (day)	5.3 ± 2.2	4.9 ± 0.9	5.2 ±2.6	0.84
rs2117032 c.I865f 19721C>T	**C/C**	**C/T**	**T/T**	
Mean peak bilirubin levels (mg/dL)	19.4 ± 2.3	19.8 ± 2.0	18.7 ± 1.1	0.19
Peak time of hyperbilirubinemia (day)	5.0 ± 1.8	5.5 ± 2.3	4.8 ± 1.5	0.47
GSTP1 rsl695 c.313A>G	**A/A**	***AJG***	**G/G**	
Mean peak bilirubin levels (mg/dL)	19.8 ±2.1	19.2 ± 1.8	18.5 ± 1.6	0.25
Peak time of hyperbilirubinemia (day)	5.5 ± 1.9	5.3 ± 2.4	4.4 ± 1.3	0.40
rsl 138272 c.341C>T	**C/C**	**C/T**	**T/T**	
Mean peak bilirubin levels (mg/dL)	19.4 ± 2.0	18.9 ± 1.4	-	0.50
Peak time of hyperbilirubinemia (day)	5.2 ± 1.9	5.2 ± 2.6	-	0.98

among the newborn infants with the different genotypes based on the *UGT1A1* gene at nt 211, variants of *SLCO1B1* gene at nt 388, 463, 521 and 1463, variants of *SLCO1B3* gene at nt 334, 727+118 and 1865+19721, variant *GSTP1* gene at nt 313 and 341 in the idiopathic hyperbilirubinemia group (Table 3). Since all newborn babies with hyperbilirubinemia were given phototherapy, no conclusion could be reached about the duration of hyperbilirubinemia in this study (Table 3).

DISCUSSION

Neonatal jaundice is a good example of a complex condition in which the usual clinical factors examined alone cannot reveal the real cause. Although a few studies have been conducted to define the mechanisms involved in NH, more attention has recently begun to be paid to genetic factors as a contributing cause [8]. This study aimed to elucidate the contribution of multiple genetic modifiers affecting bilirubin metabolism to the development of pathological hyperbilirubinemia in Turkish newborns.

UGT is the key enzyme of bilirubin conjugation. 211G > A (Gly71Arg), one of the most common *UGT1A1* gene polymorphisms in the coding region, may cause unconjugated hyperbilirubinemia by reducing enzymatic activity [9]. Recently, in a meta-analysis of 21 studies including 4738 newborns, it was reported that *UGT1A1* Gly71Arg variation may increase the risk of NH in Asian and African children [10]. There are two studies investigating the relationship between hyperbilirubinemia and the G71R variant in Turkish newborns. Kilic et al. [11] showed the frequency of heterozygous G71R variants in pathological jaundice, prolonged jaundice and control groups as 8.7%, 20.8% and 4.3%, respectively; however, these differences were not statistically significant. Narter et al. [12] reported a frequency of 33.3% and 27.1% for heterozygous G71R variants and 7.7% and 5.7% for homozygous G71R variants in the hyperbilirubinemia and control groups, respectively, and no statistically significant difference was found between these frequencies. Our study showed that the frequency of heterozygous G71R variants in idiopathic hyperbilirubinemia, prolonged jaundice, and control groups was 3.8%, 22.2%, and 7.9%, respectively. But these differences were not statistically significant. The frequency of homozygous G71R variant in idiopathic hyperbilirubinemia was 3.8%. However, no homozygous G71R variant was found in the prolonged jaundice and control groups.

SLCO1B1/B3, the gene encoding the hepatic solute transporter organic anion transporter 1B1, a putative bilirubin transporter, may also be associated with increased susceptibility to NH by limiting bilirubin uptake [6, 13]. In our study, *SLCO1B1* A388G in both the heterozygous and homozygous variants was found 72.2%, 75% and 53.7% in the idiopathic hyperbilirubinemia, prolonged jaundice and control groups, respectively. Other studies of *SCLO1B1* in different countries showed similar findings to our study in terms of the incidence of polymorphism. In studies conducted on newborns with hyperbilirubinemia, the *SLCO1B1* A388G variant was found at a rate of 77.7% in India and 87.7% in Malaysia [14, 15]. All these studies show that more than half of the studied population has the A388G variant. Our study showed that the *SLCO1B1* A388G variant was not a significant risk factor for idiopathic and prolonged hyperbilirubinemia. There is tentative evidence from different studies in different populations regarding the association between *SLCO1B1* A388G and high bilirubin levels in newborns. In a study conducted on Taiwanese newborns in 2004 by Huang et al. [16], it was reported that A388G was seen more frequently in babies with hyperbilirubinemia. Liu et al. [17] found that the A388G variant in Chinese newborns was associated with hyperbilirubinemia in the Guangdong population, but not in the Yunnan population. In our country, Büyükkale et al. [18] reported that polymorphic forms of 388 nucleotides of the *SLCO1B1* gene were risk factors for idiopathic hyperbilirubinemia. Other studies conducted in Malaysian, Brazilian, American or Thai populations also failed to prove the association of the A388G variant with NH [15, 19-21].

In our study, there was no statistically significant difference in NH risk between *SLCO1B1* T521C allele carriers (T/C+C/C) and T/T allele carriers; the same result was observed in the *SLCO1B1* T521C variant when the T allele was compared with the C allele. A meta-analysis including five case-control studies (637 subjects with hyperbilirubinemia and 918 control subjects) from three countries examined the association between the *SLCO1B1* T521C variant and NH. This meta-analysis reported that the *SLCO1B1* T521C variant conferred protection for NH in the Chinese population but not in the Malaysian, Taiwanese, Brazilian, or American populations; when the T allele was compared with the C allele, the same situation was observed in the *SLCO1B1* T521C variant [17].

Analysis of the SLCO1B1 C463A variant in our study showed a crossover variant in 14 of 61 (23%) neonates with idiopathic hyperbilirubinemia (13 heterozygous and one homozygous) and in 8 of 28 (28.6%) neonates with prolonged jaundice (7 heterozygous and one homozygous), and 7 of 39 (24.75%) control subjects (all heterozygous). The *SLCO1B1* C463A variant was not a significant risk factor for idiopathic and prolonged hyperbilirubinemia in the present study. A meta-analysis of three case-control studies involving 286 cases of hyperbilirubinemia and 456 controls from three countries examined the association between the *SLCO1B1* C463A variant and NH [17]. In studies conducted on Taiwanese and Thai newborns, carriage of the C to A substitution at nucleotide 463 was not detected [16, 21]; however, the study conducted by Watchko et al. [20] including American subjects showed that in those 31 of 153 newborns (20.26%) with hyperbilirubinemia (one homozygous and 30 heterozygous) and 74 of 299 control subjects (24.75%) (nine homozygous and 65 heterozygous). In that study (20), no statistically significant difference was found in the risk of NH between allele carriers (C/A+A/A) and (C/C) in the *SLCO1B1* C463A variant; the same situation was observed when the A allele in the SLCO1B1 C463A variant was compared with the C allele; the same situation was observed when the A allele was compared with the C allele in the *SLCO1B1* C463A variant.

There was no mutant allele in *SLCO1B1* at Nt 1463 G>C. However, according to our research, no study investigating this variant has been found in the literature.

Similar to *SLCO1B1* polymorphisms in this study, SLCO1B3 polymorphisms did not show statistical differences in genotype distribution. It was found in the present study that only the G allele at nt 344 of *SLCO1B3* may be a protective factor for idiopathic NH. However, it does not

protect against prolonged jaundice. No study investigating the relationship between *SLCO1B3* nt 334 variant and NH has been found in the literature. However, there are very few studies on the C727+118G and C1865+19721T polymorphisms of this gene. Alencastro de Azevedo et al. [19] reported that the allelic and genotypic frequencies of *SLCO1B3* gene C727+118G and C1865+19721T variants did not differ between idiopathic hyperbilirubinemia and control groups. They stated that only the T allele at nt 1865+19721 could be protective against NH in those without ABO incompatibility.

During the bilirubin conjugation process in hepatocytes, bilirubin binds to GST, also known as ligandin [22]. GST binds both bilirubin and bilirubin conjugates and reduces hepatocyte reflux into plasma [23]. The present study found that there is no statistically significant difference in the genotype and allele frequencies of *GSTP1* A313G and C341T among the idiopathic hyperbilirubinemia, prolonged jaundice, and control groups. Muslu et al. [24], from Türkiye, reported that the frequencies of *GSTM1* and *GSTT1* were similar in newborns with hyperbilirubinemia and control groups.

In this study, no significant difference was found in terms of peak total bilirubin levels and onset time of hyperbilirubinemia in newborns in the idiopathic hyperbilirubinemia group with different genotypes.

The limitation of the present study is that the need for phototherapy was taken as a criterion, which does not have a strong discriminatory feature in determining genetic variability differences. Another limitation of our study is the small number of cases.

CONCLUSION

The role of *UGT1A1*, *SLCO* and *GST* polymorphisms in neonatal jaundice is still controversial and deserves further attention. Although they do not have the power to modulate neonatal jaundice, other genes that play a role in bilirubin metabolism (heme oxygenase and biliverdin reductase) other than the genes mentioned above can be investigated.

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DECLARATION OF INTEREST

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

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CASE REPORT

EXPANDING THE PHENOTYPIC SPECTRUM: CHRONIC KIDNEY DISEASE IN A PATIENT WITH COMBINED OXIDATIVE PHOSPHORYLATION DEFECT 21

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ABSTRACT

Introduction

Pathogenic variants in *TARS2* are associated with combined oxidative phosphorylation deficiency 21 (COXPD21), an autosomal recessive disorder usually presenting as mitochondrial encephalomyopathy. Kidney impairment has been documented in a minority of COXPD21 patients, mostly with distal renal tubular acidosis.

Case report

We report on the first COXPD21 patient with generalized tubular dysfunction and early childhood progression to chronic kidney disease (CKD). Thorough diagnostic evaluation was initiated at six months of age due to failure to thrive, muscular hypotonia, motor delay and recurrent bronchiolitis. The boy was lost to follow-up until the age of two years, when he was readmitted with elevated creatinine level, reduced estimated glomerular filtrate rate, normochromic anaemia, metabolic acidosis and hyperkalaemia. Urine abnormalities pointed to generalized tubular dysfunction. Two novel heterozygous missense variants in *TARS2* gene were detected by the means of whole exome sequencing: c.1298T>G (p.Phe438Cys) of maternal origin and c.1931A>T (p.Asp644Val) of paternal origin. Currently, at 4.5 years of age, the boy has failure to thrive, severe motor and verbal delay and end stage of CKD. We referred the patient to paediatric centre that provides renal replacement therapy.

Conclusion

The overall clinical course in the patient we report on corresponds well to the previously reported cases of *TARS2* related COXPD21, especially in regard to neurological and developmental aspects of the disease. However, we point out the generalized tubulopathy and early occurrence of CKD in our patient as atypical renal involvement in COXPD21. Additionally, this is the first report of hypothyroidism and hypoparathyroidism in a COXPD21 patient.

Keywords: Mitochondrial disease; tubulopathy; *TARS2* gene

INTRODUCTION

Pathogenic variants in *TARS2* are associated with combined oxidative phosphorylation deficiency 21 (COXPD21), an autosomal recessive disorder most commonly presenting as mitochondrial encephalomyopathy (MIM# 615918) (1). The *TARS2* gene (MIM# 612805) encodes mitochondrial threonyl tRNA-synthetase. To the best of our knowledge, less than 30 patients of this particular mitochondrial disorder have been reported on to date (2,3). The main clinical features of COXPD21 include failure to thrive/growth retardation, developmental delay, axial hypotonia, hypertonus of the limbs, dystonia, seizures, and laboratory findings of lactic acidosis and elevated plasma alanine (1-4). Various pathological brain MRI findings have also been reported, such as high signal lesions in the basal ganglia and thalami, white matter volume loss, cortical atrophy, midbrain, and cerebellar atrophy (2,4,5). Metabolic crises are considered as potentially devastating

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aspects of COXPD21 (1). Early mortality was predominantly observed in children harbouring biallelic null mutations in the *TARS2* (3).

The prevalence of renal involvement in patients with primary mitochondrial disorders has been estimated to range from 25% to 50% (6). The most common renal phenotype in these patients is proximal tubulopathy with or without complete Fanconi syndrome, but a spectrum of manifestations has been described as well, including chronic kidney disease (CKD), distal tubular defects, focal segmental glomerulosclerosis, steroid resistant nephrotic syndrome, renal cysts, nephrocalcinosis and others (6-8). However, kidney impairment has been documented in only seven COXPD21 patients, presenting with distal renal tubular acidosis (2,4). A progress into chronic kidney disease (CKD) occurred in one patient at 17 years of age (4). Herein, we report the first COXPD21 patient with generalized tubular dysfunction and early childhood progression to CKD.

PATIENT REPORT

The patient is the first child of non-consanguineous parents (father has hypothyroidism and mother was treated for anxiety disorder), born from an uneventful pregnancy. Birth weight was 4.2 kg, and the perinatal period was normal. In early infancy, poor weight gain was observed. Thorough diagnostic evaluation was initiated at six months of age due to failure to thrive, muscular hypotonia, motor delay and recurrent bronchiolitis. The laboratory analyses revealed hyperlactatemia (2.53 mmol/l), hypokalaemia (2.8 mmol/l), absence of other electrolyte abnormalities in serum (table 1) and tubular loss of potassium and magnesium (table 2). The aldosterone level was normal. Serum creatine level and estimated glomerular filtration rates were normal at the time of the first evaluation. The ultrasound exam showed hyperechoic, normal sized kidneys.

After discharge he was lost to follow-up until two years of age when he was readmitted with an elevated creatinine level, reduced estimated glomerular filtrate rate, normochromic anaemia, metabolic acidosis and hyperkalaemia. Urine abnormalities pointed to tubular dysfunction (table 2). Aldosterone, renin and cortisol concentrations were within normal range, while decreased plasma concentration of parathyroid hormone (PTH) was accompanied by normal calcemia.

The association of chronic renal disease with developmental delay of unknown aetiology prompted genetic testing. Two novel heterozygous variants in the *TARS2* gene were detected by means of whole exome sequencing: c.1298T>G (p.Phe438Cys) missense variant derived from the mother and the c.1931A>T (p.Asp644Val) missense

Table 1. Key laboratory findings in serum/plasma over the course of the disease in a patient with COXPD21

Laboratory findings in blood	6 months of age	2 years of age	4.5 years of age	Reference range
Blood gases				
pH	7.4	7.39	7.42	7.35-7.45
HCO3m mmol/L	20.0	16.4	24.2	21-28
Base excess, mmol/L	-4.5	-8.5	-0.2	-2-+3
Biochemistry				
Serum creatinine, mcmol/L	24	111	274	23-37
GFR*, ml/min	116.4	32	17	≥90
Cystatin C, mg/L	1.16	2.22	4.5	0.62-1.2
Urea, mmol/L	4.9	15.0	35.0	3.3-7.5
Uric acid, mcmol/L	272	238	148	120-320
Potassium, mmol/L	2.8	6.8	5.8	3.4-4.7
Sodium, mmol/L	136	121	146	136-148
Calcium, mmol/L	2.71	2.27	2.38	2.05-2.74
Magnesium, mmol/L	0.88	0.67	0.90	0.7-1.05
Phosphorus, mmol/L	2.2	0.96	2.88	1.05-1.80
Lactate, mmol/L	2.53	2.15	2.0	0.2-2.0
Hormonal status				
Parathyroid hormone, pg/mL	26.3	3.3	17.5	15.8-68.3
25-hydroxyitamin D, nmol/L	47.8	58.9	18.0	75.0-250.0
Thyroid stimulating hormone, mIU/L	1.06	3.58	9.17	0.35-4.94
Free T4, pmol/L	16.63	12.7	8.98	9.0-19.0

*GFR – glomerular filtration rate

Table 2. Results of urinary analyses over the course of the disease in a patient with COXPD21

Urinalysis	6 months of age	2 years of age	4.5 years of age	Reference Range
pH	6.0	6.5	6.5	5-8
Urine- Specific gravity	1010	1010	1005	1010-1030
Urine protein, g/L	<0.1	<0.1	<0.1	<0.1
Urine glucose, mmol/L	Negative	Negative	5.5	Negative
Urine blood, RBC/uL	Negative	Negative	Negative	Negative
Urine white blood cells	Negative	Negative	Negative	Negative
Protein/Cr, mg/mmoL	20	30	52	<50
Aminoaciduria	Negative	Negative	Generalized	Negative
Beta-2 microglobulin/Cr, mg/mmoL	0.14	0.34	26.0	<0.35
Calcium/Cr, mmol/mmol	0.26	0.21	1.64	*
Uric acid/Cr, mmol/mmol	1.4	0.35	0.08	*
FeNa, %	0.8	3.1	32	<1
FeMg, %	6.65	4.98	38.4	<4
TTKG	9	3	2	4-6*
TRP, %	93.8	81.2	45	85-95
TmP/GFR, mmol/L	1.41	1.64	0.78	1.13-1.88

Cr – creatinine
FeNa – Fractional excretion of sodium
FeMg – Fractional excretion of magnesium
*TTKG – trans-tubular gradient of potassium (>4% suggests kidney losses in hypokalaemic patient; <7% indicate hypoaldosteronism in hyperkalaemia)
TRP – tubular reabsorption of phosphate
TmP/GFR - tubular maximum phosphate reabsorption per glomerular filtration rate
*Ca/Cr, mmol/mmol - (< 2.2 for <12 months; <1.5 for 1 to 3 years; <1.1 for 3 to 5 years)
*Uric acid/Cr, mmol/mmol - (1.5 for < 1 years; 1.3 for 1 to 3 years; 1.0 for 3 to 5 years)

variant derived from the father). At the age of 2.5 years the boy, was admitted due to generalized seizures associated with fever, severe metabolic acidosis and electrolyte imbalance (hyponatremia of 121 mmol/L, hypomagnesemia 0,5 mmol/L and hypophosphatemia 0,5 mmol/L). At the time he had elevated serum lactate (3.61 mmol/L). Pneumonia was verified, and during the treatment acute pancreatitis developed with hyperglycaemia requiring the insulin therapy. A moderate progression of chronic kidney disease was noted during this metabolic crisis (creatinine 164 mcmol/L) with subsequent polyuria. Urinary analysis showed persistent tubular dysfunction. The boy's overall condition deteriorated into encephalopathy. A brain MRI examination detected a pathologically increased signal of the brain parenchyma on T2/DVI/FLAIR sequences, in the projection of the basal ganglia, thalamus, hippocampus, substantia nigra, mesencephalic crus bilaterally, as well as in the posterior aspects of the pons and white matter of the cerebellum (figure 1). Mild supratentorial ventriculomegaly was described as well as a consequence of brain parenchyma reduction in the patient. An electroencephalogram showed diffuse slowing of basal activity of delta type without clear epileptic discharge. Slow recovery of neurologic functions ensued over the course of several weeks, while hyperglycaemia proved to be transitory, thus the insulin treatment was stopped.

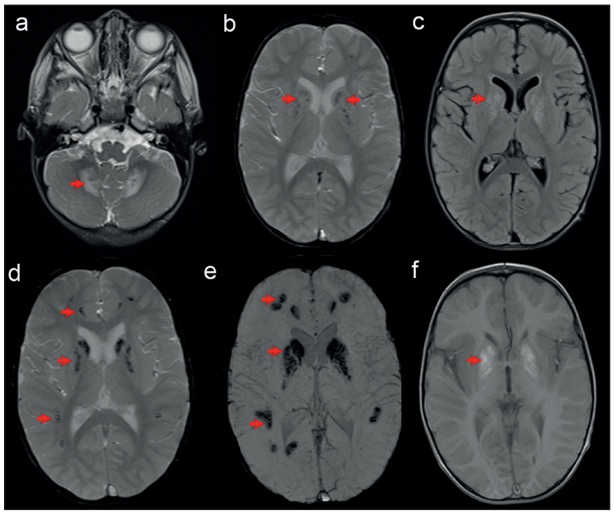

Figure 1. 1.5T Brain MRI of the patient: a-c were performed at the age of 2.5 years; a- pathologically increased signal of the brain parenchyma on T2/DVI/FLAIR sequences, in the projection of the white matter of the cerebellum; b- in the projection of nucleus caudate, globus pallidus, putamen and dentate nuclei there are punctiform and slightly linear zones of reduced signal intensity on the chemo-sensitive sequence, which are hyperintense on T1WI (c); d-f Brain MRI were performed at the age of 4 years; d- in the same regions, there are significantly larger hypointense signal lesions on T2WI/FLAIR sequences and SWI sequences (e), hyperintense on T1WI (f), corresponding to punctiform and serpiginous calcification zones. Mild ventriculomegaly is shown on these sequences.

Treatment during the disease included dietary modification, erythropoietin, calcitriol, ion-exchange resin, a "mitochondrial cocktail" of supplements and vitamins, and antiseizure medication. Thyroxine supplementation was introduced in response to hypothyroidism, detected at 4 years of age during a regular follow-up visit. Dosage of thyroxine had to be adjusted over time due to the worsening of thyroid function. Currently, at 4.5 years of age, the boy weighs 11 kg (below 3 standard deviations), has severe motor and verbal delay, and end stage CKD with generalized tubulopathy. We referred the patient to another paediatric centre that provides renal replacement therapy. The dynamics of the serum/plasma and urine laboratory findings during the course of the disease is presented in table 1 and table 2.

DISCUSSION

The clinical presentations of the patients we have reported on so far are mostly in accordance with other cases of COXPD21 (1-5.) The key clinical features of this particular mitochondrial disorder (failure to thrive, development delay, muscle tone abnormalities, epilepsy) are all present in our patient. A spectrum of brain MRI changes has been described in patients with COXPD21. Therefore, our patient's brain MRI scan revealed basal ganglia hyper intensity and generalized atrophy, resembling some of the more extensive findings thus reported (2,5).

Our report focusses on the renal aspect of a patient's phenotype, since the kidney's involvement in COXPD21 has been described in seven cases so far (2,4). In one of the reports, the male patient of Syrian descent presented with renal tubular acidosis, diagnosed at 6 months of age along with delayed psychomotor development (4). Nephrocalcinosis was observed at 12 months of age. In his early teenage years, the ultrasound showed small and scarred kidneys; the same patient was diagnosed stage III of CKD at 17 years of age. In the largest case series of COXPD21, a third of the patients had distal renal tubular acidosis (2). According to the data, none of them developed chronic kidney disease, despite half of them being of adult age. In contrast to previously reported patients, CKD was already present at two years of age and progressed into the end stage by the age of four. Moreover, the observed generalized tubulopathy differs our patient from COXPD21 patients with isolated renal tubular acidosis as the main tubular dysfunction. However, we can speculate that the origin of tubulopathy stems both from primary mitochondrial disorder and from advanced CKD itself.

Defects in the mitochondrial oxidative phosphorylation system are well-known genetic causes of renal dysfunction. Even though renal impairment can be the presenting feature of mitochondrial diseases, it is more commonly seen after the onset of neurological manifestations (9). Renal impairment can occur at any age in patients with mitochondrial disease, but the median age of this specific organ involvement has been estimated at 12 years (6). Typically, laboratory abnormalities of urine detected during regular patient check-ups are the first sign of kidney involvement in these patients. In our patient, at the very early age of six months, tubular loss of potassium and magnesium was verified. This indicated potential dysfunction of either thick ascending limb of the loop of Henle or distal tubule, even before the rise of urinary beta-2 microglobulin. Initially, there were no signs of proximal tubular dysfunction. Impairment of renal function was verified at two years of age in our patient and was consistent with both proximal and distal tubular dysfunctions. Full expression of Fanconi syndrome has been previously associated with mtDNA deletion syndromes, but also with diseases caused by nuclear DNA mutations affecting mitochondrial functioning (6,10). The presence of glycosuria, hyperphosphaturia, generalized aminoaciduria and low-molecular-weight proteinuria in our patient was detected at the end stage of CKD.

The progression of chronic kidney disease in our patient seemed to be facilitated during severe metabolic crisis occurring at the age of 2.5 years. Impairment of both proximal and distal renal tubules has been designated as generalized tubulopathy and described as such in several patients with mitochondriopathies (6). Interestingly, severe tubular dysfunction has been associated mostly with large mtDNA deletions, thereby contrasting to our patient's disease, caused by the autosomal-recessive mutations in nuclear *TARS2* gene. However, there have been reports of tubular impairment and chronic kidney disease in patients with mutations in *SARS2* gene which encodes mitochondrial seryl-tRNA synthetase (11). This autosomal recessive mitochondrial disease, caused by *SARS2* mutations, has been designated as HUPRA, an acronym based on the hallmarks of the disease (hyperuricemia, pulmonary hypertension, renal failure, alkalosis). Despite certain basic similarities in terms of abnormal mitochondrial protein translation in both HUPRA and COXPD21, clinical presentations of the two diseases are distinct. Renal impairment has been the hallmark of HUPRA, while only a quarter of COXPD21 cases published so far had kidney involvement. The patient presented herein is the second verified case of CKD in COXPD21. The overall clinical course is similar to that of our patient. Tubular dysfunction and early onset of CKD has been observed in several patients with mitochondrial genome mutations affecting tRNA synthesis (7,12). In a plethora of patients with respiratory chain assembly and function defects, kidney

involvement was noted as part of the multisystem disease, with proximal tubulopathy being the most commonly encountered renal phenotype (8).

The genotype of our patient includes two variants of unknown significance in *TARS2* gene detected by whole exome sequencing. After confirmation of combined heterozygosity of the proband, we performed a segregation analysis, proving carriership of single variants in the parents. Both variants we found in *TARS2* are designated as missense. According to one literature review of COXPD21 cases, carriers of biallelic missense variants in *TARS2* had later disease presentation with longer survival (3). However, a more recent study depicting 18 new patients with COXPD21 denied presence of any meaningful genotype-phenotype correlation (2). Although our patient had failure to thrive and delayed development early in infancy, his overall condition remained relatively stable over time, apart from one serious metabolic crisis at the age of 2.5 years. However, early and progressive CKD represents a key clinical feature in this particular patient, despite being non-typical for COXPD21.

The presence of hypothyroidism in our patient has been demonstrated by elevated thyroid stimulating hormone level and low free thyroxine in blood. A neonatal screening test for congenital hypothyroidism was previously negative. The level of thyroid peroxidase antibodies remained low, suggesting that the occurrence of hypothyroidism is most probably the part of the multisystem presentation of mitochondrial disease. Endocrine abnormalities represent one of the more prominent clinical features of mitochondrial diseases with hypothyroidism being present in approximately 6.3% of patients (13). Interestingly, defects in nuclear genes encoding mitochondrial protein pose a lower risk for hypothyroidism, when compared to mtDNA mutations (2.9% and 8.5%, respectively). The findings of the WES did not reveal any other genetic variant that could be causative to hypothyroidism. Hypothyroidism in the father is caused by Hashimoto thyroiditis and is most probably not related to the hypothyroidism in the proband. Subnormal levels of PTH found in context of overt CKD suggest hypoparathyroidism in our patient. Insufficiency of parathyroid secretion has been well established occurrence in mitochondrial disorders (14), but not in COXPD321 so far (2,3).

The overall clinical course in the patient we report on corresponds well to the previously reported cases of *TARS2* related COXPD21, especially in regard to neurological and developmental aspects of the disease. However, we point out the early occurrence CKD in our patient since it has been previously described in only a single case of COXPD21. The presence of overt hypothyroidism and hypoparathyroidism are additional phenotypic features that have not been reported on in patients with this specific mitochondrial disorder before. We hope that this case report will add to the deeper knowledge of the phenotypic spectrum of COXPD21.

AUTHOR'S CONTRIBUTION

Aleksandra Paripović wrote the manuscript and reviewed the literature. Nataša Stajić, Jovana Putnik, Slavica Ostojić, Biljana Alimpić, Nikola Ilić, Aleš Maver and Adrijan Saralija critically revised the contents and wrote specific parts of the manuscript. Aleksandra Paripović, Nataša Stajić, Jovana Putnik and Adrijan Sarajlija conceived the original idea and supervised the final draft.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflict of interest.

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CASE REPORT

EPHA4 GENETIC VARIANT IN A PATIENT WITH EPILEPSY, OPHTHALMOLOGICAL ANOMALIES, AND NEURODEVELOPMENTAL DELAY

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ABSTRACT

We present the findings of a Whole Exome Sequencing in a 2-year-old boy, conceived via *In Vitro* Fertilization with donor sperm, who suffers from an undiagnosed neurological syndrome. The following heterozygous variant in the *EPHA4* gene was identified and classified as likely pathogenic: c.1655_1656, p.(Ser552CysfsTer23). Subsequent segregation analysis showed that the variant was not inherited from the mother and the sperm donor is not accessible for genetic testing. The presented results can further expand upon the genetic variants considered when diagnosing complex neurological syndromes and shows the importance of access to biological samples from donor banks in genetically ambiguous cases.

Keywords: *EPHA4*, Whole Exome Sequencing, Epilepsy, Novel genetic variant, Ophthalmological Anomalies, Neurodevelopmental Delay

INTRODUCTION

The clinical presentation of most early-onset neurological disorders is ambiguous due to their heterogeneous manifestation and symptom non-specificity (1). In recent years, genetic testing has become a useful diagnostic tool for identifying genetic mutations associated with rare neurological disorders. However, even Whole Exome Sequencing (WES) – a technique which allows for analysis of all exons in a patient's genome – often results in the identification of multiple genetic variants which may potentially explain a patient's complex clinical picture. In such cases, segregation analysis becomes an indispensable method for clarifying the significance of the variants. The diagnostic process is further complicated if one of the parents is not available for segregation analysis, which is the case in *In Vitro* Fertilization (IVF) with donor material.

In the following case study, we present a patient with a complex neurological syndrome with accompanying facial abnormalities, who was conceived through IVF with donor sperm. Via analysis of the WES data one heterozygous genetic variant in the *EPHA4* gene was selected as a target.

The Ephrin Receptor A4 (*EPHA4*) gene, located on the long arm of human chromosome 2 (2q36.1), is a protein encoding gene producing a Protein Tyrosine Kinase (PTK) receptor. Although within the Central Nervous System (CNS) *EPHA4* has been implicated in processes such as neural migration, axonal proliferation, and synaptic plasticity (2), its pathogenicity in clinical practice is not well understood. In humans, thus far, only one germline likely pathogenic missense point mutation has been reported in a male patient with atypical cerebral palsy (3) and Van Hoecke *et al.* (4) showed that decreased *EPHA4* expression was significantly correlated with later onset of Amyotrophic Lateral Sclerosis (ALS). Light *et al.* (2) have reported several somatic genetic variants in relation to melanoma tumors. On the other hand, studies with animal models, ranging from rodents to primates, have shown that EphA4 expression plays a role in various severe CNS disorders. Fu *et al.* (5) showed that blocking EphA4 activity

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in mice had a positive effect on the hippocampal plasticity, typically ravaged by Alzheimer's disease. Goldshmit and Bourne (6) found that astrocytic upregulation of EphA4 in non-human primates has an indirect inhibitory effect on axonal regrowth and regeneration following a Traumatic Brain Injury (TBI).

CASE PRESENTATION

A 2-year-old Caucasian male was referred to our laboratory for genetic clarification of a non-specified neurological syndrome with developmental delay and facial abnormalities. The case history revealed that the patient was conceived via IVF with sperm from an unidentified Caucasian donor. The mother was healthy with no neurological disorders or genetic abnormalities, and there were no complications during pregnancy and birth (patient birth weight and height – 3490g and 50cm, respectively).

Anamnesis revealed that at approximately 4 months of age the patient's condition started deteriorating as indicated by delayed psychomotor, cognitive, and visual development. At around the same time, the patient started suffering from grand-mal seizures. At approximately 7 months of age, the patient already exhibited severe drug-resistant epilepsy, significant psychomotor retardation despite physiotherapy, limb hypertonia, loss of pupillary light response, and nearly complete loss of visual acuity, which rendered him effectively blind. He also exhibited peculiar dysmorphic facial features with hypertelorism, micrognathia, and unusually low auricles. During the following 5 months he suffered a number of additional medical complications including abnormal elevation of Vitamins B1 and B12, pneumonia, and anemia, some of these complications led to hospitalization.

Although the patient's epilepsy and eye abnormalities have continued to aggravate, multiple neurological and ophthalmological examinations have revealed no apparent cause of the patient's complex medical state. Furthermore, subsequent metabolic and biochemical blood tests were negative for lysosomal enzymes, Very Long Chain Fatty Acids (VLCFA), amino acids and acylcarnitines, and 3-O-Methyldopa (3-OMD). A dry blood spot test for Neuronal Ceroid Lipofuscinosis (NCL) was also negative. Initial genetic testing has shown a normal karyotype, no mutations in a targeted 341 gene retinal degeneration-related panel, and no mutations in the mitochondrial genome. Following from the patient's increasingly worsening condition and the absence of any effective treatment, he was referred for WES in hopes of determining his diagnosis.

METHODS

After a detailed explanation of all procedures, a written informed consent was obtained from the patient's mother, and all described medical procedures and analyses were conducted in accordance with the Declaration of Helsinki and the ethical guidelines of Medical University Sofia. The Ethics Committee of Medical University Sofia has approved this study.

A blood sample was taken from the patient and subjected to DNA extraction by standard salting-out procedure (7). WES was performed and the patient's genetic profile was analyzed via the GenesearchNGS software (Phenosystems). The detected variants were interpreted with respect to their pathogenicity following the recommendations of the American College of Medical Genetics and Genomics/ Association for Molecular Pathology (ACMG/AMP) (8). Direct Sanger sequencing was performed with BigDye® Terminator cycle sequencing kit v.3.1 (Applied Biosystems, Foster City, CA, USA) for confirmation of the WES findings and for Segregation analysis in order to determine the variants' inheritance. Due to the ethical standards of IVF with donor sperm, no genetic analyses of the biological father were possible.

RESULTS

We identified a heterozygous frameshift variant c.1655_1656del, p.(Ser552CysfsTer23) in the *EPHA4* gene (NM_004438.5). This variant is classified as likely pathogenic (categories: PVS1 and PM2) (8). Segregation analysis showed that the patient's mother is not a carrier of the genetic variant. The biological father is not accessible for genetic testing due to ethical and legislative issues as well as the anonymous process of sperm donation for IVF procedures.

DISCUSSION

Mutations in the *EPHA4* gene are compatible with optic nerve defects and severe abnormalities in the central nerve system. The reasons behind our focus on the genetic variant c.1655_1656del, p.(Ser552CysfsTer23) in the *EPHA4* gene are described below.

MetaDome (9) indicates that the identified *EPHA4* genetic variant is located in the beginning of the protein's transmembrane domain (IPR027936; Figure 1). This type of domain is characteristic for ephrin receptors and is responsible for the oligomerization of these receptors and for successful signaling (IPR027936) (10). Due to this

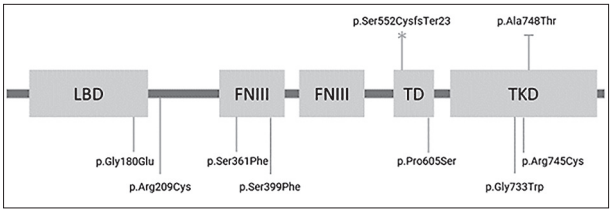

Figure 1. The genetic variant found in our patient and reported genetic variants presented in relation to the Epha4 protein domains. Above the protein domains are shown the genetic variant found in our patient (marked with *) and the likely pathogenic variant reported in ClinVar (marked with -), and below the protein domains - the somatic genetic variants reported by Light *et al.* (2). Domain names are the following: LBD – Ligand Binding Domain; FNIII – Fibronectin type III domain; TD – Transmembrane Domain and TKD – Tyrosine Kinase Domain. The protein domain structure is created based on MetaDome (9).

localization, the frameshift variant could affect the protein's ability to integrate into the cell membrane and function as an ephrin receptor in the CNS. To the best of our knowledge, this is the first identification of a frameshift genetic variant in the transmembrane domain of the EphA4 protein. The only reported likely pathogenic variant in ClinVar (3) falls within the kinase region of the protein.

Moreover, the genetic variant is not found in the gnomAD v2.1.1 controls and *EPHA4*'s pLI score is 1, which indicates that the gene is highly intolerant to loss of function variants (11,12). As the genetic variant does not abide the 50-55 nt boundary rule (13), we can assume that the produced mRNA undergoes nonsense-mediated decay. It has been suggested previously that mutations in ephrin receptor genes, causative of nonsense-mediated decay, lead to pathogenesis (14).

Recently, a number of *EPHA4*-cases emerged in the GeneMatcher platform (15), helping understand the pathophysiology of severe neurological cases having *EPHA4* gene variants in common. Based on this new understanding, the variation in *EPHA4* seems highly compatible with our patient's clinical manifestation. Unfortunately, we cannot confirm that the genetic variant has occurred *de novo*, due to the lack of a paternal sample. Furthermore, Oliver *et al.* (16) have published a comprehensive list of epilepsy-related genes, wherein *EPHA4* is not included. With this study we bring attention to the *EPHA4* gene as a potential target for additional functional studies in association with neurodevelopmental disorders, including epilepsy. Moreover, we emphasize on the difficulties in classifying genetic variants when DNA from donors is not available for genetic testing.

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CONFLICT OF INTEREST STATEMENT

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

DATA AVAILABILITY STATEMENT

The genetic variant reported in this study is openly available in ClinVar with accession number: SCV003925746.

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CASE REPORT

MISDIAGNOSIS OF TRACHER-COLLINS SYNDROME INITIALLY ATTRIBUTED TO DRUG TERATOGENICITY: A MOROCCAN CASE REPORT

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ABSTRACT

Background

Treacher Collins syndrome (TCS) is a rare congenital disorder of craniofacial development characterized by numerous developmental anomalies that are restricted to the head and neck. Most TCS cases are inherited in an autosomal dominant manner. The diagnosis of TCS relies on clinical and radiographic findings. The four genes involved in TCS are *TCOF1*, *POLR1D, POLR1C,* and *POLR1B*.

Case presentation

In this report, we present the case of a 7-year-old Moroccan boy who exhibited distinctive dysmorphic features, including coloboma and zygomatic bone hypoplasia. Upon genetic analysis, a mutation in the *TCOF1* gene was identified, conclusively confirming the presence of Treacher Collins Syndrome. It is worthy that the correct etiological diagnosis was significantly delayed due to the initial misperception that the observed malformation syndrome was a result of drug teratogenicity.

Conclusions

This case highlights the importance of seeking pharmacovigilance advice if any adverse event occurs following medication use. Furthermore, requesting a genetic consultation to establish a confirmed etiological diagnosis for any malformation syndrome can significantly reduce the protracted social and psychological suffering that patients and their families may endure.

Keywords: Genetic consultation, Pharmacovigilance, *TCOF1* gene, Teratogenicity, Treacher Collins syndrome

INTRODUCTION

Treacher Collins syndrome (TCS, OMIM # 154500), also known as mandibulofacial dysostosis and Franceschetti-Zwahlen-Klein syndrome, is a rare congenital disorder of craniofacial morphogenesis with an estimated prevalence of 1/50000 births [1]. Most TCS cases are inherited in an autosomal dominant manner. Typical cases of TCS are characterized by four major clinical manifestations**,** hypoplasia of the zygomatic bones and mandible, external ear abnormalities, lower eyelid abnormalities, and family history consistent with autosomal dominant inheritance. The four genes involved in TCS are *TCOF1*, *POLR1B, POLR1C,* and *POLR1D*.

In this report, we present the case of a 7-year-old Moroccan boy exhibiting characteristic traits of TCS. Unfortunately, an initial misdiagnosis, which erroneously attributed the child's condition to the mother's medication, led to profound psychological and social challenges for the family. Subsequent molecular genetic testing definitively confirmed the presence of TCS.

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CASE PRESENTATION

The patient is a 7-year-old male, first child of Moroccan consanguineous parents (first-degree), aged of 29-year-old for the mother and 37-year-old for the father. Pregnancy and delivery were normal, and the child was born at term with normal physical measurements. The mother had no history of abdominal trauma or radiographic examination, but she had been taking an antidepressant treatment based on trimipramine Surmontil® during the first two months of pregnancy. The child had normal psychomotor development and has been schooled with good follow-up.

Upon general examination at 7 years, the patient's body weight was 23 Kg (50th percentile), head circumference 45 cm (50th percentile), and height 123 cm (70th percentile). He was dysmorphic with coloboma of the lower eyelids, downslanting palpebral fissures, missing eyelashes, and bilateral symmetrical hypoplasia of the zygomatic bones (Fig. 1). The rest of his physical examination was normal; in particular, he has no ear abnormalities. Ophthalmological examination revealed a corneal ulcer with palpebral coloboma, otorhinolaryngol-scopic and dental examinations were normal.

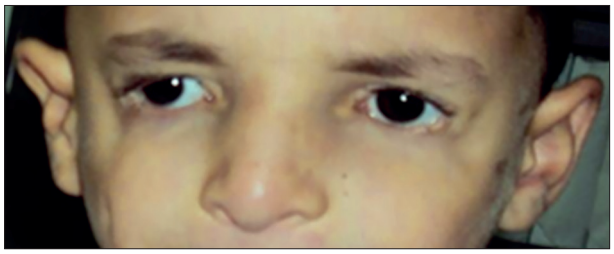

Figure 1. Patient photograph showing dysmorphic facies.

From the very first months of their child's life, the parents keenly observed facial dysmorphia. Upon seeking medical advice, the consulting pediatrician attributed this dysmorphia to the antidepressant treatment that the mother had undergone during her pregnancy. At that time, neither a genetic consultation nor a pharmacovigilance assessment was sought.

The attribution of the child's condition to the mother's medication has precipitated profoundly distressing psychological and social consequences. This includes a deterioration in the mother's depressive syndrome, characterized by self-blame, self-indignation, and a substantial decline in her self-esteem. Adding to her distress, the child's father has consistently expressed a threat of divorce, associating the mother with misfortune, even extending to their own child.

It was not until the child reached the age of seven that the mother, realizing the existence of a national pharmacovigilance center, made the decision to formally report her child's case. Her primary motivation was to share her personal ordeal and to raise awareness about the potential risks associated with the medication.

Upon receiving the case report, the physician conducted a causality assessment using the French method of imputability study [2]. To establish a comprehensive semiotic score and eliminate potential differential diagnoses, a genetic consultation was deemed necessary. Consequently, a genetic consultation was sought, ultimately leading to the accurate rectification of the etiological diagnosis of dysmorphia. According to the French method for assessing the causality of adverse drug reactions, the pharmacologist assigned a level of doubt regarding the drug's origin, as indicated by an Intrinsec Score of I2. The chronological score was noted as C2, and the semiological score as S1. The extrinsic score, designated as B1, was based on the available data for trimipramine, which although limited, appeared to suggest the absence of a specific malformation risk associated with trimipramine antidepressants [3].

During the parental evaluation, it was observed that the father exhibited discreet coloboma and mild hypoplasia of the zygomatic bones.

Treacher Collins syndrome was considered a potential diagnosis due to the presence of characteristic dysmorphic features and a family history consistent with autosomal dominant inheritance. Prior to conducting genetic studies, informed consent was obtained from the proband's parents.

The genetic testing conducted involved a multigene panel, including genes *TCOF1, POLR1B, POLR1C*, and *POLR1D*. The results revealed a heterozygous frameshift mutation NM_001371623.1(*TCOF1*): c.4372_4376del (p.Lys1458fs) in exon 24 of the *TCOF1* gene for the proband.

However, the father declined to participate in the genetic study, as he was unwilling to accept that he could be the carrier of the disease.

DISCUSSION

Treacher Collins syndrome is named after the English surgeon Edward Treacher Collins, who initially described the syndrome's traits in 1900. It is a rare congenital disorder of craniofacial development with an estimated prevalence of 1/50000 births [1]. Most TCS is inherited in an autosomal dominant manner; a small portion (~4%) is inherited in an autosomal recessive manner [4]. TCS is characterized by major clinical manifestations including hypoplasia of the zygomatic bones and mandible resulting in midface hypoplasia, micrognathia and retrognathia; lower eyelid abnormalities including coloboma and partially or totally absent lashes; external ear abnormalities comprising absent

or malformed ears; and a family history consistent with autosomal dominant inheritance. Minor clinical features related to TCS are atresia or stenosis of the external auditory canals; conductive hearing loss; ophthalmologic defects; airway abnormalities comprising tracheostoma and choanal stenosis or atresia; cleft palate; preauricular hair displacement; and delayed motor or speech development. Da Silva Dalben et al. found dental anomalies in 60% of TCS patients, with one to eight anomalies per individual [5]. These anomalies consist in tooth agenesis, enamel deformities and malposition of the maxillary first molars. In some cases, dental anomalies in combination with mandible hypoplasia result in a malocclusion, thus possibly leading to problems with food intake and the ability to close the mouth [5]. Our patient has none of these dental anomalies. Less commonly, TCS has been associated with heart defects, malformed or absent thumbs and cryptorchidism [6].

To date, four genes have been identified in TCS whose diagnosis is established by detection of a heterozygous (autosomal dominant) pathogenic variant in *TCOF1*, *POLR1D* or *POLR1B* [7,8], or biallelic (autosomal recessive) pathogenic variants in *POLR1C* or *POLR1D* [7,9].

TCOF1, located on the 5q32-q33.1 region, is the major gene involved with heterozygous mutation in up to 93% of individuals with TCS [4].

TCOF1 encodes a nucleolar phosphoprotein called treacle, thought to play a central role in various cellular processes such as ribosome biogenesis, rRNA transcription, and potentially neural crest cell migration. Pathogenic variants in the *TCOF1* gene lead to haploinsufficiency of treacle, disrupting its normal functions. This would affect nuclear localization signals and triggers apoptosis of cephalic neural crest cells during embryogenesis, thereby contributing to the symptoms observed in Treacher Collins Syndrome [10,11,12]. The specific variant identified in our patient, c.4372_4376del, results in a premature stop codon, producing a truncated protein. Already described in the literature, this variant is classified as pathogenic according to American College of Medical Genetics and Genomics (ACMG) guidelines. In individuals with TCS, hundreds of pathogenic variants within *TCOF1* have been documented [13], and while some have been observed more than once, our patient's variant is noteworthy for its recurrence in 16% of cases [4]. The genes *POLR1D, POLR1C*, and *POLR1B*, located at 13q12.2, 6p21.1, and 2q14.1, respectively, exhibit limited mutations, being associated with a small subset of TCS patients.

These three genes are also expressed in neural crest cells, impacting ribogenesis and potentially disrupting cell division. *POLR1D* and *POLR1C* encode subunits that are integral to both the RNA polymerase I and RNA polymerase III complexes, critical for the synthesis of ribosomal RNA precursors and small RNA, and *POLR1B* encodes the RNA polymerase I subunit B [7,8].

Roughly 40% of individuals with autosomal dominant TCS have an affected parent [4], and this is the case of our patient, whose father exhibits a mild expression of TCS. The risk to the siblings is 50%, the specific malformations or their severity cannot be predicted because significant inter- and intrafamilial clinical variability is common in TCS.

Treatment should be customized to meet the unique requirements of each person, ideally carried out by a comprehensive craniofacial management team. Such a team typically includes a medical geneticist, plastic surgeon, head and neck surgeon, otolaryngologist, oral surgeon, orthodontist, audiologist, speech pathologist, and psychologist, ensuring a holistic approach to care.

CONCLUSION

In conclusion, it is crucial to emphasize two key points that hold immense significance for healthcare professionals in general, and pediatricians, in particular. Firstly, a profound awareness of the social and psychological implications of any malformation syndrome in children is paramount. It is essential to recognize that seeking a genetic consultation to establish a confirmed etiological diagnosis can significantly alleviate the prolonged social and psychological distress that patients and their families may endure. This step can lead to more targeted and effective interventions, ultimately improving the quality of life for affected individuals. Secondly, healthcare practitioners must act swiftly in seeking pharmacovigilance guidance whenever adverse events emerge after medication administration. This proactive approach is essential to ensure a precise assessment of causality. It also safeguards against prematurely attributing blame to the medication, as establishing drug causation should be a diagnosis of exclusion, requiring a thorough and meticulous process of elimination.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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CASE REPORT

SEVERE FORM OF SALIH MYOPATHY CAUSED BY COMBINATION OF TWO HETEROZYGOUS TTN MUTATIONS

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ABSTRACT

Salih myopathy is autosomal recessive hereditary early-onset myopathy with fatal cardiomyopathy. It is a rare and heterogeneous form of congenital titinopathies (TTN). Affected children have delayed motor development, normal mental development, and in further course dilated cardiomyopathy. Motor functions have a tendency to improve, but death occurs most often before 20 years of age due to arrhythmias.

Our patient is a 2-year-old girl, born in severe perinatal asphyxia, with global hypotonia and poor spontaneous movements. She required immediate endotracheal intubation and mechanical ventilation was initiated without the possibility of cessation. Improvement in her neurological status was not observed. Due to her clinical presentation, we performed genetic testing and a diagnosis of Salih myopathy caused by combination of two heterozygous TTN mutations was confirmed.

This case illustrates that Salih myopathy may have severe presentation from birth, with continuous necessity for mechanical ventilation, without any motor improvement.

Keywords: cardiomyopathies, congenital hypotonia, exome sequencing, muscular diseases, Salih myopathy, titinopathy

INTRODUCTION

Salih myopathy is also known as early-onset myopathy with fatal cardiomyopathy, which is a rare and heterogeneous form of congenital titinopathies (TTN). It is an autosomal recessive hereditary neuromuscular disorder with early onset of its clinical manifestations in the neonatal period or in early childhood (1,2). Affected children have delayed motor development, with joint contractures and scoliosis at a later age. Most cases have normal mental development. Further progression of the disease leads to the development of dilated cardiomyopathy. Death occurs due to arrhythmias, most often between 8 and 20 years of age (3,4).

Salih myopathy is ultra-rare disease. It was first described in two Arab families, in 2007 (5). The prevalence of Salih myopathy is unknown, as it occurs in a small number of families of Moroccan and Sudanese origin.

The diagnosis is made in the proband by identifying biallelic pathogenic variants in the first three exons encoding the M-line (*Mex1*, *Mex2*, and *Mex3*) of TTN, the only gene for which pathogenic variants are known to cause Salih myopathy (6).

Treatment requires a multidisciplinary approach (3).

CASE REPORT

In this manuscript we describe a 2-year-old Roma origin girl who was diagnosed with Salih myopathy. She is the fourth child of nonconsanguineous parents. The second child from the same parents was born in severe perinatal asphyxia, with hypotonia, congenital heart defect (partial anomalous inflow of the pulmonary veins) and multiple joint contractures and died in the 5th month of life before a definitive diagnosis was made. Our patient is from a regularly monitored pregnancy, but oligohydramnios was noted near the end of the pregnancy which was terminated by caesarean section at 38+ 5/7 weeks of gestation. The baby was born in severe perinatal asphyxia (Apgar score 2/2). The somatometric parameters at birth were as follows: BW 3090 g (50.p), BL 50 cm (50.p), HC 37 cm (>99.p). After birth she was cyanotic, bradycardic, without spontaneous breaths and movement, with global hypoto-

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nia and hypermobility of the elbows, hips and ankles and deformity of foot (equinovarus). Upon first examination, the following was observed: macrocephaly, short nose, low set ears, low set hairline, up-slanting palpebral fissures, excess skin at the back of the neck. On the radiographic image of the locomotor system, multiple fractures of the long bones were established (fracture of the body of the left humerus, the body of both femurs and the right tibia in the proximal part). She was referred to the Department of Intensive Care and Therapy immediately after birth due to respiratory failure. She required endotracheal intubation and since then the child has been on mechanical ventilation without the possibility of cessation, while maintaining a neurological finding dominated by global hypotonia and poor spontaneous movements. Fractures of long bones were treated with immobilization. No new fractures were registered during further hospitalization.

Due to signs of global muscular hypotonia and a positive family history of congenital hypotonia, a laboratory, metabolic, neuroradiological and genetic evaluation have been conducted. The findings are shown in Table 1.

According to clinical manifestations, conducted examinations, including genetic testing, a diagnosis of Salih myopathy was made. A segregation analysis was done for the parents and a heterozygous genetic variant c.15218-2A>G in the TTN gene was detected in the child's mother, and a heterozygous genetic variant c.56572C>T (p.Arg18858Ter) in the TTN gene was detected in the child's father.

DISCUSSION

Salih myopathy is relatively new entity, so it is difficult for clinicians to distinguish congenital titinopathies from congenital myopathies associated with other genes (7,8). With that in mind, the diagnostic approach excluded some of the more common causes of hypotonia in children and suspected a rare cause.

Salih myopathy is characterized by muscle weakness, hypotonia that manifests itself in the neonatal period or early childhood. According to Hackam et al., affected children walk between the ages of 20 months and 4 years, with a tendency for motor functions to improve (3). In contrast, our reported child is 2 years old, without spontaneous movements, with the same motor functions as at birth, and is dependent on invasive respiratory support.

Contractures and deformities of the foot are common in patients with titinopathies, localized distally and affecting more than two joints, appearing in the first decade of life (7, 9). Unlike joint contractures and foot deformities, multiple fractures are rare. According to data from the literature, fractures were described only in two patients until now (10). So far, there are no reported multiple fractures in the neonatal period as our patient had.

Cases are described where mechanical support was needed only at birth and with further progression of the disease. This was performed intermittently only during the night (10). Unlike patients found in the literature, our patient has been on invasive respiratory support from birth.

Table 1.

Diagnostics	Results
Creatine kinase	13.69 μkat/l (reference range 0.72-7.9 μkat/l)
Karyotype	46,XX Normal female karyotype
Plasma and urine amino acid concentration	Normal finding
Organic acids in urine	Normal finding
Genetic testing for SMA	Negative
TORCH	Normal finding
Echocardiography	Normal finding
MRI of the head	Volume reduction of brain parenchyma at the expense of white matter and corpus callosum and diffuse hyperintensity of supratentorial white matter periventricularly.
EMNG	The finding indicates myopathically altered pattern, slightly prolonged and polyphasic. Denervation potentials were not registered. Neurographic parameters are obtained as expected for age.
Muscle biopsy	Examination of muscle biopsy sample showed the presence of small, oval muscle fibers of abnormal size with accentuated interfascicular fibrous weft. Signs of necrosis and inflammation were not observed. The presence of centrally located nuclei and perinuclear halo was not observed. Neurofibrillary tangle with myelinated nerve fibers was clearly observed. Mitochondria were normal. Small groups of type 2 atrophic fibers was observed.
Psychologist	Gross delay of psychomotor development.
Genetic testing	Two heterozygous pathogenic genetic variants c.56572C>T (p.Arg18858Ter) and c.15218-2A>G in the TTN gene were detected.

SMA - Spinal muscular atrophy; TORCH - Toxoplasma, Rubella, Cytomegalovirus, Herpes simplex; EMNG – Electromyoneurography

According to data from the literature, dilated cardiomyopathy can occur in patients at the age of 4 months, but most often between 5 and 16 years of age (11,12,13). The echocardiographic examination of our patient is still normal.

There are no specific laboratory and radiological findings that can be used to diagnose Salih myopathy. Creatine kinase may be at the upper limit of normal or elevated (generally 1.5-7x elevated) (3). Similarly, creatine kinase in our case was initially elevated, 1.7x above the upper limit, and later normal. In the literature, EMNG findings of patients are reported, indicating a polyphasic potential of low amplitude of short duration (3). The same finding was obtained in our patient.

The findings of muscle biopsies in patients with titinopathies are pathological. The changes that can be observed are increased fiber size variation, centrally placed cores and cores with additional structural abnormalities (10). In Salih myopathy, electron microscopy of skeletal muscles reveals multiple foci of sarcomere disruption and mitochondrial depletion (3). In our patient's muscle biopsy, no pathognomonic changes were registered to the extent that they were diagnostic.

The intellectual development of children with Salih myopathy is usually normal (3). Our patient is conscious, establishes social contact, and a psychological examination shows gross retardation in psychomotor development, most likely because of severe perinatal asphyxia (MRI), most likely due to severe congenital hypotonia.

CONCLUSION

Congenital titinopathies are increasingly recognized as a potentially severe form of congenital myopathies. In case of congenital hypotonia, it is necessary to think about Salih myopathy as a possible underlying cause. By establishing an earlier diagnosis, it is possible to improve the development of motor functions with early habilitation treatment, regular monitoring and treatment of cardiac and respiratory disorders. Prenatal diagnosis is possible, including preimplantation genetic testing for monogenic gene defects (PGT-M) in some centers.

DECLARATION OF INTEREST

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

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CASE REPORT

EXPERIENCE WITH THE KETOGENIC DIET IN A BOY WITH *CLCN4* RELATED NEURODEVELOPMENTAL DISORDER

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ABSTRACT

Raynaud-Claes syndrome is rare condition characterized with intellectual disability and is caused by X-linked pathogenic variants in *CLCN4* gene. Hemizygous missense variant NM_001830.4: c.1597G>A (p.V533M) was detected in a 6-year-old male followed up with intellectual disability, dysmorphism, and epileptic encephalopathy. The mother and one sister of the patient were also carrying the same variant. The clinical picture of the patient was significantly more severe, and the patient exhibited nonconvulsive status. Tonic status was observed with benzodiazepine treatment and the patient was successfully treated with a ketogenic diet. Many types of seizures can be seen in Raynaud-Claes syndrome, some of which can be life-threatening. *CLCN4* variants can be investigated in patients who exhibit an increase in tonic seizures with benzodiazepine treatment. However, ketogenic dietary therapy as first-line treatment can be lifesaving in resistant epilepsy cases caused by the *CLCN4* gene.

Key words: ClC-4, epilepsy, lennox gastaut, ketogenic diet, Raynaud-Claes

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INTRODUCTION

Raynaud-Claes syndrome is a rare syndrome linked to the X chromosome. People with this syndrome exhibit facial dysmorphism (long face, prominent chin, flat midface, downslanting palpebral fissures, strabismus), hypotonia, mild to severe intellectual disability, epilepsy, epileptic encephalopathy, behavioral problems, and cerebral atrophy [1]. Some heterozygous females are unaffected; however, mild to severe intellectual disability can be seen in some heterozygous girls. As expected, male patients exhibit a more severe clinical picture [2].

The chloride channel (CLC) gene family comprises nine different channel proteins in mammals, four of which encode plasma membrane CLCs (ClC-1, ClC-2, ClC-Ka, ClC-Kb) and the other five encode intracellular 2Cl−/H+ exchangers (ClC-3–7). The ClC-4 channel protein encoded by the *CLCN4* gene in chromosome Xp22.2 is a voltage-dependent 2Cl−/H+ exchanger. Pathogenic variations in the *CLCN4* gene cause Raynaud-Claes syndrome (MIM:#300114) listed in the Online Mendelian Inheritance in Man database. ClC-4 is expressed in the brain as well as in striped muscle tissue, heart, intestine, and kidney. CIC-4 is probably involved in the ion homeostasis of endosomes and intracellular trafficking, but its physiological function is still unknown [3].

A study conducted with a meta-analysis revealed that neurodevelopmental disorders associated with *CLCN4* have been identified in 122 individuals from 67 families so far [4].

The traditional ketogenic diet is characterized by its high-fat, adequate-protein (1 gram/kg), and low-carbohydrate composition, inducing metabolic alterations reminiscent of a state of starvation. Shifts in plasma ke-

tones, insulin, glucose, glucagon, and free fatty acids may manifest within hours of initiating the diet, exhibiting significant and rapid changes [5].

The effectiveness of ketogenic diet therapy extends to patients with epilepsy across various ages and seizure types, solidifying its status as a beneficial treatment option [6].

Here, we report on three cases of Raynaud-Claes syndrome in a family with a missense variant in the *CLCN4* gene.

CASE PRESENTATIONS

Case 1

A 6-year-old male was evaluated in the pediatric neurology department due to multidrug resistance epilepsy. Perinatal history was uneventful (caesarean section; birth weight of 2850 g) and was born from a non-consanguineous marriage.

The patient exhibited mild hypotonia during the first year of life. Eye contact was partial and social interaction was poor. The patient learned to walk at the age of 24 months and started speaking with few single words at the age of 3.5 years but could not form a sentence. The patient had severe intellectual disability and was receiving special training for the same. The patient's height was 110 cm, in the 10 percentile (p), weight was 20 kg (50p), and head circumference was 49.6 cm (3-10p). Dysmorphological examination revealed a round face, bitemporal narrowing, depressed nasal bridge, narrow and downslanting palpebral fissures, and strabismus. Cerebellar examination revealed intentional tremor and ataxia; the extrapyramidal system examination was normal. Moreover, the cranial nervous system examination was normal, but strabismus was present.

The patient's first seizure, as cyanosis and motor arrest, occurred at 12 months of age. The electroencephalogram (EEG) findings at that time were multifocal and accompanied by generalized spike slow wave activity, slow background activity, and paroxysmal rapid rhythms, which were found to be compatible with epileptic encephalopathy. After 20 months of age, the patient's generalized tonic and atypical absence seizures continued intermittently. From a phenotypical perspective, Lennox–Gastaut Syndrome was considered. Metabolic scans of blood amino acids, organic acid analysis, creatine kinase, lactate, ammonia, and tandem mass spectrometry were normal (amino acids and acyl-carnitine profile). Cerebrospinal fluid examination for amino acid and glucose content were also normal. Past medical history included valproic acid, levetiracetam, and phenobarbital therapy. Topiramate was discontinued due to ineffectiveness, ethosuximide, and clobazam treatment increased tonic seizures in approximately 10 days of use, lamotrigine was discontinued due to an allergic reaction.

The patient presented to our clinic with complaints of continuous absence seizure, non-responsiveness, and inability to walk. Continuous generalized 2.5–3 Hz spike slow wave activity was detected in the patient's EEG (Figure 1a). The patient was admitted to the intensive care unit with the diagnosis of atypical absence status. The patient was taking oral valproic acid, levetiracetam, and phenobarbital medications. IV benzodiazepine infusion was started. However, the patient's seizures assumed a tonic status after benzodiazepine infusion (Figure 1b); therefore, thiopental infusion was initiated. Thiopental infusion reduced the seizures; therefore, rufunamide and cannabidiol (CBD) oil therapy were added to the oral treatment. However, seizure activity increased with the reduction in thiopental infusion. Further, CBD oil therapy was terminated, and ketogenic diet therapy was initiated. Thiopental infusion began to be reduced at the 72nd hour of the ketogenic diet.

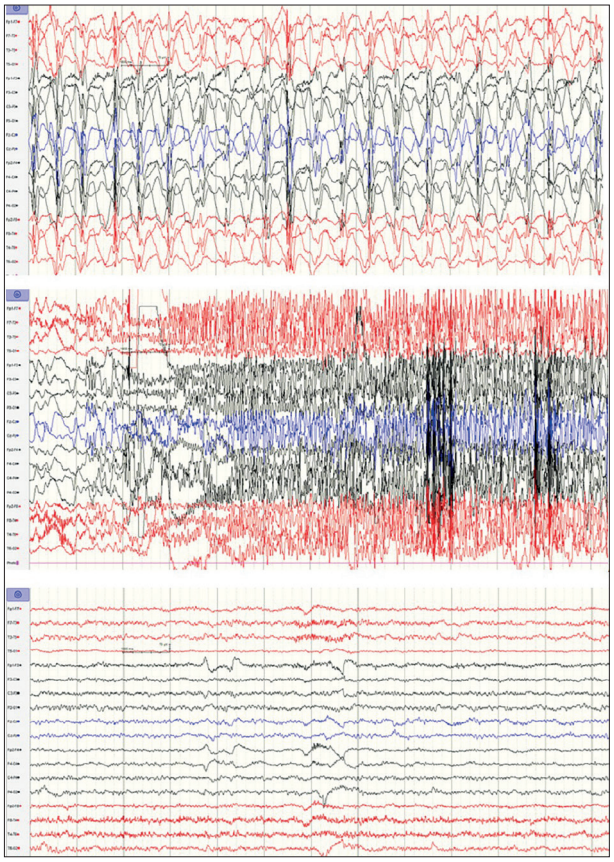

Figure 1. a) Top EEG shows atypical absence status epilepticus with ictal generalized spike-and-wave discharges with a frequency of 3 Hz. b) Middle EEG shows ictal generalized paroxysmal fast activity accompanied by an intense tonic contraction induced by benzodiazepine infusion c) Last EEG shows ,slow background activity superimposed with fast rhythms caused by phenobarbital use without ictal or inter-ictal discharges. Patient was on the 8 months of his ketogenic diet.

The patient did not have any further seizures under the ketogenic diet. The patient was discharged from intensive care after 17 days with oral valproic acid, rufinamide, phenobarbital, levetiracetam, and a ketogenic diet therapy. EEG taken 8 months after discharge from the intensive care unit showed slow background without epileptic discharges. The patient is being followed up without seizures for 8 months (Figure 1c).

Cranial MRI of the patient showed a thin corpus callosum, ventriculomegaly, and white matter atrophy (Figure 2). Chromosome analysis and array-CGH analyses of the patient were normal. Furthermore, the patient was evaluated using WES analysis, and a maternal hemizygous missense variant NM_001830.4: c.1597G>A (p.V533M) was detected in exon 11 of the *CLCN4* gene.

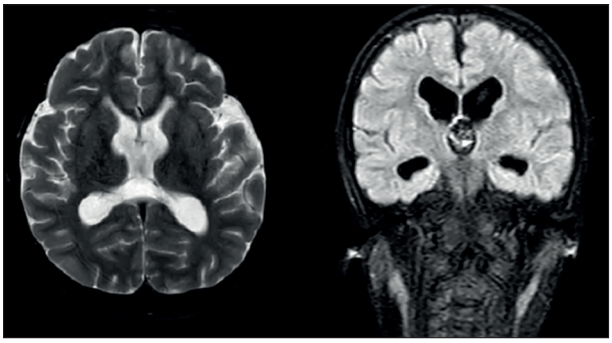

Figure 2. Axiel T2 and Coronal T2 flair Cranial MRI images of the patient showed a thin corpus callosum, ventriculomegaly, and white matter atrophy.

Cases 2-3

Thise index case has two sisters, and the same variant was found as heterozygous in one and wild-type in the other.

The seven years old sister with the heterozygous variant was born with term birth weight of 3200 g. She started walking at 18 months of age and began to speak at 24 months of age. She received special education due to learning difficulties. The patient had no seizures with a normal EEG. Neurological examination of the patient was normal. Her height was 115 cm (10p), weight was 22 kg (25p), and head circumference was 50 cm (3-10p). The Wechsler Intelligence Scale for Children-Revised (WISC-R) evaluation revealed borderline mental retardation. A heterozygous variant was detected in the mother. The neurological evaluation of the mother was normal. The patient's Porteus Maze Test score was 89, her Kent Egy intelligence test score was 64 (the average score was 77), and the mother was diagnosed with borderline mental retardation. The mother had no history of epilepsy. Moreover, it was found that the patient's aunt had poor school achievement and the son of the aunt had epilepsy and learning difficulties.

DISCUSSION

CLCN4 variants have been associated with X-linked dominant intellectual disability and epilepsy phenotype. Epilepsy is seen in 56% of these patients, and 25% of patients suffer from epilepsy-related deaths. Reported epilepsies are mostly drug resistant and range from absence to epileptic encephalopathy [1,7]. Our patient also has drug-resistant epilepsy accompanied by various types of seizures, such as atypical absence and tonic seizures, and exhibited characteristics of Lennox-Gastaut epilepsy phenotype from both an EEG and clinical perspective [8].

The *CLCN4* family of voltage-dependent *CLC* genes comprises nine members (*CLCN-1–7, Ka,* and *Kb*), which demonstrate quite diverse functional characteristics while sharing significant sequence homology. *CLCN4* is a voltage-dependent 2Cl−/H+ exchanger. Its precise physiological function is unclear, but *ClC-4* is probably involved in the ion homeostasis of endosomes and intracellular trafficking. Additionally, *CLCN4* has a significant effect on neuronal differentiation. It was reported that the number and length of dendritic branches decreased significantly in primary hippocampal neurons of *CLCN4*-null mice and hippocampal or cortical neurons of *CLCN4* knock down mice. However, the epilepsy mechanism of *CLCN4* variants is still unclear [1,9,10].

So far, 18 missense, 2 frameshift, 1 splice-site, and 1 exonic deletion mutations have been detected in the *CLCN4* gene in the literature [1,2,11]. The NM_001830.4: c.1597G>A (p.V533M) variant in the *CLCN4* gene detected in this family was previously reported by Fernandez-Marmiesse et al. in a 14-year-old male with Dravet syndrome-like phenotype whose seizures were taken under control by topiramate. This variation in the exon 11 of the *CLCN4* gene is known to be located at helical-intramembrane domains, which play an important role in CLC activity of the *CLCN4* protein. This variation was shown to be co-segregated with the disease in our family. This variation is not currently available in population databases (ExAC, gnomAD, 1000 Genomes Project) and was predicted as disease-causing in in-silico analyses (Mutation Taster, Polyphen2, SIFT, CADD). Comparative amino acid sequence alignment of *CLCN4* across different species at https://www.ncbi.nlm.nih.gov/homologene revealed that the glycine at position 533 is highly conserved. Considering these data, this variation is thought to be responsible for phenotype [12].

Literature evidence shows that missense variants are more severe than frameshift and intragenic deletions in terms of epilepsy. Our patient also carried a missense variant and had polytherapy-resistant epilepsy. Two separate studies reported that one patient benefited from carbam-

azepine and one patient benefited from levetiracetam treatment. However, atypical absence seizures were predominant in our patient; therefore, carbamazepine treatment was not initiated. Lamotrigine was reported as beneficial in the literature and treatment could not be continued due to an allergic reaction in our patient. Studies have reported that the effect of valproic acid is limited [1]. Atypical absence status developed under the use of 30 mg/kg/day valproic acid in our patient. It can be concluded that anti-epileptic treatment as first-line therapy is unsuccessful in severe cases. Our patient developed a benzodiazepine-resistant tonic status. There are reports of some Lennox–Gastaut patients developing tonic status with benzodiazepine and the molecular etiopathogenesis of this condition is unclear [13]. This case can lead us to believe that the chlorine channels encoded by *CLCN4* caused this. Our patient prominently benefited from ketogenic diet treatment and showed improvement in interictal discharges on EEG. The patient had no seizures for 8 months.

All benzodiazepines enhance the binding of gamma-aminobutyric acid (GABA) to the (GABA) receptor and increase the threat of CLC conductance triggered by the GABA-GABAa receptor interaction following greater chloride influx mediated by an increased frequency of CLC opening [14]. Interestingly, while benzodiazepines do not directly activate channels but only modify GABA binding affinity, phenobarbital can directly promote channel opening in the presence and absence of GABA [15]. The shift in seizure characteristics into tonic status with benzodiazepine use may give us an opportunity to explain the mechanisms of action of *CLNC4* on the nervous system.

The male patient ad moderate to severe ID. He had no seizures in the past 8 months under a ketogenic diet. Improvement in social interaction skills and gait were observed. The sister of the patient, who carried the same mutation as heterozygous, has mild ID and her clinical picture is significantly better than her brother. The sister has never had epilepsy. Studies in the literature report normal–moderate ID in female cases and epileptic EEG disorders in some cases. EEG was normal in the sister. The patient's mother also had mild ID and did not finish primary school. The mother had no history of epilepsy or febrile seizures [2].

Cranial MRI revealed a thin corpus callosum, ventriculomegaly, and white matter atrophy in our patient. Among the reported cases, ventriculomegaly, cortical atrophy, and white matter lesions were reported in 9 patients and no correlation was found between epilepsy severity and cranial abnormality [16].

Ketogenic dietary therapy emerges as a viable treatment option for patients who have not responded to at least two antiseizure medications. There are several conditions in which ketogenic dietary therapy shows notable effectiveness, and it can be considered early in the treatment process. These conditions encompass Doose syndrome, Dravet syndrome, glucose transporter 1 (GLUT-1) deficiency, infantile spasms, pyruvate dehydrogenase deficiency, and tuberous sclerosis complex. Moreover, ketogenic dietary therapy may prove particularly beneficial for individuals with drug-resistant epilepsy who rely on a gastrostomy tube or formula for nutrition.

According to the existing evidence, a consensus panel of experts in 2018 recommended the consideration of ketogenic dietary therapy for children facing drug-resistant epilepsy when two antiseizure medication trials have proven unsuccessful.

Our case is the first one in which a ketogenic diet was applied and yielded successful results in epilepsy cases associated with *CLCN4.*

In conclusion, many types of seizures can be seen in Raynaud-Claes syndrome, some of which can be life-threatening. *CLCN4* variants can be investigated in patients who exhibit an increase in tonic seizures with benzodiazepine treatment. However, ketogenic dietary therapy as the first-line treatment can be lifesaving in resistant epilepsy cases caused by the *CLCN4* gene mutations.

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AUTHOR CONTRIBUTIONS

Gunes Sager and Ufuk Yukselmis examined and treated the patient, conceiving the idea for the case report, leading the case report writing, Merve Akcay provided data collection, Orkide Guzel provided the ketogenic diet treatment and revised the manuscript. Ayberk Turkyilmaz completed the WES examination and genetic counseling, supervised the entire case report and revised the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to disclose.

CONSENT

Written informed consent was obtained from the legal guardian of the patient (father) for publication of this case report and any accompanying images. We confirm that

we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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