UNIVERZITET CRNE GORE
MEDICINSKI FAKULTET
Broj:
Podgorica, _____. godine

Na osnovu člana 64 i 65 Statuta Univerziteta Crne Gore, a u vezi sa realizacijom dopisa Univerziteta Crne Gore br.01-4097 od 08.12.2022. godine, Vijeće Medicinskog fakulteta na elektronskoj sjednici održanoj 16-19.12.2022. godine, utvrdilo je

P R I J E D L O G

Predlaže se prof. dr Olivera Miljanović, vanredna profesorica Medicinskog fakulteta Univerziteta Crne Gore, **za dodjelu priznanja za poseban doprinos razvoju naučno-istraživačkog rada i medjunarodnom pozicioniranju Univerziteta Crne Gore, za 2022. godinu.**

O b r a z l o ž e nj e

Rektor Univerziteta Crne Gore obratio se dopisom br. 01-4097 od 08.12.2022. godine, u kojem navodi da Vijeće organizacione jedinice treba da formira prijedlog za dodjelu priznanja, jednom kandidatu, odnosno zaposlenom nastavniku sa te organizacione jedinice, za doprinose u razvoju naučno-istraživačkog, ili umjetničko-istraživačkog rada i medjunarodnom pozicioniranju Univerziteta Crne Gore.

Vijeće Medicinskog fakulteta je saglasno da u odnosu na impresivnu biografiju i izuzetan doprinos naučno-istraživačkom i stručnom radu, predloži prof. dr Oliveru Miljanović, za dodjelu navedenog priznanja, u kom smislu je odlučeno kao u dispozitivu.

VIJEĆE MEDICINSKOG FAKULTETA
PREDSJEDAVAJUĆI,

prof. dr Miodrag Radunović, dekan

BIOGRAFIJA

Rođena sam u Titogradu (sada Podgorica), 25. oktobra 1960. godine. Osnovnu školu i gimnaziju završila sam u Titogradu.

PODACI O OBRAZOVANJU

Mediicinski fakultet Univerziteta u Beogradu upisala sam školske l979/80. godine, a na istom diplomirala juna 1985. godine i stekla zvanje *doktor medicine*. Specijalizaciju iz pedijatije, u trajanju od četiri godine, započela sam 1988, a specijalistički ispit iz pedijatrije položila marta. 1993. na Medicinskom fakultetu u Beogradu i stekla zvanje *specijalista pedijatar*. Specijalističke studije iz uže oblasti *klinička genetika* završila sam 2006. na Medicinskom fakultetu Univerziteta u Beogradu i stekla zvanje *specijalista kliničke genetike*.

Poslediplomske studije iz oblasti *medicinska genetika* pohađala sam na Medicinskom fakultetu Sveučilišta u Zagrebu od 1989. do 1991. godine. Magistarsku tezu odbranila sam na Medicinskom fakultetu Univerziteta u Beogradu 1998. i stekla zvanje *magistra medicinskih nauka*. Doktorsku disertaciju odbranila sam na Medicinskom fakultetu Univerziteta u Kragujevcu 2010. godine i stekla zvanje *doktor medicinskih nauka*.

U cilju profesionalnog i naučnog usavršavanja više puta sam bila na kraćim studijskim usavršavanjima i trening programima u inostranstvu i pohađala međunarodne škole iz oblasti pedijatrije i kliničke genetike, od kojih izdvajam:

- 1990: Studijski boravak u Zavodu za medicinsku genetiku Pedijatrijske klinike «Rebro» – Zagreb, 6 mjeseci.
- 1990. i 1991: Škola medicinske genetike "Nedelje genetike", Medicinski fakultet Novi Sad.
- 1991: Međunarodna škola medicinske genetke "Recent advances in humman genetics", Interuniverzitetski centar Dubrovnik.
- 1995: UNICEF-ova škola za međunarodne spoljašnje procenitelje BFHI programa, Budimpešta.
- 1997. i 1995: Studiski boravak na Kliničkom institutu za laboratorijsku dijagnostiku – Odeljenje za medicinsku genetiku KBC Zvezdara – Beograd - 6 mjeseci.
- 1997: Međunarodna poslediplomska škola "Emergency care in pediatrics" Fonda za otvoreno društvo, Američko-Auatrijske fondacije i Dječje univerzitetske bolnice iz Filadelfije, Salzburg.
- 2000: Studijki boravak na Medicinskom fakultetu Univerziteta u Bariju (Italija) – Institut za medicinsku genetiku (interuniverzitetska saradnja Univerziteta Crne Gore i Univerziteta regije Puglija) – 2 mjeseca.
- 2001: Cochran - Biotechnology Science training (biotehnologija genetski modifikovanih organizama) – USDA Washington – 3 sedmice.
- 2002: Studijski boravak na Qeen·s University – Canada.
- 2007. i 2006: Škole TIORCAS projekta: “Tehnike molekularne genetike u analizi patogenih mutacija kod genetskih bolesti”, Univerzitet Campobasso, Italija; “Molekularna - genetska osnova kancera”, Univerzitet Campobasso, Italija; “Biohemija i molekularna biologija u humanoj medicini”, Predavači sa Univerziteta Campobasso u Italiji, Podgorica.
- 2008: XIII ECPD International summer school ”Management of health care institutions - Monitoring of health risks in Europe”, Miločer.

PODACI O RADNIM MJESTIMA I IZBORIMA U ZVANJA

Stručni rad doktora medicine započela sam 1985. godine, kao pripravnik, potom kao ljekar opšte prakse u školskom dispanzeru Zavoda za zaštitu zdravlja narodnog podmlatka (sada Institut za bolesti djece Kliničkog centra Crne Gore). U istoj ustanovi u kontinuitetu radim i dalje kao specijalista pedijatrije i uži specijalista kliničke genetike. Bila sam direktorica Instituta za bolesti djece Kliničkog centra Crne Gore u periodu od 1999. do 2005. godine, a direktorica Kliničkog centra Crne Gore u periodu od 2007. do 2011. godine. Bila sam rukovodilac tima za osnivanje Centra za medicinsku genetiku i imunologiju Kliničkog centra Crne Gore, na čijem se čelu nalazim od otvaranja 2000. godine do danas. Zvanje *primarius* stekla sam 2008, a licencu iz prakse Ljekarske komore Crne Gore 2007. godine

Na Medicinskom institutu u Podgorici birana sam 1988. godine u zvanje istraživač saradnik i bila sam glavni istraživač u dva nacionalna i jednom saveznom (SFRJ) naučnoistraživačkom projektu Ministarstva nauke Crne Gore.

U zvanje asistenta za predmet Pedijatrija na Medicinskom fakultetu Univerziteta Crne Gore izabrana sam 2001. godine i od tada redovno učestvujem u izvođenju nastave na ovom fakultetu. U zvanje docenta izabrana sam 2011. godine, a u zvanje vanrednog profesora 2017. U periodu 2013 – 2018. godina bila sam prodekanica za nastavu na Medicinskom fakultetu Univerziteta Crne Gore. Od 2017. godine predsjedavam Komitetu za medicinsku etiku i bioetiku na Medicinskom fakultetu UCG. Aktuelno sam odgovorna za organizaciju nastave na predmetima; Pedijatrija, Bioetika i biomedicina i Klinička genetika na studijskim programima Medicina, Stomatologija i Visoka medicinska škola. Od 2018. godine rukovodim Centrom za naučno-istraživački rad Medicinskog fakulteta, bila sam rukovodilac jednog nacionalnog, a sada sam rukovodilac bilateralnog projekta Ministarstva nauke Crne Gore i član Savjetodavnog borda i Upravnog komiteta u dva međunarodna Horizon projekta Evropske Unije.

Više od 25 godina obavljam dužnosti mentora za ljekare na specijalizaciji iz pedijatrije. Odlukom Nastavno naučnog vijeća Medicinskog fakulteta u Beogradu imenovana sam 1994. godine za mentora za specijalizaciju pedijatrije, a nekoliko godina kasnije i metora za specijalizaciju medicinke genetike. Status mentora za navedene oblasti potvrđen mi je i Odlukom Vijeća Medicinskog fakulteta UCG.

U dosadašnjem profesionalnom radu višekratno sam angazovana u radu brojnih komisija i stručnih tijela Minisatrstva zdravlja i drugih državnih institucija, kao stručnjak u oblasti medicinske genetike, pedijatrije i biomedicine, od čega izdvajam angažman na izradi zakona i ostale zakonske regulative u oblasti biomedicine. Bila sam ili sam i dalje članica brojnih stručnih tijela, kao što su: Nacionalni savjet za bezbjednost hrane, Upravni odbor Fonda zdravstva Crne Gore, Medicinski odbor Kliničkog centra Crne Gore, Upravni odbor Udruženja pedijatara Crne Gore, Uprava Pedijatrijske škole Srbije i Crne Gore, Savjet Asocijacije za preventivnu pedijatriju Crne Gore, Nacionalna komisija za kvalitet i bezbjednost zdravstvene zaštite, Komisija za primjenu postupaka asistiranih reproduktivnih tehnologija, Komisije za verifikaciju odstranjivanja i iskorjenjivanja pojedinih zaraznih bolesti, Nacionalnog savjeta za Rijetke bolesti Crne Gore.

Članica sam Odbora za medicinska istraživanja Crnogorske akademije nauka i umjetnosti od 1998. godine. Od 2014. godine članica sam Internacionalmog Foruma predavača (IFT) UNESCO-ve katedre za bioetiku na Univerzitetu u Haifi. Članica sam Komiteta za bioetiku Savjeta Evrope od 2013. godine.

Kontinuirano učestvujem u radu brojnih naučnih i stručnih skupova izlaganjem sopstvenih rezultata i angažmanom u svojstvu predavača i članice naučnih i organizacionih odbora.

Polymorphisms of *ACE* and thrombophilic genes: risk for recurrent pregnancy loss

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ABSTRACT

Background Recurrent pregnancy loss (RPL) affects up to 5% of pregnancies, but with no consensus on the definition. Inherited thrombophilia has been postulated as a risk factor for RPL. The aim of this study was to investigate the association of RPL with polymorphisms of five genes that influent the coagulation and fibrinolysis.

Methods This study was conducted on total of 224 women, 129 women with ≥2 early RPL or ≥1 late pregnancy loss, 95 women with at least two normal life births and no history of pregnancy loss. Five gene polymorphisms *F2* 20 210G>A (rs1799963), *F5* 1691G>A (rs6025), *MTHFR* 677C>T (rs1801133), *SERPINE1* −675 4G/5G (rs1799762) and *ACE* I/D (rs1799752) were genotyped by PCR-based methods.

Results A significant relationship was found between *SERPINE1* 4G/4G and *ACE* D/D polymorphisms and RPL (p<0.001 both, OR 2.91 and 3.02, respectively). In contrast, no association was found between *F2* 20 210G>A, *F5* 1691G>A and *MTHFR* 677C>T polymorphisms and risk for RPL. A combination of hypofibrinolytic homozygotes *SERPINE1* 4G/4G+*ACE* D/D was observed as a highly associated with RPL (Cochran-Armitage test, p<0.001), and their strong independent association with RPL risk was confirmed by logistic regression analysis (both p values <0.001, OR 3.35 and 3.43, respectively).

Conclusion Our data have demonstrated that *SERPINE1* and *ACE* gene polymorphisms, individually or in combination, appear to be a significant risk for RPL. This data may be useful in adding to the knowledge on inherited thrombophilia as an important contributor to RPL pathogenesis.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Recurrent pregnancy loss (RPL) aetiology remains unclear in about 50% of cases. Polymorphisms in genes that affect coagulation and fibrinolysis have been postulated as a risk factor for RPL.

⇒ Conflicting results and large geographical variations were shown in previous research.

WHAT THIS STUDY ADDS

⇒ Individual and synergistic hypofibrinolytic effects of SERPINE1 and ACE gene polymorphisms have been shown to be a significant risk factor for RPL in a coherent Montenegrin population.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The findings of the study may add to the data on hereditary thrombophilia role in the pathogenesis of RPL and may serve to address whether SERPINE1 and ACE gene polymorphisms should be included in the diagnostic work-up of women with RPL.

INTRODUCTION

Recurrent pregnancy loss (RPL) affects up to 5% of pregnancies, but with no international consensus on the definition, which includes three or more consecutive losses, including non-visualised pregnancies, after the 6th and until 24th week of gestation and also applies to two or more failed pregnancies, confirmed by ultrasound or histopathologic examination.[1–4] The term intrauterine fetal death (IUFD) refers to a fetus without signs of life in the uterus after 24 weeks of gestation and has great clinical significance, even in the case of a single loss.[5] It is widely accepted that RPL and IUFD are heterogeneous conditions, with various factors postulated to be the cause of these outcomes, such as chromosomal abnormalities, uterine anomalies, cervical weakness, endocrine dysfunctions, antiphospholipid syndrome and infections.[1–3] Advanced maternal age and the number of previous pregnancy losses (PLs) have been found to be a strong independent determinants of RPL risk, but maternal life style risk factors, like alcohol, cigarette and drug abuse and maternal obesity (BMI (Body Mass Index) ≥30), are also identified as factors that increase risk for RPL and IUFD.[6] However, in about 50% of RPLs, the underlying pathophysiological mechanisms remain undetermined.[1–6]

Inherited thrombophilia, secondary to an alteration in different genes that encode functional proteins included in coagulation and fibrinolysis cascade, has been postulated as a factor for susceptibility to deep vein thrombosis as well as an adverse pregnancy outcome, including RPL and IUFD.[7 8] The coagulation factor V (*F5*) Leiden 1691G>A (NM_000130.4:c.1601G>A (p.Arg534Gln)) and the coagulation factor II (*F2*) prothrombin 20 210G>A (NM_000506.5:c.*97G>A) gene polymorphisms, known as the most common genetic pro-thrombotic factors in Caucasian population, have been widely investigated.[9–11] It has also been shown that hyper homocysteinemia and reduced activity of methylenetetrahydrofolate reductase enzyme (MTHFR) by up to 40%, as a result of 677C>T polymorphism in *MTHFR* gene (NM_005957.5:c.665C>T (p.Ala222Val)), may increase homocysteine plasma levels, predisposing

to thrombosis.[9 11] The association of these three polymorphisms with RPL has been extensively studied, but the results obtained are quite inconsistent.[8–11]

Previous reports have also implied the link between RPL and IUFD and impaired fibrinolysis or defected fibrin stabilisation.[12] Serpin family E member 1 (SERPINE1) is a crucial regulator of proteolysis, controlling the fibrinolytic cascade and maternal tissue remodelling during the trophoblast invasion. Elevation of SERPINE1 plasma levels inhibits fibrinolysis, increasing the risk for thrombosis. The *SERPINE1* promoter 4G/5G insertion polymorphism (NM_000602.4:c.-820_−817G(4_5)) modulates the expression of *SERPINE1* gene and determines SERPINE1 plasma levels, which is the highest in the presence of homozygous 4G allele.[12 13] Plasma SERPINE1 levels are also increased by angiotensin II, which imply that the angiotensin I converting enzyme (ACE) is also an important factor in controlling the fibrinolytic process, converting inactive angiotensin I to the active form of angiotensin II, which controls *SERPINE1* expression, accordingly increasing *SERPINE1* messenger RNA and plasma SERPINE1 levels. ACE activity is determined by I/D polymorphism (287 bp insertion/deletion polymorphism) in intron 16 of the *ACE* gene (NM_000789.3:c.2306–117_2306-116insAF118569.1:g.14094_14382). The presence of *ACE* D allele, or DD genotype, enhances the production of angiotensin I and leads to an increased *SERPINE1* expression, increasing the risk of thrombotic events.[13 14] Both, the *SERPINE1* 4G and *ACE* D polymorphisms, may compromise placental formation and trophoblast invasion. Studies focused on this topic have yielded diverse results, some confirming the association of *SERPINE1* and *ACE* polymorphisms with RPL and IUFD, while others do not.[12–15] It was also shown that a combination of several pro-thrombotic gene polymorphisms may additionally increase the risk for RPL and IUFD.[8]

The aim of this study was to examine the association of RPL/IUFD with polymorphisms of five genes that, directly or indirectly, influent coagulation and fibrinolysis (*F5* 1691G>A, *F2* 20210G>A, *MTHFR* 677C>T, *SERPINE1* −675 4G/5G and *ACE* I/D), and their possible cumulative or synergistic effect on PL in the population of Montenegrin women.

PARTICIPANTS AND METHODS

Study population

This study included a total of 224 women who had experienced pregnancy, of which 129 with a history of PL (case group) and 95 fertile women with no PL (control group).

The participants of case group were recruited from women referred to genetic counselling and examination after RPL, in the period 2018–2020. A total of 129 women, who had at least two unexplained consecutive RPL within the first trimester and women with history of at least one PL in the second trimester or IUFD after 24 weeks of gestation, were included to the case group. The participants with known causes of PL, such as antiphospholipid syndrome, thyroid dysfunction, diabetes mellitus, polycystic ovary syndrome, chronic hypertension, uterine abnormalities, cervical weakness, parental chromosome abnormalities, fetal anomalies, protein C and S deficiencies, were excluded from the study.

Additionally, the participants of case group were stratified into three subgroups according to the number and period of PL.[1–3] The first group (2EPL group) included women with only two early PLs within the first trimester (up to completed 13th weeks of gestation), the second group (≥3 EPL group) included women with three or more early PLs within the first trimester; and the third group (≥1 LPL/IUFD group) consisted of women with one or more late PL in second trimester or IUFD after 24th week (with or without PLs in the first trimester).

The control group included 95 age-matched women, who had at least two normal term deliveries after uneventful pregnancies and no history of PL, venous thromboembolism and serious chronic diseases. All participants in the case and control group were Caucasians, with similar ethnic background.

The study was carried out at the Center for Medical Genetics and Immunology at state University hospital of Montenegro (Clinical Center of Montenegro).

DNA extraction and genotype analyses

Peripheral blood samples (5 mL) were collected in EDTA tubes and stored at −40°C until DNA extraction. Genomic DNA was extracted using the commercial QIAamp DNA Blood Mini Kit (Qiagen, Germany).

F2 20210G>A (prothrombin, rs1799963; NM_000506.5:c.*97G>A), *F5* 1691G>A (Leiden, rs6025; NM_000130.4:c.1601G>A (p.Arg534Gln)), *MTHFR* 677C>T (rs1801133; NM_005957.5:c.665C>T (p.Ala222Val)) and *SERPINE1* −675 4G/5G [rs1799762; NM_000602.4:c.-820_−817G(4_5)) genotyping was performed by Attomol Quicktype kits (Attomol GmbH, Molekulare Diagnostika, Germany) for allele-specific PCR. Analysis of *ACE* I/D polymorphism (rs1799752; NM_000789.3:c.2306–117_2306-116insAF118569.1:g.14094_14382) was done by PCR (F: 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3' and R: 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3'; 30 cycles: 1 min at 94°C, 30 s at 59°C and 1 min at 72°C). Insertion (I) variant was represented by 490 bp band and deletion (D) variant by 190 bp band.[16] All PCRs were performed using the Mastercycler Gradient (Eppendorf, Germany). After electrophoresis in 2.5% agarose gel (stained with ethidium bromide), all PCR products were visualised by ultra violet transilluminator (LKB, Sweden).

Statistical analyses

For statistical analysis, frequencies of genotypes and alleles between the groups were compared with the Pearson χ^2 test or with the two-tailed Fisher exact test. To describe the strength of the association between the polymorphisms and RPL/IUFD, dominant genetic model was used,[14 15] and calculation of ORs, with corresponding 95% CIs was performed for each polymorphism. The association of RPL/IUFD with combined the *ACE* I/D and PAI1 4G/5G polymorphisms was tested by the Cochran-Armitage trend test. The most prominent factors for RPL/IUFD were identified by the logistic regression analysis (Wald test). In all testing, $p<0.05$ was considered to be statistically significant. Statistical analyses were performed by SPSS for Windows Statistics V.20.0 (SPSS, Illinois) and EZR 1.36 (Saitama Medical Center Jichi Medical University, Japan).

RESULTS

Distribution of *MTHFR* 677C>T, *SERPINE1* −675 4G/5G and *ACE* I/D genotypes did not deviate from Hardy-Weinberg equilibrium in both groups (χ^2 test, all p values >0.05), while no assessment was performed for *F5* 1691G>A and *F2* 20210G>A genotypes, due to the absence of variant homozygotes among the studied population (zero values).

Demographic data and maternal behaviour risk factors of participants included in the study are presented in table 1. The study group participants were slightly younger, had significantly fewer children (p=0.0001) and showed higher presence of

Table 1 Demographic data and maternal behavioural risk factors of study population

	Case group N° 129	Control group N° 95	Test and p value
Age (years)	Mean 34.50±5.89 Range 23–50	Mean 35.47±3.31 Range 24–44	p=0.191†
N° of PL	Total 387 Mean 3±1.09 Range 1–9	Σ 0	
N° of children	Σ 57 Mean 0.44±0.84 Range 0–4	Σ 249 Mean 2.62±0.75 Range 2–5	p=0.0001***†
Maternal behaviour risks, n (%)			
Cigarette smoking	42 (32.5)	17 (7.9)	p=0.014*‡
Obesity	24 (18.6)	7 (7.4)	p=0.016*‡
Preeclampsia/hypertension	6 (4.6)	1 (1.0)	p=0.243§
Potential teratogenicity	8 (6.2)	2 (2.1)	p=0.196§

*p<0.05; **p<0.01; ***p<0.001.
†t-test.
‡Pearson χ^2 test.
§Two-tailed Fisher exact test.
PL, Pregnancy loss.

maternal behaviour risk factors: cigarette smoking (p=0.014) and obesity (p=0.016).

A low frequency of *F2* 20210G>A and *F5* 1691G>A polymorphic genotypes (heterozygotes only) and variant A allele was found in both, the case and the control group. The *MTHFR* 677C>T, *SERPINE1* −6754 G/*5G* and *ACE* I/D polymorphic genotypes (variant homozygotes and heterozygotes) and variant alleles were common in both groups (table 2). There was no statistical difference in the distribution of *F2* 20210G>A, *F5* 1691G>A and *MTHFR* 677C>T genotypes and alleles between women with RPL/IUFD and control fertile woman group. In contrast, variant homozygotes *SERPINE1* −675 4G/4G and *ACE* D/D as well as 4G and D alleles were significantly more frequent (all p values <0.001) in the case group compared with controls (table 2).

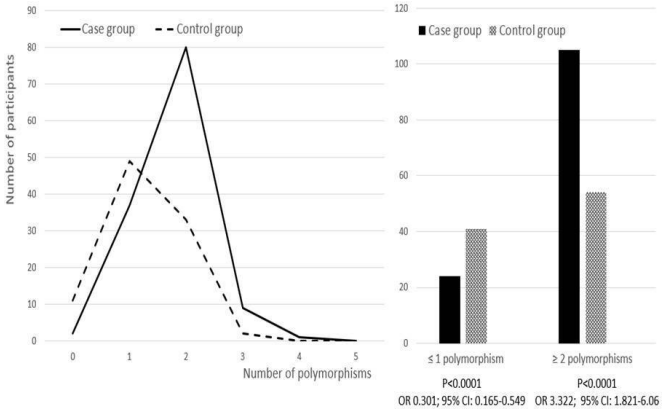

Figure 1 Distribution of case and control group subjects according to the total number of detected polymorphisms.

Polymorphic genotypes (heterozygotes and variant homozygotes) were registered among participants of both groups, but the distribution of women according to the total number of detected polymorphisms was different among these groups (figure 1). The presence of ≥2 polymorphisms was significantly more frequent in the case group compared with the control (p<0.0001; OR 3.322; 95% CI 1.821 to 6.06).

On the dominant genetic model (variant homozygotes+heterozygotes vs wild type homozygotes) both, *SERPINE1* −675 and *ACE* polymorphic genotypes (4G/5G+4G/4G and I/D+D/D) were significantly more prevalent in the case group than in the controls (both p values <0.001, table 3), with a three times higher probability for RPL/IUFD outcome if the woman is a carrier of *SERPINE1* or *ACE* polymorphic genotypes (*SERPINE1*: OR 2.917; 95% CI 1.594 to 5.33 and *ACE*: OR 3.026; 95% CI 1.665 to 5.497). Although both heterozygous genotypes occurred more frequently in the case group than in the controls (*F2*: 2.3% vs 1.0%; *F5*: 5.4% vs 2.1%), no significant difference was found in the distribution of *F2* and *F5* heterozygotes (no variant homozygotes were found). For *MTHFR*

Table 2 Genotype distribution and allele frequencies of *F2*, *F5*, *MTHFR*, *SERPINE1* and *ACE* genes in case and control group

Genotypes and alleles	Case group N°129 N° (%)	Control group N°95 N° (%)	P value
F2 20210G>A (rs1799963)			
GG/GA/AA	126/3/0 (97.7/2.3/0)	94/1/0 (98.9/1.1/0)	–
G allele/A allele	255/3 (98.8/1.2)	189/1 (99.5/0.5)	p=0.641†
F5 1691G>A (rs6025)			
GG/GA/AA	122/7/0 (94.6/5.4/0)	93/2/0 (97.5/2.1/0)	–
G allele/A allele	251/7 (97.3/2.7)	188/2 (99/1.0)	p=0.313†
MTHFR 677C>T (rs1801133)			
CC/CT/TT	83/37/9 (64.3/28.7/7)	48/41/6 (50.5/43.1/6.3)	p=0.078‡
C allele/T allele	203/55 (78.7/21.3)	137/53 (72.1/27.9)	p=0.108§
SERPINE1 4G/5G (rs1799762)			
5G/5G-4G/5G-4G/4G	24/64/41 (18.6/49.6/31.8)	38/45/12 (40/47.3/12.7)	p=0.002**‡
5G allele/4G allele	112/146 (43.4/56.6)	121/69 (63.7/36.3)	p<0.0001***§
ACE I/D (rs1799752)			
I/I-I/D-D/D	25/46/58 (19.4/35.6/45)	40/33/22 (42,1/34,7/23,2)	p=0.0002***‡
I allele/D allele	96/162 (37.2/62.8)	113/77 (59.5/40.5)	p<0.0001***§

*p<0.05; **p<0.01; ***p<0.001.
†Fisher exact two-tailed test.
‡χ^2 test.
§Pearson χ^2 test.

Table 3 Analysis of genotype distribution in the case and the control group on the dominant genetic model

Genotypes	Case group N° 129 N° (%)	Control group N° 95 N° (%)	p value	OR (95% CI)
***F2* 20210G>A (rs1799963)** AA+GA vs GG	3 (2.3) vs 126 (97.7)	1 (1.1) vs 94 (98.9)	p=0.639†	OR 2.238 (0.229 to 21.858)
***F5* 1691G>A (rs6025)** AA+GA vs GG	7 (5.4) vs 122 (94.6)	2 (2.1) vs 93 (97.5)	p=0.308†	OR 2.668 (0.542 to 13.142)
***MTHFR* 677C>T (rs1801133)** TT+CT vs CC	46 (35.7) vs 83 (64.3)	47 (49.5) vs 48 (50.5)	=0.038*‡	OR 0.566 (0.33 to 0.971)
***SERPINE1* 4G/5G (rs1799762)** 4G/4G+4G/5G vs 5G/5G	105 (81.4) vs 24 (18.6)	57 (60.0) vs 38 (40.0)	p=0.0004***‡	OR 2.917 (1.594 to 5.337)
***ACE* I/D (rs1799752)** D/D+I/D vs I/I	104 (80.6) vs 25 (19.4)	55 (57.9) vs 40 (42.1)	p=0.0002***‡	OR 3.026 (1.665 to 5.497)

*p<0.05; **p<0.01; ***p<0.001.
†Pearson χ^2 test.
‡Fisher exact two-tailed test.

677C>T polymorphism, more frequent occurrence of polymorphic genotypes (CT+TT) was found in the control group (p=0.038), while there was no difference in the frequency of variant homozygotes TT, between the study and control groups (table 3).

Further analyses showed a similar difference in distribution of polymorphic genotypes between the subgroups and the control group, as when compared the entire case group with the controls (online supplemental table 1). In all subgroups, *SERPINE1* 4G/5G and *ACE* I/D polymorphisms were significantly more prevalent compared with the controls, except for the *ACE* polymorphism in the group with ≥1 LPL/IUFD and group under 35 years and *F5* polymorphism, which was found to be significantly more frequent in the 2EPL group alone (p=0.038).

Given the synergic influence of *SERPINE1* −675 4G/5G and *ACE* I/D polymorphisms on *SERPINE1* gene expression, we also analysed their co-occurrence (table 4).

A significant difference was found in the prevalence of hypofibrinolytic combination of variant homozygotes (*SERPINE1* 4G/4G+*ACE* D/D), which was present in 15.5% in the case group, but was not found within control group at all (p=0.00007). In contrast, combination of wild type genotypes (*SERPINE1* 5G/5G+*ACE* I/I) was significantly higher among controls (p=0.0007). A moderate statistical significance was also found for the combinations of variant homozygote in one and heterozygote in another gene (p<0.05). The association between the combination of hypofibrinolytic *SERPINE1* and *ACE* gene polymorphisms and RPL was confirmed by the Cochran-Armitage trend test (p<0.001 for the combination of both variant homozygotes; p<0.05 for the combination of variant homozygote and heterozygote).

Logistic regression analysis was used to evaluate the predictive value of all examined polymorphisms and maternal behaviour risk factors for RPL and IUFD (age, cigarette smoking, obesity, preeclampsia/hypertension, potential teratogenicity) in 224 women. The obtained results showed that *SERPINE1* 4G/5G and *ACE* I/D polymorphisms are independent factors significantly associated with the occurrence of RPL and IUFD (both p values <0.001, Wald test) (table 5). Among maternal behaviour risk factors for RPL and IUF, only obesity showed a tendency to be statistically significant (p=0.058).

DISCUSSION

The aim of this study was to evaluate the association between RPL and five gene polymorphisms, which affect coagulation and fibrinolysis (*F2* 20210G>A, *F5* 1691G>A, *MTHFR* 677C>T, *SERPINE1* −675 4G/5G and *ACE* I/D) in Montenegrin women with a history of RPL and IUFD.

Table 4 Distribution of combined *SERPINE1*−675 4G/5G and *ACE* I/D genotypes in recurrent pregnancy loss (N° 129) and control group (N° 95)

Genotypes	*ACE* *SERPINE1*	I/I N	 %	I/D N	 %	D/D N	 %	Cochran armitage trend test
Case	5G/5G	3	2.3	7	5.4	14	10.8	3.897
Control		14	14.7	9	9.5	15	15.8	p=0.0484*
p value		0.0007***†		0.245‡		0.277‡		
Case	5G/4G	15	11.6	25	19.4	24	18.6	6.019
Control		17	17.9	21	22.1	7	7.4	p=0.014*
p value		0.163‡		0.617‡		0.016*‡		
Case	4G/4G	7	5.4	14	10.8	20	15.5	15.693
Control		9	9.5	3	3.1	0	0	p=0.0001***
p value		0.245‡		0.04*†		0.00007***†		
Cochran Armitage trend test		2.4625 p=0.1166		5.1333 p=0.235*		16.3967 p=0***		

*p<0.05; **p<0.01; ***p<0.001.
†Pearson χ^2 test.
‡Fisher exact two-tailed test.

Table 5 Logistic regression analysis for outcome of RPL and IUFD in 224 women with RPL/IUFD and fertile control group

Predictive factors for outcome dominant genetic model	95% CI ratio (lower to upper)	OR test	Wald test p value
Age	0.446 to 1.53	0.827	0.546
Cigarette smoking	0.971 to 3.88	1.94	0.061
Obesity	0.968 to 6.63	2.53	0.058
Preeclampsia/hypertension	0.628 to 63.3	6.3	0.118
Potential teratogenicity	0.382 to 15.8	2.45	0.344
F2 20210G>A(rs1799963)	0.281 to 41.8	3.43	0.334
F5 1691G>A (rs6025)	0.603 to 18.4	3.34	0.167
MTHFR 677C>T (rs1801133)	0.591 to 0.323	1.08	0.088
SERPINE1 4G/5G(rs1799762)	1.7 to 6.58	3.35	0.00046***
ACE I/D (rs1799752)	1.75 to 6.73	3.43	0.000323***

For all environmental/host risk factors: 1—risk factor present versus 0—risk factor was not present.
For each gene genotypes: 1—genotypes containing at least one polymorphic allele versus 0—wild type genotypes.
*p<0.05; **p<0.01; ***p<0.001.
IUFD, intrauterine fetal death; RPL, recurrent pregnancy loss .

The most extensively investigated polymorphisms of procoagulatory factors are the *F5* 1691G>A and *F2* 20210G>A gene polymorphisms. Due to *F5* 1691G>A transition and consequent substitution of arginine by glutamine at amino-acid residue 506, as a result of 'gain of function', the coagulation factor V becomes resistant to degradation by protein C, increasing 3–5 times risk of venous thromboembolism. A transition G>A at 20210 position in the gene encoding F2 increases prothrombin level and was found as 3–4-fold increased risk for venous thrombosis in heterozygotes.[17] The prevalence of *F2* 20210G>A and *F5* 1691G>A polymorphisms in the Caucasian population is 2–3% for *F2* and 3–5% for *F5*, with a very rare occurrence of homozygosity for both (0.014% for *F2* and 0.02 for *F5*).[9 10 17]

Our study showed that the prevalence of heterozygous *F2* 20210G>A and *F5* 1691G>A polymorphisms in the control fertile population of Montenegrin women was lower (1.1% and 2.1%, respectively) than it was found for the European – Caucasian population, but it was in the range of reported results within different populations. We previously reported a similar prevalence for *F2* and *F5* heterozygotes (both 2.5%) in the general Montenegrin population,[18] but neither the present, nor our previous study showed the presence of variant homozygotes in these genes among general population. The prevalence of *F2* 20210G>A and *F5* 1691G>A heterozygotes among women with RPL and IUFD in our study was lower than reported in the studies that confirmed the association between these polymorphisms and early/late RPL and late non-recurrent PL and IUFD.[9 10 19–21] Despite the fact that the prevalence of *F2* 20210G>A and *F5* 1691G>A heterozygotes among women with RPL and IUFD more than two times as high as in the control group, we failed to show statistical significance. The only significance was found for *F5* 1691G>A polymorphism in subjects with only two RPLs in the first trimester compared with fertile controls, while for the other subgroups of the case group, no significant difference was demonstrated. A quite number of studies and meta-analyses have showed no association of these polymorphisms with PL, especially at early pregnancy stages.[11 12 22] Rey *et al*,[9] in their meta-analysis, presented the results of studies that confirmed association between first-trimester RPL and *F5* 1691G>A and *F2* 20210G>A polymorphisms, but majority of studies reported a positive association mainly with the late second and third trimester PL and IUFD.

Present study did not show any association between the *MTHFR* 677C>T polymorphism and PL.

The presence of T allele and CT genotype was even more common in the control group than in the group of women with RPL/IUFD, but with no difference in the frequency of variant homozygotes TT. Applying the dominant genetic model, we found a more frequent presence of polymorphic genotypes (CT+TT) in the control group than in women with RPL/IUFD, but in the subgroup analysis, this significance was only confirmed for women with only two RPLs. Our results suggest that carriers of both, TT and CT genotypes of *MTHFR* gene do not have an increased risk of PL. Moreover, their prevalence is common in the general Montenegrin population. Results of previous studies and meta-analyses, conducted on various population are conflicting, with some studies that confirmed the association between *MTHFR* TT homozygosity and RPL[11 21] and the others which did not reported any association at all,[9 12 22 23] which concurs with our results. Several meta-analyses showed strong association between *MTHFR* TT homozygosity and RPL in Chinese population, but not in any other ethnicities or not at least in Caucasians.[24]

Since trophoblastic invasion, crucial for efficient placentation and successful pregnancy require metalloproteinase activity, which is mainly regulated by plasminogen activators,[12] the *SERPINE1* 4G/5G and *ACE* I/D gene polymorphisms were also investigated in this study.

Our findings demonstrated a significantly higher frequencies of the *SERPINE1* 4G and *ACE* D alleles and their polymorphic genotypes among women with a history of PL compared with the controls (all p values <0.001 for both genes). However, the *SERPINE1* 4G and *ACE* D alleles and polymorphic genotypes were also found to be widespread among the control fertile female population in Montenegro, which is in line with data from other similar studies of the European and worldwide population.[14 15 25–32] Given the significantly higher prevalence of polymorphic genotypes, found under the dominant genetic model, for both genes in entire case group and in all subgroups of women with PL, our results indicate a strong association between polymorphic *SERPINE1* 4G/5G and *ACE* I/D genotypes with early and late PL or IUFD, pointing out an almost 3–5 times higher probability of PL if the woman is a carrier of one of these polymorphisms.

A number of studies reported that the *SERPINE1* −675 4G/5G polymorphism causing elevated plasma SERPINE1 levels and hypo-fibrinolysis appear to be an independent significant factor for PL and other pregnancy complications.[11 12 21] On the contrary, meta-analysis by Su *et al*,[14] and also several individual case–control studies,[13 25–28] did not observe significant differences in genotype distribution between the RPL group and controls. The findings from present study are in concordance with reports that confirmed the association of the *SERPINE1* 4G homozygosity with RPL in different populations, like Coulam *et al*[12] in USA, Shakarami *et al*[29] in Iran, Yalcintepe *et al*[30] in Turkey or Pereza *et al*[11] and Li *et al*[33] in their meta-analysis on worldwide population.

Hypofibrinolysis may also be caused by increased levels of angiotensin II, which increases plasma SERPINE1 levels. The insertion/deletion polymorphism in the *ACE* gene has been shown to affect the activity of angiotensin II, which production is wo times as high in the presence of D allele or D/D genotype in the *ACE* gene.[13] Su *et al*[14] and Yang *et al*[15] in their meta-analyses confirmed a significant association between *ACE* I/D

polymorphism and more than two RPLs. Like these authors and the other case–control studies,[31 32] we have found a significant association between *ACE I*/D polymorphism and PL. Subgroup analysis in our study showed a strong association with early RPL and with advanced maternal age. Women with two early PLs were three times more likely, and women with three or more early PLs were four times more likely to have RPL, if they were carriers of ACE I/D polymorphism. Furthermore, carriers of *ACE* I/D polymorphism with advance age (over 35 years) have over three times increased risk for RPL, which is in line with observation of Su *et al*,[14] and Yang *et al*[15] who have reported that maternal age could enhance the effects of genetic polymorphisms. Some other studies, on the contrary, have not confirmed any association between RPL and *ACE* I/D polymorphism.[13 25–27]

There are a several studies that have examined the cumulative risk associated with joint presence of *SERPINE1* 4G/5G and *ACE* I/D polymorphisms, based on the premise that both polymorphisms affect the same downstream pathway and that their combined effects may increase the incidence of macroangiopathy. Buchholz *et al*[27] showed that although individually homozygous genotypes *SERPINE1* 4G/4G and *ACE* D/D were not associated with RPL, their co-occurrence was strongly associated with RPL. On the contrary, Kim *et al*[13] and Goodman *et al*[25] failed to show such association. Investigating the presence of the combination of hypofibrinolytic *SERPINE1* 4G/5G and *ACE* I/D polymorphisms, this study revealed their significantly higher prevalence among women with RPL. Our findings imply that the simultaneously occurrence of both variant homozygotes (4G/4G and D/D), and also the combination of variant homozygote in one gene and heterozygote in another, and their synergic effect on increased *SERPINE1* gene expression and consequent hypofibrinolysis, could have a significant biological impact on susceptibility to PL. This association was further confirmed by the Cochran-Armitage trend test ($p<0.001$ for both homozygote variants and $p<0.05$ for the combination of variant homozygote and heterozygote), implying that the risk for PL may depend on variant allele dose.

Considering that the presence of multiple polymorphisms may, by synergistic or cumulative polygenic effects on coagulation and fibrinolysis, contribute to a higher risk of RPL, results of this study have shown that the presence of two or more polymorphisms is significantly more common in women with PRL and IUFD, pointing out that the risk for PL is over three times higher if a woman is a carrier of two or more thrombophilic polymorphisms. According to the findings of Coulam *et al*,[12] the risk of PL could be more associated with the concomitant effects of more thrombophilic polymorphisms in pregnant women, than with a particular polymorphism. On the other hand, Poursadegh Zonouzi *et al*[26] failed to confirm such correlation between the presence of multiple polymorphisms and RPL.

Logistic regression analysis in our study also demonstrated the strong independent influence of *SERPINE1* 4G/5G and *ACE* I/D polymorphisms on early RPL and later PL and IUFD (both p values <0.001). Buchholz *et al*[27] reported an association of RPL and combination of *SERPINE1* and *ACE* homozygosity but failed to show by stepwise logistic regression analysis any individual association of these genes as well as for *F2* 20210G>A, *F5* 1691G>A and *MTHR* 677C>T gene polymorphisms, while Torabi *et al*[21] reported that *F5* 1691G>A and *MTHFR* 677C>T polymorphisms increase the risk for RPL. Among maternal behaviour risk factors, in our study, only maternal obesity appeared to be of some importance for PL, which is in concordance with systematic review by El Hachem *et al*,[6] which stated that increases in maternal BMI over 30 kg/m2 were associated with increased risk of PL.

Similar association that we have found between investigated thrombophilic gene polymorphisms and risk for PL after two or three and more RPLs, strongly support the recommendations[2–4 6 7 34 35] that the diagnostic protocol regarding inherited thrombophilia should be administered after second PLs. The number of participants in our study could be considered as a limitation, but this is the first study of thrombophilic gene polymorphisms and PL in Montenegrin population (total approximately 650 000) and our results highlight the possible role of *SERPINE1* 4G/5G and *ACE* I/D polymorphisms in PL.

In conclusion, our results showed that both individual and co-occurrence of polymorphisms in the *SERPINE*1 and *ACE* genes were significant risk factors for both, recurrent early PL and late PL and IUFD. It has also been shown that the multiple polymorphism occurrence further amplifies the risk of PL, which is best confirmed by the joint presence of *SERPINE*1 and *ACE* polymorphisms in women with RPL and IUFD. The findings presented in this study may contribute to addressing the question of whether *SERPINE1* and *ACE* gene polymorphisms should be included in diagnostic work-up of women with RPL and may be useful in adding to the data on inherited thrombophilia as an important contributor to RPL pathogenesis.

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Supplemental table. Analysis of genotype distribution in 2EPL, ≥3EPL, ≥1LPL/IUGD and under/over 35 years subgroups of case group compared to the controls upon the dominant genetic model

Genotypes	n (%)	n (%)	p value	OR CI 95%
	2EPL group Nº 33	**Control group Nº 95**		
***F2* 20210G>A (rs1799963)** AA+GA vs GG	1 (3.0) vs 32 (97.0)	1 (1.1) vs 94 (98.9)	ᵅ p=1	OR 2.938(0.179-48.342)
***F5* 1691G>A (rs6025)** AA+GA vs GG	4 (12.1) vs 29 (87.9)	2 (2.1) vs 93 (97.5)	ᵅ p=0.038*	OR 6.414 (1.117-36.83)
***MTHFR* 677C>T (rs1801133)** TT+CT vs CC	4 (12.1) vs 29 (87.9)	47 (49.5) vs 48 (50.5)	ᵅ p=0.0001***	OR 0.141(0.046-0.432)
***SERPINE1* 4G/5G (rs1799762)** 4G/4G+4G/5G vs 5G/5G	28 (84.8) vs 5 (15.2)	57 (60.0) vs 38 (40.0)	ᵞ p=0.009**	OR 3.733 (1.324-10.524)
***ACE* I/D (rs1799752)** D/D+I/D vs I/I	27 (81.8) vs 6 (18.2)	55 (57.9) vs 40 (42.1)	ᵞ p=0.014*	OR 3.273 (1.236-8.667)
	≥3EPL group Nº 72	**Control group Nº 95**		
***F2* 20210G>A(rs1799963)** AA+GA vs GG	0 vs 72	1 (1.1) vs 94 (98.9)	ᵅp=1	-
***F5* 1691G>A (rs6025)** AA+GA vs GG	2 (2.8) vs 70 (97.2)	2 (2.1) vs 93 (97.5)	ᵅp=1	OR 1.329 (0.183-9.665)
***MTHFR* 677C>T (rs1801133)** TT+CT vs CC	30(41.7) vs 42(58.3)	47 (49.5) vs 48 (50.5)	ᵞp=0.317	OR 0.73 (0.393-1.353)
***SERPINE1* 4G/5G (rs1799762)** 4G/4G+4G/5G vs 5G/5G	56(77.8) vs 16(22.2)	57 (60.0) vs 38 (40.0)	ᵞp=0.015*	OR 2.33 (1.169-4.656)
***ACE* I/D (rs1799752)** D/D+I/D vs I/I	61(84.7) vs 11(15.3)	55 (57.9) vs 40 (42.1)	ᵞ p=0.0002***	OR 4.033 (1.886-8.627)
	≥1LPL/IUFD Nº 24	**Control group Nº 95**		
***F2* 20210G>A(rs1799963)** AA+GA vs GG	2 (8.3) vs 22 (91.7)	1 (1.1) vs 94 (98.9)	ᵅp=0.103	OR 8.546 (0.741-98.531)
***F5* 1691G>A (rs6025)** AA+GA vs GG	1 (4.2) vs 23 (95.8)	2 (2.1) vs 93 (97.5)	ᵅp=0.495	OR 2.022 (0.176-23.277)
***MTHFR* 677C>T (rs1801133)** TT+CT vs CC	12(50.0) vs 12(50.0)	47 (49.5) vs 48 (50.5)	ᵞp=1	OR 1.021 (0.417-2.501)
***SERPINE1* 4G/5G (rs1799762)** 4G/4G+4G/5G vs 5G/5G	21 (87.5) vs 3 (12.5)	57 (60.0) vs 38 (40.0)	ᵞp=0.011*	OR 4.667 (1.301-16.742)
***ACE* I/D (rs1799752)** D/D+I/D vs I/I	16 (66.7) vs 8 (33.3)	55 (57.9) vs 40 (42.1)	ᵞp=0.435	OR 1.455 (0.567-3.729)
	Case <35 yr Nº 73	**Control <35 yr Nº41**		
***F2* 20210G>A(rs1799963)** AA+GA vs GG	1 (1.4) vs 72 (98.6)		ᵅp=1	-
***F5* 1691G>A (rs6025)** AA+GA vs GG	7 (9.6) vs 66 (90.4)	2 (4.9) vs 39 (95.1)	ᵅp=0.485	OR 2.068 (0.409-10.456)
***MTHFR* 677C>T (rs1801133)** TT+CT vs CC	23(31.5) vs 50(68.5)	20 (48.8) vs 21 (51.2)	ᵞp=0.068	OR 0.483 (0.22-1.061)
***SERPINE1* 4G/5G (rs1799762)** 4G/4G+4G/5G vs 5G/5G	60(82.2) vs 13(17.8)	24 (58.5) vs 17 (41.5)	ᵞp=0.006**	OR 3.269 (1.379-7.753)
***ACE* I/D (rs1799752)** D/D+I/D vs I/I	61(83.6) vs 12(16.4)	28 (68.3) vs 13 (31.7)	ᵞp=0.058	OR 2.36 (0.957-5.824)
	Case ≥35 yr Nº 56	**Control ≥35 yr Nº54**		
***F2* 20210G>A(rs1799963)** AA+GA vs GG	2 (3.6) vs 54 (96.4)	1 (1.8) vs 53 (98.2)	ᵅp=1	OR 1.963 (0.173-22.302)
***F5* 1691G>A (rs6025)** AA+GA vs GG	0 vs 56	0 vs 54	ᵅP=1	-
***MTHFR* 677C>T (rs1801133)** TT+CT vs CC	23(41.1) vs 33(58.9)	27 (50) vs 27 (50)	ᵞp=0.348	OR 0.697 (0.328-1.481)
***SERPINE1* 4G/5G (rs1799762)** 4G/4G+4G/5G vs 5G/5G	45(80.4) vs 11(19.6)	33 (61.1) vs 21 (38.9)	ᵞp=0.026*	OR 2.603(1.105-6.132)
***ACE* I/D (rs1799752)** D/D+I/D vs I/I	3 (76.8) vs 13 (23.2)	27 (50) vs 27 (50)	ᵞp=0.004**	OR 3.308 (1.46-7.496)

PL: pregnancy loss

2EPL group; two PLs in the first trimester; ≥3EPL group; ≥PLs in the first trimester; ≥1LPL/IUFD group;≥ late pregnancy loss in 2nd trimester or IUFD after 24th week; yr: years

ᵞ Pearson chi-squared test; ᵅFisher exact two tailed test

* p<0.05; **p<0.01; ***p<0.001

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Maternal *MTHFR* 677C>T, 1298A>C gene polymorphisms and risk of offspring aneuploidy

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Abstract

Objective: The objective was to investigate the association between maternal methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms, crucial for DNA methylation, and risk of offspring aneuploidy.

Methods: *MTHFR* gene polymorphisms 677C>T and 1298A>C were determined by polymerase chain reaction based method, in 163 women with offspring aneuploidy and 155 women with healthy children. Five genetic models were used to assess risk, according to the type of aneuploidy and the age of women at conception.

Results: *MTHFR* 677TT genotype and T allele were significantly more prevalent among women with offspring aneuploidy, with an increased risk of aneuploidy demonstrated under a recessive (OR 3.499), homozygote (OR 3.456) and allele contrast model (OR 1.574). The more prominent association was found with sex chromosome aneuploidies and trisomy 13/18, and also in women ≤35 years at conception. No association was observed between 1298A>C polymorphism and risk of offspring aneuploidy, although synergistic effect of two polymorphisms increase the risk of aneuploidy, primarily amplifying the 677T allele effects ($p < 0.001$).

Conclusion: Maternal *MTHFR* 677C>T gene polymorphism, alone or in combination with another 1298A>C polymorphism, appears to be a substantial risk factor for offspring aneuploidy in Montenegro population, especially for sex chromosome aneuploidies and trisomy 13/18, and among younger women.

Key points

What's already known about this topic?

- Polymorphisms in folate pathway genes, especially the *MTHFR* 677C>T, with consequent DNA hypometilation, have been identified as possible risk factors for trisomy 21 in certain populations.
- Large geographical variations with conflicting results are shown.

What does this study add?

- This is the first clinical and genetic study on Montenegrin population and one of several in Mediterranean region, providing evidence of maternal *MTHFR* 677C>T gene polymorphism as a substantial risk factor for offspring aneuploidy, not only 21 trisomy but also trisomy 13/18 and sex chromosome aneuploidies.

1 | INTRODUCTION

Aneuploidies, as the most common chromosomal disorders (0.3%–0.5% of live births), have a devastating impact on child health, from mental retardation and congenital anomalies to cognitive impairment and reproductive failure in adulthood, but also severely limit survival in less frequently viable trisomies. 13 and 18. An improper chromosome segregation, leading to aneuploidy, mainly occurs (95%) in maternal, primarily meiosis I.[1,2] The most frequent aneuploidies in live births are free trisomy 21 and sex chromosome aneuploidies, while other autosomal trisomies are unviable, with the exception of trisomy 13 and 18, which are rarely observed in live births.[3,4] Despite detrimental clinical consequences and extensive research, the cellular and molecular mechanisms involved in chromosome non-disjunction are still poorly understood. Advanced maternal age at conception is the most convincing clinical factor for autosomal trisomies, although the underlying mechanisms of the aging effects on chromosomal malsegregation are still not clear enough.[5,6] Moreover, a significant number of aneuploidies, particularly of sex chromosomes, are more common among younger women,[7] requiring the search for other risk factors that predispose to aneuploidy in young women.

An association between folate metabolism and chromosomal segregation has been shown, suggesting the influence of folate pathway gene polymorphisms on DNA hypomethylation and abnormal chromosomal disjunction.[8] Enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is the major regulator of cellular DNA methylation, by catalyzing the conversion of 5,10-methylenetetrahydrofolate to the dominant circulating form of folate, 5-methylenetetrahydrofolate, which provides the single carbon needed for nucleotide synthesis, remethylation of homocysteine to methionine, synthesis of *S*-adenosylmethionine and DNA methylation.[9] It has been shown by James and al. that *MTHFR* gene polymorphisms can cause decreased MTHFR activity, disturbing homocysteine re-methylation, which is considered a risk factor for chromosome instability and non-disjunction.[10] Among more than 40 currently described *MTHFR* gene polymorphisms the most frequently investigated are two single nucleotide polymorphisms: rs1801133 (c.677C>T) and rs1801131 (c.1298A>C).[2,11]

The *MTHFR* c.677C>T transition results in alanine to valine substitution (p.Ala222Val), subsequently causing the enzyme thermolability and reduction of its activity by 70% in variant TT homozygote, and 35% in heterozygote CT carriers.[9,10] The transfersion of c.1298A>C in the *MTHFR* gene results in a glutamate to alanine substitution (p.Glu429Ala), with reduced in vitro enzyme activity up to 40%, in the presence of the 1298C allele.[12,13] In addition, the complex heterozygous state of the *MTHFR* gene 677T and 1298C alleles has been shown to reduce enzyme activity by 40%–50% in vitro, with similar biochemical characteristics as 677TT homozygotes.[9] A number of studies, starting from James and al.[10] over the past decade have supported maternal *MTHFR* polymorphisms as a risk of trisomy 21, other aneuploidies, and various adverse reproductive outcomes, while other studies have highlighted their influence only in certain populations, or have shown no association at all.[14] Given the controversial results and geographical variations, the clinical importance of polymorphisms in the *MTHFR* gene remains doubtful and requires a broader assessment in different populations.

To date, there are no data on maternal *MTHFR* gene polymorphisms in relation to offspring aneuploidy in the Montenegrin population, and to our knowledge, a relatively small number of studies have been conducted in the southern European-Caucasian population.[15–18] This case-control study was conducted to determine whether the two maternal *MTHFR* gene polymorphisms are associated with an increased risk of offspring aneuploidy in a homogeneous Caucasian population of Montenegrin women.

2 | METODS

2.1 | Study design

This case-control study was conducted on a total of 318 women, of whom the *Case group* included 163 women with offspring aneuploidy (pregnancy or childbirth with chromosomal aneuploidy) and a *Control group* of 155 women who gave birth to at least two healthy children without chromosomal or congenital abnormalities.

The participants of the case group were recruited from women with offspring chromosomal aneuploidy confirmed by invasive prenatal or postnatal cytogenetic diagnostics. A total of 163 women with fetus (76) or child (87) with free chromosomal aneuploidy, were included in the case group. Due to chromosomal instability and non-disjunction caused by *MTHFR* gene polymorphism and a similar mechanism of DNA hypomethylation in meiosis or in early postzygotic cell divisions, 12 cases of mosaic aneuploidy (7.3%). The participants with offspring structural chromosomal aberrations were excluded from the study. The case group participants were stratified into subgroups according to type of aneuploidy in their offspring: *Trisomy 21 group*; *Trisomy 13/18 group* and Sex *chromosome aneuploidy* group (XXX, XXY trisomy or monosomy X). All the participants of case and control group were also divided into two groups according to the age of women at the time of conception: *Under 35 years* (≤35) and *Over 35 years* (>35) group. The control group included 155 age-matched women, who had at least two healthy children without chromosomal or congenital abnormalities and with no history of pregnancy loss. All participants in the case and control group were Caucasians, with similar ethnic background.

The study was carried out at the Center for Medical Genetics and Immunology, tertiary hospital of Montenegro (University Clinical Center of Montenegro), and was approved by the Institutional Ethics Committee (code no 03/01–5005/1). All the participants were enrolled in the study after obtaining written informed consent for participation in the research.

2.2 | DNA extraction and genotype analyses

Peripheral blood samples (5ml) were collected in EDTA tubes and stored at −40°C until DNA extraction. Genomic DNA was extracted using the commercial QIAamp DNA Blood Mini Kit (Qiagen,

Germany). *MTHFR* 677C>T [rs1801133; NM_005957.5:c.665C>T (p.Ala222Val)] and *MTHFR* 1298A>C [rs1801131; NM_005957.5:c.665C>T (p.Ala222Val)] genotyping was performed by Attomol® Quicktype kits (Attomol GmbH, Molekulare Diagnostika) for allele-specific polymerase chain reaction (PCR). After electrophoresis in 2.5% agarose gel (stained with ethidium bromide), all PCR products were visualized by ultra-violet (UV) transilluminator (LKB).

2.3 | Statistical analyses

Statistical analyses were performed by SPSS for Windows Statistics 20.0 (SPSS, Inc.) and EZR 1.36 (Saitama Medical Center Jichi Medical University). In all testing, a p value < 0.05 was considered to be statistically significant. Distributions of genotypes and frequencies of alleles in both polymorphisms were compared between groups, using Pearson's chi-square, two-tailed Fisher's exact test and Bonferroni's correction for multiple comparisons in small subgroups. Chi-square test was also conducted to assess the Hardy–Weinberg equilibrium (HWE) for each polymorphism in cases and controls. To evaluate the maternal *MTHFR* gene polymorphisms as a risk for offspring aneuploidy five genetic models were used: *dominant* (variant homozygote plus heterozygote vs. wild type homozygote); *recessive* (variant homozygote vs. heterozygote plus wild type homozygote); *homozygote* (variant homozygote vs. wild type homozygote); *codominant* (heterozygote vs. wild-type homozygote) and *allele contrast* (variant allele vs. wild type allele).[11,19] Calculation of odds ratios (ORs), with corresponding 95% confidence intervals (CIs) was performed for each genetic model to estimate the relative risk regarding different genotypes. Post-hoc power (PHP) analysis (2 × 2 analysis) and 95% confidence interval (95% CI) for the difference between two population proportions were used to indicate the power of the study in genetic model subgroup analyses. The Cochran-Armitage trend test was used to explore possible interactions of two assessed polymorphisms.

3 | RESULTS

The relationship between maternal *MTHFR* c.677C>T (rs1801133) and *MTHFR* c.1298A>C (rs1801131) gene polymorphisms (hereinafter *MTHFR* 677C>T and 1298A>C) and risk for aneuploidy conception was examined in the group of women with offspring aneuploidy (case group) and compared to fertile control women. Analyzes of case group subgroups with regard to the type of aneuploidy and the age of the woman at the time of conception were also performed.

The mean age of the case and control group women were 33.3 years (range 20–45 years) and 35.4 years (range 18–42 years) respectively, with no statistical difference among the groups ($p = 0.25$). Distribution of *MTHFR* 677C>T and *MTHFR* 1298A>C genotypes didn't deviate from Hardy–Weinberg equilibrium in both groups (chi-square test, all p values > 0.05).

Variant homozygote TT and T allele of *MTHFR* 677C>T polymorphism were significantly more prevalent in the case group than in the control ($p < 0.001$ and $p < 0.01$ respectively; Table 1). Significantly more prevalent distribution of variant homozygote 677TT and 677T allele was found in all subgroups of case group compared to the controls, except for the T allele in the *Trisomy 21* subgroup. The prevalence of TT homozygote and variant T allele was higher and with stronger statistical significance among participants of *Trisomy 13/18* and *Sex chromosome aneuploidies* subgroups. For the *MTHFR* 1298A>C polymorphism, no statistically significant difference was found in the distribution of the variant homozygote CC and C allele between the case group and its subgroups compared to controls.

Genetic model analysis demonstrated a significantly increased risk of offspring aneuploidy in the presence of maternal variant homozygote TT and T allele of *MTHFR* 677C>T polymorphism (Table 2) under three out of five investigated genetic models (recessive: OR = 3.499; homozygote: OR = 3.456, and allele contrast: OR = 1.574). The same three genetic models illustrated the association between homozygote TT and T allele and trisomy 13/18 and sex chromosome aneuploidies upon recessive model, and additionally upon allele contrast model for sex chromosome aneuploidies (PHP ≥80%). A significantly increased risk for trisomy 21 was found under recessive model (OR 2.58), but with PHP trend (71.2%). The association of *MTHFR* 2198A>C polymorphism with an increased risk of offspring aneuploidy has not been demonstrated in any of the applied genetic models, with CC and AC polymorphic genotypes even more prevalent among in control group compared to the *sex chromosome aneuploidy* subgroup (Supplementary 1).

Significant association between maternal variant genotype 677TT and 677T allele and risk of offspring aneuploidy was demonstrated in women under 35 according to dominant (OR 2.504), recessive (OR 4.97), homozygote (OR 6.452) and allele contrast (OR 3.506) model (PHP ≥80% for recessive and allele contrast models in case group, and additionally for dominant model in other aneuploidies subgroup; Table 3). Among women above 35, the association of 677C>T polymorphism and risk for aneuploidies was found for *other aneuploidies*, under recessive and homozygote models (PHP ≥80% for recessive model), but not for *trisomy 21* subgroup. No association was found between the *MTHFR* 2198A>C polymorphism and aneuploidy in women over and under 35 years (Supplementary 2).

The presence of two variant alleles (homozygote 677TT or 1298CC or 677CT+1298AC) as a risk combination was significantly higher in the case group ($p < 0.001$) compared to the control group (Figure 1A). Two variant alleles were observed in 55% (89/163) of the case group, while 72% of the control group (111/155) had all four wild type alleles or only one variant allele. No participant in either group had more than two variant alleles, that is, two variant homozygous genotypes (677TT and 1298CC), or variant homozygote of one and heterozygote of the other polymorphism. Two heterozygotes combination 677CT+1298AC, was also significantly higher in the case group ($p < 0.003$; Figure 1B). An increased risk of offspring aneuploidy in the combination of two maternal *MTHFR* gene polymorphisms was additionally confirmed by the Cochran-Armitage trend test ($p < 0.01$ for the combination of *MTHFR* 677CT

TABLE 1 Genotype distribution and allele frequencies of maternal *MTHFR* 677C>T and 1298A>C gene polymorphisms in case group, subgroups with different offspring aneuploidy and control group of women

Genotypes and alleles	*n* (%) Case group No 163	*n* (%) Control group No 155	χ^2 test/*p* value
MTHFR 677C>T			
CC/CT/**TT**	58/68/37 (35.6/41.7/22.7)	65/78/12 (41.9/50.3/7.4)	***p* = 0.001****
C allele/T allele	184/142 (56.4/43.6)	208/102 (67.1/32.9)	†***p* = 0.006****
MTHFR 1298A>C			
AA/AC/**CC**	88/61/14 (54/37.4/8.6)	89/58/8 (57.4/37.4/5.2)	*p* = 0.468
A allele/C allele	237/89 (72.7/27.3)	236/74 (76.1/23.9)	†*p* = 0.322
	Trisomy 21 No 118	**Control group No 155**	
MTHFR 677C>T			
CC/CT/**TT**	48/49/21 (40.7/41.5/17.8)	65/78/12 (41.9/50.3/7.4)	***p* = 0.034***
C allele/T allele	145/91 (61.4/38.6)	208/102 (67.1/32.9)	†*p* = 0.17
MTHFR 1298A>C			
AA/AC/**CC**	61/46/11 (51.7/39/9.3)	89/58/8 (57.4/37.4/5.2)	*p* = 0.348
A allele/C allele	168/68 (71.2/28.8)	236/74 (76.1/23.9)	†*p* = 0.192
	Trisomy 13/18 No 24	**Control group No 155**	
MTHFR 677C>T			
CC/CT/**TT**	7/10/7 (29.2/41.6/29.2)	65/78/12 (41.9/50.3/7.4)	***p* = 0.018***[a]
C allele/T allele	24/24 (50/50)	208/102 (67.1/32.9)	†***p* = 0.021***
MTHFR 1298A>C			
AA/AC/**CC**	10/12/2 (41.6/50/8.3)	89/58/8 (57.4/37.4/5.2)	*p* = 1[a]
A allele/C allele	32/16 (66.7/33.3)	236/74 (76.1/23.9)	†*p* = 0.159
	‡**Sex chromos. aneupl. No 21**	**Control group No 155**	
MTHFR 677C>T			
CC/CT/**TT**	3/9/9 (14.2/42.9/42.9)	65/78/12 (41.9/50.3/7.4)	***p* < 0.003****[a]
C allele/T allele	15/27 (35.7/64.3)	208/102 (67.1/32.9)	†***p* < 0.001****
MTHFR 1298A>C			
AA/AC/**CC**	17/3/1 (80.9/14.3/4.8)	89/58/8 (57.4/37.4/5.2)	*p* = 0.306[a]
A allele/C allele	37/5 (88.1/11.9)	236/74 (76.1/23.9)	†*p* = 0.081

Note: Bold values indicate statistically significant values ($p < 0.5$).

[a]Bonferroni's correction.

†Pearson chi square.

‡Sex chromosome aneuploidies: trisomy XXX, XXY and monosomy X.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

genotype with different *MTHFR* 1298A>C genotypes and $p < 0.05$ for the combination of *MTHFR* 1298AC genotype with different *MTHFR* 677C>T genotypes; Figure 2).

4 | DISCUSSION

Evaluation of maternal *MTHFR* 677C>T and 1298A>C gene polymorphisms in this study showed that 677C>T polymorphism, but not 1298A>C, is a meaningful risk factor for chromosomal malsegregation in the group of Montenegrin women with offspring aneuploidy. The analyzes were performed by applying five genetic models, in relation to different types of aneuploidy and the age of the woman at conception.

Recent research on elucidating chromosomal non-disjunction, started by James and al.[10] opens up a new perception of aneuploidy as a metabolic disorder caused by DNA hypomethylation and consequent chromosome instability affected by polymorphisms in folate metabolism genes.[13] Intensive biomedical research has provided evidence of folate metabolism crucial role in DNA synthesis

TABLE 2 Estimates of genotype distribution and odds ratio (OR) of the *MTHFR* 677C>T polymorphism according to five genetic models in the case group subgroups with different offspring aneuploidy and control group of women

[§]Genetic models	*n* (%) Case group No 163	*n* (%) Control group No 155	*p* value	OR (95% CI)
TT+CT versus CC	105 (64.4) versus 58 (35.6)	90 (58.1) versus 65 (41.9)	[†]$p = 0.245$	1.308 (0.832–2.055)
TT versus CT+CC	37 (22.7) versus 126 (77.3)	12 (7.7) versus 143 (92.3)	[†]**$p < 0.001$***	**3.499 (1.748–7.003)**
TT versus CC	37 (22.7) versus 58 (35.6)	12 (7.7) versus 65 (41.9)	[†]**$p < 0.001$***	**3.456 (1.647–7.251)**
CT versus CC	68 (41.7) versus 58 (35.6)	78 (50.3) versus 65 (41.9)	[†]$p = 0.925$	0.977 (0.604–1.58)
T versus C	142 (43.6) versus 184 (56.4)	102 (32.9) versus 208 (67.1)	[†]**$p = 0.006$****	**1.574 (1.14–2.173)**
	Trisomy 21 No 118	**Control group No 155**		
TT+CT versus CC	70 (59.3) versus 48 (40.7)	90 (58.1) versus 65 (41.9)	[†]$p = 0.842$	1.053 (0.648–1.713)
TT versus CT+CC	21 (17.8) versus 97 (82.2)	12 (7.7) versus 143 (92.3)	[†]**$p = 0.012$*[CI]**	**2.58 (1.213–5.487)**
TT versus CC	21 (17.8) versus 48 (40.7)	12 (7.7) versus 65 (41.9)	[†]**$p = 0.0322$***	**2.37 (1.063–5.281)**
CT versus CC	49 (41.5) versus 48 (40.7)	78 (50.3) versus 65 (41.9)	[†]$p = 0.538$	0.851 (0.508–1.426)
T versus C	91 (38.6) versus 145 (61.4)	102 (32.9) versus 208 (67.1)	[†]$p = 0.17$	1.28 (0.899–1.822)
	Trisomy 13/18 No 24	**Control group No 155**		
TT+CT versus CC	17 (70.8) versus 7 (29.2)	90 (58.1) versus 65 (41.9)	[†]$p = 0.235$	1.754 (0.688–4.473)
TT versus CT+CC	7 (29.2) versus 17 (70.8)	12 (7.7) versus 143 (92.3)	[¶]**$p = 0.006$**[p,CI]**	**4.907 (1.702–14.15)**
TT versus CC	7 (29.2) versus 7 (29.2)	12 (7.7) versus 65 (41.9)	[¶]**$p = 0.008$**[CI]**	**5.417 (1.607–18.26)**
CT versus CC	10 (41.6) versus 7 (29.2)	78 (50.3) versus 65 (41.9)	[†]$p = 0.74$	1.191 (0.429–3.303)
T versus C	24 (50) versus 24 (50)	102 (32.9) versus 208 (67.1)	[†]**$p = 0.021$***	**2.039 (1.104–3.766)**
	[Φ]**Sex chrom. aneupl. No 21**	**Control group No 155**		
TT+CT versus CC	18 (85.7) versus 3 (14.3)	90 (58.1) versus 65 (41.9)	[†]**$p = 0.015$*[CI]**	**4.333 (1.225–15.326)**
TT versus CT+CC	9 (42.9) versus 12 (57.1)	12 (7.7) versus 143 (92.3)	[¶]**$p < 0.001$***[p,CI]**	**8.938 (3.14–25.436)**
TT versus CC	9 (42.9) versus 3 (14.3)	12 (7.7) versus 65 (41.9)	[¶]**$p < 0.001$****	**16.25 (3.833–68.89)**
CT versus CC	9 (42.9) versus 3 (14.3)	78 (50.3) versus 65 (41.9)	[†]$p = 0.17$	2.5 (0.65–9.619)
T versus C	27 (64.3) versus 15 (35.7)	102 (32.9) versus 208 (67.1)	[†]**$p < 0.001$***[p,CI]**	**3.671 (1.87–7.203)**

Note: Bold values indicate statistically significant values ($p < 0.5$).

[†]Pearson chi square.

[‡]Sex chromosome aneuploidies: trisomy XXX, XXY and monosomy X.

[¶]Fisher exact two tailed.

[§]Genetic models: dominant TT+CT versus CC; recessive TT versus CT+CC; homozygote TT versus CC; codominant CT versus CC and allele contrast model T Versu C.

[P]PHP ≥80%.

[CI]95% CI significant (no zero value).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

and repair, nucleic acid methylation, gene expression control and other fundamental processes involved in conception, cell division, embryo development, and successful pregnancy outcome.[20,21] Disturbances of folate metabolism, caused by folate gene polymorphisms can lead delayed DNA replication and DNA hypometilation, which epigenetically increase the risk of improper oocyte maturation, DNA damage, chromosome breakage and malsegregation in meiosis.[8,10,14]

It has been demonstrated that two maternal *MTHFR* gene polymorphisms (677C>T and 1298A>C), lead to decreased activity of this pivotal enzyme in folate metabolism, with direct impact on DNA hypomethylation, indicating that *MTHFR* is one of the few known human genes with the ability to modulate chromosomal abnormality rates.[2,9,13,22] Research on cellular biomarkers of chromosome breakage or whole chromosome loss, has shown an increased frequency of micronuclei in the lymphocytes of women with *MTHFR* 677C>T polymorphism, who had offspring with Down syndrome, suggesting a direct correlation between chromosome damage and this polymorphism, that may cause hypomethylation of pericentromeric repetitive DNA sequences, leading to a greater predisposition

TABLE 3 Estimates of genotype distribution and odds ratio (OR) of the *MTHFR* 677C>T polymorphism according to five genetic models in the case group, subgroups, and control group under and over 35 years at conception

	***n* (%)**	***n* (%)**		
	Under 35 years subgroups (≤35)			
§Genetic models	**Case group No 90**	**Control group No 44**	***p* value**	**OR (95% CI)**
TT+CT versus CC	59 (65.6) versus 31 (34.4)	19 (43.2) versus 25 (56.8)	†**$p = 0.014^{*CI}$**	**2.504 (1.197–5.239)**
TT versus CT+CC	24 (26.7) versus 66 (73.3)	3 (6.8) versus 41 (93.2)	†**$p = 0.007^{**p,CI}$**	**4.97 (1.407–17.554)**
TT versus CC	24 (26.7) versus 31 (34.4)	3 (6.8) versus 25 (56.8)	†**$p = 0.003^{**CI}$**	**6.452 (1.739–23.93)**
CT versus CC	35 (38.9) versus 31 (34.4)	16 (36.4) versus 25 (56.8)	†$p = 0.158$	1.764 (0.799–3.895)
T versus C	97 (53.9) versus 83 (46.1)	22 (25) versus 66 (75)	†**$p < 0.001^{***p,CI}$**	**3.506 (1.994–6.166)**
	Trisomy 21 No 67	**Control group No 44**		
TT+CT versus CC	39 (58.2) versus 28 (41.8)	19 (43.2) versus 25 (56.8)	†$p = 0.121$	1.833 (0.849–3.955)
TT versus CT+CC	15 (22.4) versus 52 (77.6)	3 (6.8) versus 41 (93.2)	†**$p = 0.029^{*CI}$**	**3.942 (1.069–14.545)**
TT versus CC	15 (22.4) versus 28 (41.8)	3 (6.8) versus 25 (56.8)	†**$p = 0.022^{*}$**	**4.464 (1.155–17.253)**
CT versus CC	24 (35.8) versus 28 (41.8)	16 (36.4) versus 25 (56.8)	†$p = 0.488$	1.339 (0.583–3.075)
T versus C	54 (40.4) versus 80 (59.6)	22 (25) versus 66 (75)	†**$p = 0.019^{*CI}$**	**2.025 (1.119–3.665)**
	‡Other aneuploidies No 23	**Control group No 44**		
TT+CT versus CC	19 (82.6) versus 4 (17.4)	19 (43.2) versus 25 (56.8)	†**$p = 0.002^{**p,CI}$**	**6.25 (1.823–21.431)**
TT versus CT+CC	9 (39.1) versus 14 (60.9)	3 (6.8) versus 41 (93.2)	¶**$p = 0.002^{**p,CI}$**	**8.786 (2.08–37.106)**
TT versus CC	9 (39.1) versus 4 (17.4)	3 (6.8) versus 25 (56.8)	**$p < 0.001^{***CI}$**	**18.75 (3.496–100.576)**
CT versus CC	10 (43.5) versus 4 (17.4)	16 (36.4) versus 25 (56.8)	†**$p = 0.036^{*}$**	**3.906 (1.045–14.6)**
T versus C	28 (60.9) versus 18 (39.1)	22 (25) versus 66 (75)	†**$p < 0.001^{***p,CI}$**	**4.667 (2.174–10.017)**
	Over 35 years subgroups (>35)			
	Case group No 73	**Control group No 111**		
TT+CT versus CC	47 (64.4) versus 26 (35.6)	71 (64) versus 40 (36)	†$p = 1$	1.018 (0.55–1.886)
TT versus CT+CC	13 (17.8) versus 60 (82.2)	9 (8.1) versus 102 (91.9)	†**$p = 0.047^{*}$**	2.456 (0.991–6.087)
TT versus CC	13 (17.8) versus 26 (35.6)	9 (8.1) versus 40 (36)	†$p = 0.108$	2.222 (0.832–5.938)
CT versus CC	34 (46.6) versus 26 (35.6)	62 (55.9) versus 40 (36)	†$p = 0.6$	0.844 (0.442–1.611)
T versus C	60 (41.1) versus 86 (58.9)	80 (36) versus 142 (64)	†$p = 0.327$	1.238 (0.807–1.901)
	Trisomy 21 No 51	**Control group No 111**		
TT+CT versus CC	31 (60.8) versus 20 (39.2)	71 (64.0) versus 40 (36)	†$p = 0.699$	0.873 (0.441–1.728)
TT versus CT+CC	6 (11.8) versus 45 (88.2)	9 (8.1) versus 102 (91.9)	¶$p = 0.56$	1.511 (0.508–4.499)
TT versus CC	6 (11.8) versus 20 (39.2)	9 (8.1) versus 40 (36)	†$p = 0.624$	1.333 (0.416–4.271)
CT versus CC	25 (49.0) versus 20 (39.2)	62 (55.9) versus 40 (36.0)	†$p = 0.554$	0.807 (0.397–1.64)
T versus C	37 (36.3) versus 65 (63.7)	80 (36) versus 142 (64)	†$p = 1$	1.01 (0.62–1.646)
	‡Other aneuploidies No 22	**Control group No 111**		
TT+CT versus CC	16 (72.7) versus 6 (27.3)	71 (64) versus 40 (36)	†$p = 0.431$	1.502 (0.544–4.146)
TT versus CT+CC	7 (31.8) versus 15 (68.2)	9 (8.1) versus 102 (91.9)	¶**$p = 0.006^{**p,CI}$**	**5.289 (1.714–16.318)**
TT versus CC	7 (31.8) versus 6 (27.3)	9 (8.1) versus 40 (36)	¶**$p = 0.015^{*}$**	**5.185 (1.401–19.187)**

(Continues)

TABLE 3 (Continued)

	‡Other aneuploidies No 22	Control group No 111		
CT versus CC	9 (40.9) versus 6 (27.3)	62 (55.9) versus 40 (36)	†$p = 1$	0.968 (0.32–2.927)
T versus C	23 (52.3) versus 21 (47.7)	80 (36) versus 142 (64)	†$p = 0.043$	1.944 (1.013–3.731)

Note: Bold values indicate statistically significant values ($p < 0.5$).

†Pearson chi square.

‡Other aneuploidies: trisomy 13/18, trisomy XXX, XXY and monosomy X.

¶Fisher exact two tailed.

§Genetic models: dominant TT+CT versus CC; recessive TT versus CT+CC; homozygote TT versus CC; codominant CT versus CC and allele contrast model T versu C.

PPHP ≥80%.

CI95% CI significant (no zero value).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

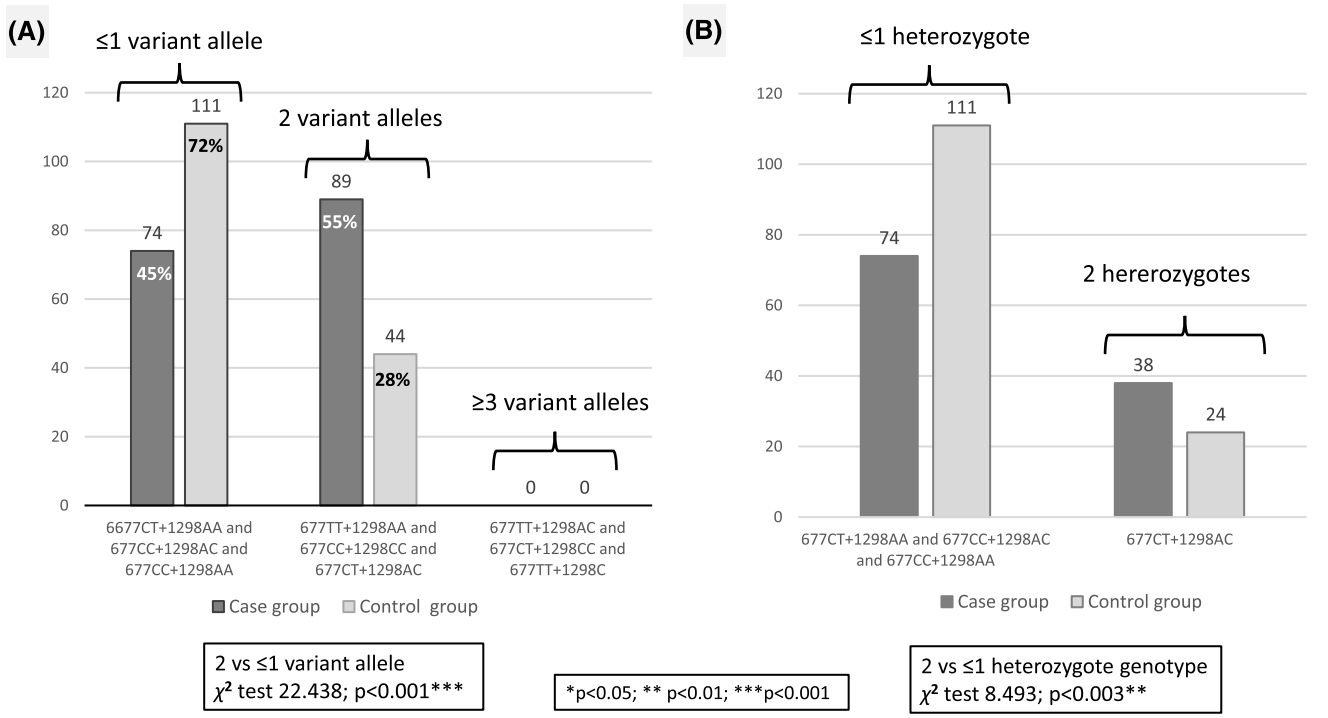

FIGURE 1 The distribution of alleles and genotypes of *MTHFR* 677C>T and 1298A>C gene polymorphisms in women with offspring aneuploidy (No 163) and control group of women with healthy children (No 155). (A) The presence of two variant alleles 677TT or 1298CC or the combination of two heterozygotes 677CT+1298AC, as a risk combination is significantly more prevalent in women with offspring aneuploidy (55%) in contrast to 28% of women in control group ($p < 0.001$). No participant of either group had more than two variant alleles that is, two variant homozygous genotypes (677TT+1298CC), or variant homozygote of one and heterozygote of the other polymorphism (677TT+128AC or 677CT+1298CC). (B) The combination of two heterozygotes 677CT+1298AC, was also significantly higher in the case group ($p < 0.003$)

to offspring aneuploidy.[1,23] Research on this topic become of increasing interest worldwide, but the presented results are conflicting and with large variations among populations.

4.1 | *MTHFR* 677C>T gene polymorphism

A significant, over 3 times higher prevalence of variant homozygote TT and 1.4 times higher variant T allele frequency of *MTHFR* 677C>T gene polymorphism was observed in our study among women with offspring aneuploidy (22.7% and 43.6% respectively) with respect to the control population of Montenegrin women (7.4% and 32.9%, respectively). Variant TT genotype was 2.4-fold more prevalent among women with free trisomy 21 in offspring; almost 4-fold in trisomy 13/18 and over 5-fold higher in women with sex chromosome aneuploidies in offspring compared to control population. Notable variations in the frequency of the *MTHFR* 677T allele in mothers of children with Down syndrome have been reported

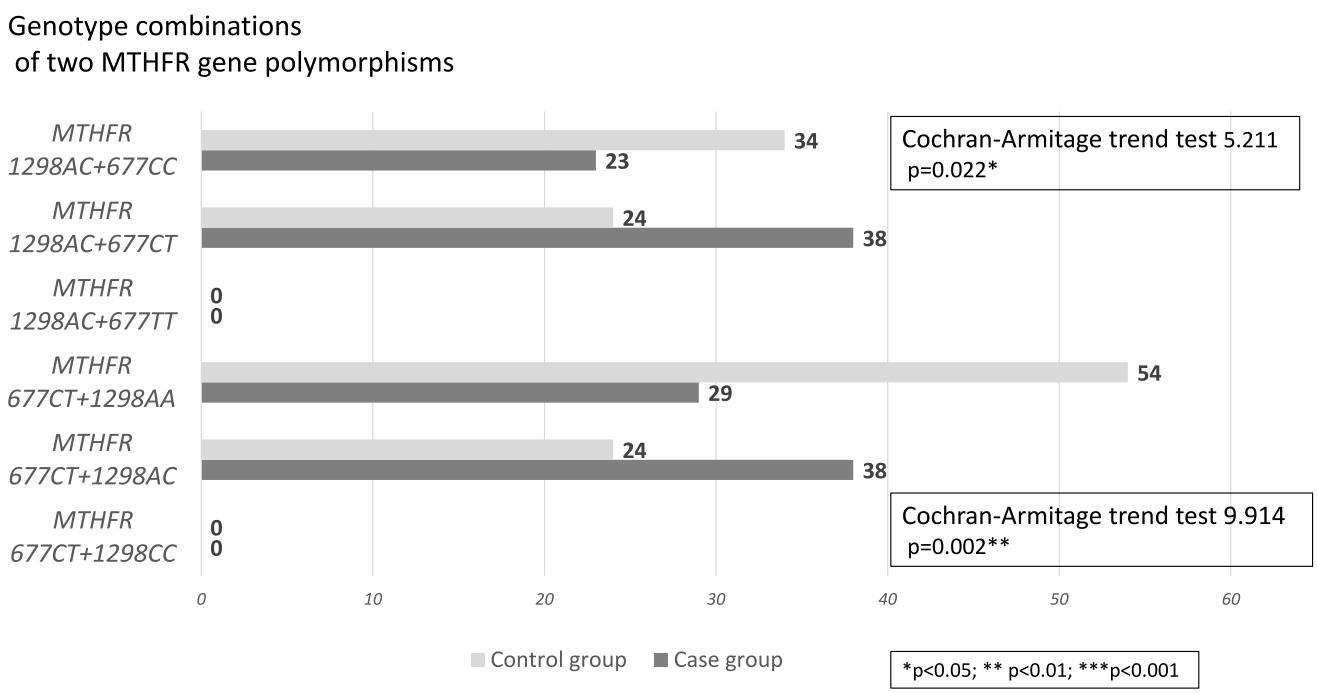

FIGURE 2 The distribution of combined *MTHFR* 677C>T and 1298A>C genotypes in women with offspring aneuploidy (No 163) and control group of women with healthy children (No 155). The Cochran-Armitage trend test demonstrate an increased risk of offspring aneuploidy in the combination of two maternal *MTHFR* gene polymorphisms, with significance of $p < 0.01$ for the combination of *MTHFR* 677CT genotype with different *MTHFR* 1298A>C genotypes and $p < 0.05$ for the combination of *MTHFR* 1298AC genotype with different *MTHFR* 677C>T genotypes

worldwide, with the highest prevalence found among the Euro-Caucasian population, ranging from 35.6% to 41.4%, increasing with less latitude, with the highest prevalence and pointed risk for Down syndrome in populations belonging to subtropical regions.[19] This zonal association was explained as the result of photolysis of folates due to high emission of specific wavelength UV radiation in subtropical regions.[24] Given that Montenegro is a country in the Mediterranean, South-Eastern Europe area and predominantly falls within the subtropical region, the frequency of T allele among women with trisomy 21 (38.6%), but also among women with trisomy 13/18 or sex chromosome aneuploidies (50% and 64.3% respectively), are as it is expected, within the reported range for the Euro-Caucasian population.

Applying five genetic models to demonstrate the association of maternal 677C>T polymorphism with the risk of offspring aneuploidy, we showed a significant, over 3-fold higher risk under recessive and homozygote models (both p values < 0.001), and above 50% higher risk if the woman is a carrier of the variant T allele (allele contrast model; $p < 0.01$). The risk of offspring with trisomy 21 was 2.5times higher, with PHP trend under recessive model, and even higher risk for trisomy 13 and 18 under recessive and allele contrast models (ORs in the range 5.417–2. 039; PHP ≥80% for recessive model). The highest risk was demonstrated for sex chromosome aneuploidies under all but the co-dominant model (all ORs above 4; PHP ≥80% for recessive model).

Unlike individual case-control studies showing conflicting outcomes within different geographic populations,[1,2,10,11,13,15–18,26–32] several meta-analyzes conducted over the past decade have highlighted a significant association between *MTHFR* 677C>T maternal polymorphism and Down syndrome risk in offspring.[19,24,25,33,34] Using different genetic models, a stable association was shown for *MTHFR* 677C>T maternal polymorphism in Caucasians, which concurs with our results. Our results confirm the genetic association between the *MTHFR* 677C>T maternal polymorphism and the risk of trisomy 21 and other aneuploidies, in a homogeneous Caucasian Montenegrin population belonging to the Mediterranean basin, where, to our knowledge, not so many studies have been conducted.

In contrast to numerous studies on trisomy 21, a limited number examined the association of maternal *MTHFR* polymorphisms with other viable aneuploidies. An association of 677C>T polymorphism with trisomy 18 and 13 was reported,[4,14] while an association with Turner syndrome was observed for *MTHFR* 1298A>C, but not for 677C>T polymorphism.[35,36] Our data strongly support the significance of maternal polymorphism *MTHFR* 677C>T as a risk factor for sex chromosome aneuploidies, including monosomy X, which we have not been able to find in the available literature. Several studies have shown that, in addition to hypomethylation of the repetitive DNA sequences, elevated homocysteine levels cause endoplasmic oxidative stress and the formation of free radicals that cause altered cellular activity and DNA damage,[30,37] which could explain the strong association of *MTHFR* 677T with sex chromosome aneuploidies in our study.

Our findings also highlighted the maternal 677T allele as a risk factor for offspring aneuploidy in younger women (≤35 years at

conception), with 3-6-fold higher risk under recessive, homozygous and allele contrast models, suggesting that chromosomal nondisjunction in young women may be the result of complex gene-environmental interactions involving the lifestyle and genotypes of mother and maternal grandmother, as shown in previous studies on maternal meiosis I and II.[4,14] A stronger association with other chromosome aneuploidies (PHP ≥80% for dominant, recessive and allele contrast models), than with trisomy 21 (PHP trends 59.8% for recessive and 66.3% for allele contrast models), in our study is consistent with recent reports on significantly higher prevalence of sex chromosome aneuploidies in younger women.[6,7] Our data do not support the 677T allele as a risk for trisomy 21 in women older than 35 years, but an increased risk has been observed for other offspring aneuploidies in presence of maternal TT homozygote (recessive model, PHP ≥80%).

4.2 | *MTHFR* 1298A>C gene polymorphism

The present study did not show any association between maternal *MTHFR* 1298A>C polymorphism and risk for aneuploidy in offspring, which is consistent with the findings of vast majority of case-control studies,[2,17,18,22,27,28,31] and almost all meta-analyzes showing that *MTHFR* 1298A>C polymorphism is not an independent risk factor for trisomy 21 in offspring,[19,24,33,38] especially among Euro-Caucasian population. However, some studies demonstrate an association of maternal *MTHFR* 1298A>C polymorphism with trisomy 21[9,13,15,26,30,31] or monosomy X certain populations.[34,35]

4.3 | Interaction between *MTHFR* variant genotypes and risk for aneuploidy

A growing research on folate pathway gene polymorphisms reported their interaction as an additional risk for aneuploidy in offspring. Several studies have reported an additive risk effect of *MTHFR* 677T in combination with the 1298C allele, suggesting that two distinct variants in the *cis* position may functionally interact causing a decrease in enzyme activity with a stronger effect on the observed phenotype.[14,15,27,28,31] It is also noticed that co-occurrence of ≥3 folate metabolism polymorphisms present an increased risk for trisomy 21.[9,13,14]

The combination of maternal *MTHFR* 677T and 1298C alleles and 677CT and 1298AC genotypes was identified as an additive risk for offspring aneuploidy in this study (Figure 1A,B). The interactive and dosage effects of the two polymorphisms are best illustrated by evidence of two variant alleles in 55% of women with offspring aneuploidy, as opposed to 28% in control mothers, who had predominantly all four wild type alleles or only one variant allele. No subject carrying three or four variant alleles was identified. Our findings are consistent with previous reports,[9,27] confirming the independent presence of these polymorphisms on two different wild-type alleles. Enciso et al.[9] hypothesized that the ancestral *MTHFR* gene had a haplotype of 677C/1298A. The mutation at the 677 nucleotide position and, independently, the mutation at the 1298 position, later produced the 677T/298A and 677C/1298C haplotypes, thus two alleles are always in trans-configuration, and the 677TT genotype always goes with the 1298AA genotype or vice versa. Accordingly, 677T/1298C haplotypes can only be formed by recombination within the *MTHFR* gene, which is extremely unlikely given the small distance (2.1 kb) between these polymorphisms. An increased risk of combined maternal genotypes 677CT/298AC, confirmed by the Cochran-Armitage trend test in our study, implies that the synergistic effect of maternal variant alleles may increase the risk of chromosome malsegregation and aneuploidy in offspring, primarily amplifying the effects of 677T allele.

There are few limitations of the present study: two polymorphism effects of only one folate pathway gene were evaluated; no gene-environmental interaction has been considered. Small subgroups and control group <35 years at conception could also be a limitation of the study, although PHP was ≥80% in almost all subgroups upon the recessive model and upon the allele contrast model in subgroups <35 years. However, the following could be considered advantages: controls were age-matched and with healthy children, providing the same likelihood of aneuploidy conceiving for both groups, five genetic models were applied for the risk assessment and subgroup analysis included all viable aneuploidies and the age of women at conception.

5 | CONCLUSION

The present study has demonstrated that maternal *MTHFR* 677C>T gene polymorphism is associated with increased risk of offspring aneuploidy in the Montenegrin population. The impact of *MTHFR* 677 T allele and TT genotype on aneuploid conception was more pronounced in younger women (≤35 years) and was more strongly associated with the risk of sex chromosome aneulpoidies and trisomy 13/18, than trisomy 21. Although no association was observed between the 1298A>C polymorphism and the risk of offspring aneuploidy, the synergistic effect of the two polymorphisms may increase the risk of aneuploidy, primarily by amplifying the 677T allele effects. It is hoped that these results may contribute to the understanding of the *MTHFR* gene relevance, in the complex etiology of chromosomal aneuploidies, and thus to potential future interventions to increase the efficiency of normal conceptions and reduce the aneuploidy rate.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated by this study are presented in the manuscript, including supplemental data.

ETHICS STATEMENT

This study received ethical approval from University Clinical Center of Montenegro Ethics Committee (code no 03/01-5005/1). Written informed consent was obtained from all participants included in the study.

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SUPPORTING INFORMATION

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Current State of Compulsory Basic and Clinical Courses in Genetics for Medical Students at Medical Faculties in Balkan Countries With Slavic Languages

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Introduction: In this study we aimed to perform the first research on the current state of compulsory basic and clinical courses in genetics for medical students offered at medical faculties in six Balkan countries with Slavic languages (Bosnia and Herzegovina, Croatia, Montenegro, North Macedonia, Serbia, and Slovenia).

Materials and Methods: The study was conducted from June to September 2021. One representative from each country was invited to collect and interpret the data for all medical faculties in their respective country. All representatives filled a questionnaire, which consisted of two sets of questions. The first set of questions was factual and contained specific questions about medical faculties and design of compulsory courses, whereas the second set of questions was more subjective and inquired the opinion of the representatives about mandatory education in clinical medical genetics in their countries and internationally. In addition, full course syllabi were analysed for course aims, learning outcomes, course content, methods for student evaluation and literature.

Results: Detailed analysis was performed for a total of 22 medical faculties in Bosnia and Herzegovina (6), Croatia (4), Montenegro (1), North Macedonia (3), Serbia (6), and Slovenia (2). All but the two medical faculties in Slovenia offer either compulsory courses in basic education in human genetics (16 faculties/courses) or clinical education in medical genetics (3 faculties/courses). On the other hand, only the medical faculty in Montenegro offers both types of education, including one course in basic education in human genetics and one in clinical education in medical genetics. Most of the basic courses in human genetics have similar aims, learning outcomes and content. Conversely, clinical courses in medical genetics are similar concerning study year position, number of contact hours, ECTS (European Credit Transfer and Accumulation System) and contents,

but vary considerably regarding aims, learning outcomes, ratio of types of classes, teaching methods and student evaluation.

Conclusion: Our results emphasise the need for future collaboration in reaching a consensus on medical genetics education in Balkan countries with Slavic languages. Further research warrants the analysis of performance of basic courses, as well as introducing clinical courses in medical genetics to higher years of study across Balkan countries.

Keywords: genetic education, medical genetics, human genetics, medical education, compulsory course, genomic medicine, medical students, medical faculty

INTRODUCTION

Medical genetics is one of the most complex, comprehensive and multidisciplinary medical specialties covering all stages of life and organ systems, simultaneously placing a special emphasis on ethical, legal and social implications of genetic testing. Moreover, the integration of the fascinating advancements in the development of genetic and genomic testing methods into various parts of medicine occurs at an accelerated pace. Therefore, most countries in Europe, especially Western Europe, have long recognised not only the importance of introducing medical and laboratory genetics as separate medical specialties, but have also put effort into raising the level of genetic literacy among medical students as the future health professionals who will be involved in the care of patients with genetic disorders (Tobias et al., 2021).

The Balkan area is a geographical region in the south-eastern part of the European continent, associated with different cultural and historical classifications. One of these includes the classification according to the languages spoken in specific countries, such as Slavic, Romance, Turkish and other languages. Countries with Slavic languages include Bosnia and Herzegovina, Bulgaria, Croatia, Montenegro, North Macedonia, Serbia and Slovenia. In fact, these countries are not only associated by Slavic language but also similar higher education and health systems.

Unfortunately, Balkan countries with Slavic languages have encountered many historical obstacles that have left inevitable consequences in terms of significant delays in both introducing medical and laboratory genetics as medical specialties, as well as recognising genetic education at medical faculties as an indispensable tool for future physicians of the 21st century. Consequently, the advances in medical genetics internationally have not been accompanied always by an adequate level of application in clinical practice nor raising genetic literacy among medical students locally in the Balkans. Furthermore, most countries have not yet introduced medical or laboratory genetics as medical specialties, which inevitably reflects on the (poor) position of genetic education in the integrated undergraduate and graduate medical education system.

Genetic education of medical students is a critical prerequisite for appropriate care for patients with genetic disorders (Bennett et al., 2017; Hyland and Dasgupta, 2019). Because medical genetics is both a basic science and a clinical specialty, appropriate genetic education of medical students should include the literacy on basic concepts in human genetics, as well as clinical concepts in medical genetics (Robinson and Fong, 2008). However, the current situation for genetic education opportunities for medical students at medical faculties in the afore-mentioned Balkan countries is not known. Considering this, as well as the fact that Balkan countries with Slavic languages are associated by more similarities than separated by simply geographical boundaries, the aim of this study was to analyse the current state of compulsory basic and clinical courses in genetics for medical students offered at medical faculties in these countries.

MATERIALS AND METHODS

Inclusion of Representatives From Different Balkan Countries

This retrospective study was conducted from June to September 2021. To investigate the current state of basic and clinical compulsory courses in genetics for medical students at medical faculties in Balkan countries in which Slavic languages are spoken, the study was designed so that one representative from each of the selected countries was invited to collect and interpret the data for all medical faculties in their respective country.

An additional four representatives from four different Balkan countries with Slavic languages (Bosnia and Herzegovina, Montenegro, North Macedonia, and Serbia) were contacted *via* e-mail in June 2021 with a letter of invitation to participate in the study. The representatives were chosen based on their expertise, as well as national and international excellence in the field of both basic human genetics and clinical medical genetics. The letter of invitation contained all the relevant information regarding the research, including an explanation of the background, aims, materials and methods. In addition, in this invitation letter, the representatives were sent and asked to fill a questionnaire about the basic and clinical compulsory courses in genetics offered in their respective countries at medical faculties for medical students and a due date was provided. All six representatives (Bosnia and Herzegovina, Croatia, Montenegro, North Macedonia, Serbia and Slovenia) filled the questionnaire and were sent a second e-mail with the request to send the full syllabi for each course mentioned in the

questionnaire. The second e-mail also contained a detailed explanation of the reasons for requesting the full course syllabi (evaluation of course aims, learning outcomes, course content, methods for student evaluation and mandatory literature).

All representatives participated in the research voluntarily. Considering that this research is a retrospective study, no approval of ethical committees was necessary.

Questionnaire

A short questionnaire was designed with the aim of collecting the relevant data about basic and clinical compulsory courses in genetics for medical students at medical faculties in Balkan countries in a concise and uniform manner. The questionnaire consisted of two sets of questions.

The first set of questions was factual and contained specific questions about mandatory education, including the names of the medical faculties in their respective countries and titles of basic and clinical compulsory courses in genetics offered at each medical faculty for medical students. In addition, for each course, the representatives were asked to grade the appropriateness of the study years on which the courses are offered at each faculty (level too low/appropriate/too high), number of contact hours (insufficient/appropriate/too high), and ECTS (underestimated/appropriate/overestimated).

The second set of questions was more subjective and inquired the opinion of the representatives about mandatory education in clinical medical genetics in their countries and internationally. The questions were: "Do you think that there should be a single, uniform curriculum for all compulsory courses in medical genetics in your country?", "Do you think that there should be a single, uniform curriculum for all compulsory courses in medical genetics internationally?", "Is medical genetics recognized as a medical specialty in your country? If yes, from which year", "Is laboratory genetics recognized as a medical specialty in your country? If yes, from which year", and "What are the main obstacles for optimization of the courses in your country?".

Full Course Syllabi

Data extracted, analysed and compared from full course syllabi were course aims, learning outcomes, course content, methods for student evaluation and literature.

RESULTS

Representatives of six Balkan countries with Slavic languages (Bosnia and Herzegovina, Croatia, Montenegro, North Macedonia, Serbia, and Slovenia) participated in the research. Detailed analysis was performed for the total number of medical faculties in these countries, which is 22 (Bosnia and Herzegovina 6, Croatia 4, Montenegro 1, North Macedonia 3, Serbia 6, Slovenia 2). All but two medical faculties (Faculty of medicine, Universities of Ljubljana and Maribor in Slovenia) offer either compulsory courses in basic education in human genetics (16 faculties/courses) or clinical education in medical genetics (3 faculties/courses). On the other hand, only one medical faculty offers both types of education, including one course in basic education in human genetics and one in clinical education in medical genetics (Faculty of Medicine, University of Montenegro, Podgorica). Data on the 20 medical faculties that offer compulsory courses in genetics for medical students is shown in **Tables 1**, **2**.

Basic Courses in Human Genetics

General Features

A total of 17 compulsory basic courses in human genetics are offered at 17 medical faculties in five countries (Bosnia and Herzegovina 6, Croatia 1, Montenegro 1, North Macedonia 3, Serbia 6) (**Table 1**). While most courses are similar according to their position in the study years (15 in the first year, two in the second year), the courses vary considerably regarding the number of contact hours (45–135) and ECTS (4–9). Furthermore, the representative of Croatia stated that a small percentage of the compulsory course "Medical biology", which is offered on all four medical faculties in the country, is dedicated to the basics of human genetics but this is not reflected in the title of the courses and is therefore not presented in **Table 1**.

Representatives of Bosnia and Herzegovina, Montenegro and North Macedonia agree that the position of the courses regarding the study years is too low. On the contrary, the representative of Serbia considers that the positions for the basic courses are appropriate in their country but emphasises the importance of introducing additional mandatory education in clinical genetics in the later study years. A special emphasis should be placed on the Faculty of Medicine, University of Split (Croatia), where the title of the basic course "Immunology and Medical Genetics" does not reflect its content, which is a mixture of both basic and clinical topics.

In addition, representatives of Bosnia and Herzegovina (regarding medical faculties in Banja Luka, East Sarajevo and Mostar), Croatia, Montenegro and Serbia agree that the number of contact hours and ECTS is appropriate for the respective courses. On the other hand, the representatives of Bosnia and Herzegovina (regarding medical faculties in Sarajevo, Tuzla, Zenica and Mostar) and North Macedonia state that the number of contact hours and ECTS in insufficient.

Analysis of Full Course Syllabi

The analysis of full course syllabi across different Balkan countries (indicated in **Table 1**) revealed many similarities with only a few differences, which can be attributed to the freedom of each course coordinator, as well as specificities of the faculties' full curricula. The only exception is the course "Immunology and Medical Genetics" at the Faculty of Medicine, University of Split (Croatia), which contains mostly basic topics with a hint of practical topics, and a consequently unclear aim and learning outcomes of the course and was therefore excluded from further comparison. Also, the title of the course "Medical genetics" at the Faculty of Medicine, University of Mostar (Bosnia and Herzegovina) would correspond more to a "Human Genetics" type of course according to their aims, learning outcomes and contents. The mandatory literature is similar for all courses (Cooper, 2000), and, additionally, at certain medical faculties, the course coordinators have their own accredited handbooks.

TABLE 1 | Basic courses in genetics offered at medical faculties for medical students in Balkan countries with Slavic languages.

Country	Names of medical faculties in country	Titles of the compulsory courses offered at each medical faculty	Number of contact hours in course	Number of ECTS for the course	Study year at which the course is offered
Bosnia and Herzegovina	Faculty of Medicine, University of Banja Luka	Human Genetics	75	6	1st
	Faculty of Medicine Foca, University of East Sarajevo	Cell Biology and Human Genetics	135	9	1st
	Faculty of Medicine, University of Sarajevo	Cell Biology and Human Genetics	75	6	1st
	Faculty of Medicine, University of Tuzla	Biology with Human Genetics	75	7	1st
	Faculty of Medicine, University of Zenica	Medical Biology with Human Genetics	50	5	1st
	School of Medicine, University of Mostar	Medical Genetics	45	4	2nd
Croatia	Faculty of Medicine, University of Split	Immunology and Medical Genetics	95	6	2nd
Montenegro	Faculty of Medicine, University of Montenegro, Podgorica	Human genetics	90	6	1st
North Macedonia	Faculty of Medicine, SS. Cyril and Methodius University, Skopje	Human genetics	60	5	1st
	Faculty of Medical Sciences, Goce Delcev University, Stip	Human genetics	45	4	1st
	Faculty of Medical Sciences, State University, Tetovo[a]	Human genetics	45	4	1st
Serbia	Faculty of Medicine, University of Belgrade	Human Genetics	75	6	1st
	Faculty of Medical Sciences, University of Kragujevac	Human Genetics	60	6	1st
	Faculty of Medicine, University of Novi Sad	Biology with Human Genetics	75	8	1st
	Faculty of Medicine, University of Niš	Molecular and Human Genetics	75	7	1st
	Faculty of Medical Sciences, University of Prishtina[b]	Human Genetics	75	7	1st
	Medical Faculty of the Military Medical Academy, University of Defence in Belgrade	Human Genetics	75	7	1st

[a]*Teaching in performed in Albanian language.*
[b]*Temporary headquarers in Kosovska Mitrovica.*

TABLE 2 | Clinical courses in medical genetics offered at medical faculties for medical students in Balkan countries with Slavic languages.

Country	Names of medical faculties in country	Titles of the compulsory courses offered at each medical faculty	Number of contact hours in course	Number of ECTS for the course	Study year at which the course is offered
Croatia	Faculty of Medicine, University of Zagreb	Medical Genetics	45	4	6
	Faculty of Medicine, University of Rijeka	Medical Genetics	45	3	5
	Faculty of Medicine, University of Osijek	Medical Genetics	45	4	6
Montenegro	Faculty of Medicine, University of Montenegro, Podgorica	Clinical genetics	60	4	5

The aims were highly similar between courses, and mostly referred to the basic principles of modern biology and genetics (e.g. cell, biology, molecular biology, developmental biology and human genetics), focusing on the important molecular mechanisms that are important to human health, as well as the diagnosis and therapy of human diseases. Furthermore, learning outcomes were also comparable regarding knowledge, skills, and attitudes, although the biggest differences can be attributed to the level of performance required from the student. Moreover, the course content is again similar with certain specificities; however, the topics are relevant for medical students and up to date for the field of modern human genetics. The topics cover a wide array of content, from the structure of nucleic acids and chromosomes to the basics of genetic disorders aetiology (e.g. gene mutations, chromosome aberrations, epigenetic modifications) and modern methods for detection of genetic disorders. Finally, the biggest differences are present in the methods for student evaluation, especially in terms of grading and number of tests used. Although student evaluation is based mostly on the assessment of knowledge, some courses use only written exams, whereas others use both written and oral exams. With a few exceptions, the acquisition of skills is not assessed in most courses, i.e., assessment does not reflect the expected learning outcomes regarding skills.

Clinical Courses in Medical Genetics

General Features

A total of four compulsory basic courses in medical genetics are offered in two countries (Croatia—Faculties of Medicine,

University in Rijeka, Osijek and Zagreb, and Montenegro—Faculty of Medicine, University in Podgorica) (**Table 2**). Two of the courses are offered at the fifth year and two at the sixth year of study. All four studies are similar according to the number of contact hours (45–60) and ECTS (3–4).

All representatives agree that the position of the respective courses in the study year is appropriate. On the other hand, the representative of Montenegro stated that the number of contact hours and ECTS in insufficient for their course, whereas the representative of Croatia agrees that it is appropriate.

Finally, an additional course offering mandatory education in clinical genetics is integrated with pediatrics at the Faculty of medicine, University in Maribor (Slovenia). However, the program is focused only on genetics in the paediatric period and was therefore excluded from further analysis. In addition, in Slovenia at the Faculty of Medicine, University of Ljubljana some of the medical genetic topics are included in other basic or clinical courses.

Analysis of Full Course Syllabi

Unlike the basic courses in human genetics, the four clinical mandatory courses in medical genetics (**Table 2**) are similar only regarding the course contents, whereas they vary considerably with respect to the aims, learning outcomes, types of classes, ratio of types of classes, teaching methods and methods for student evaluation. The mandatory literature for the courses offered at the medical faculties of Zagreb, Osijek and Podgorica is the same (Turnpenny and Ellard, 2012), whereas the course "Medical Genetics" offered at the Faculty of Medicine, University of Rijeka has its own accredited mandatory literature.

The course "Medical Genetics" offered at the Faculty of Medicine, University of Rijeka (Croatia) consists of 17 h of lectures, 15 h of seminars and 13 h of practicals. The entire course is conducted exclusively through active learning methods and is designed and performed through case-based reasoning, thus achieving both clinical reasoning and a simulation of the actual physician-patient relationship in practice. The learning outcomes were determined and derived in accordance with key competencies according to Core Competences in Genetics for Health Professionals in Europe published by the European Society of Human Genetics specifically for physicians who are not specialists in medical genetics (ESHG European Society of Human Genetics, 2008; Čargonja et al., 2021). The final exam is delivered in the form of patient management problems, evaluating knowledge, skills, and attitudes at the same time.

The course "Medical Genetics" offered at the Faculty of Medicine, University of Zagreb (Croatia) consists of 20 h of lectures, 5 h of seminars and 20 h of practicals. Practicals are conducted at the clinics for pediatrics and the final exam is a written test. On the other hand, the third course delivered in Croatia, "Medical Genetics" at the Faculty of Medicine, University of Osijek (Croatia) consists of 27 h of lectures and 18 h of seminars.

Finally, the course "Clinical Genetics", which is delivered at the Faculty of Medicine, University of Podgorica (Montenegro) resembles the course "Medical Genetics" at the Faculty of Medicine, University of Rijeka regarding the aim and learning outcomes, although it has more contact hours, thus enabling a wider approach in topics. The final exam consists of the practical and oral part.

Reflections on Uniform Curricula Locally and Internationally

The representatives of all six countries agree that there should be a single, uniform curriculum for all compulsory courses in medical genetics in their respective countries. The representative of Bosnia and Herzegovina believes that it would allow easier cooperation and coordination of program. However, the representatives of Croatia and Slovenia believe that although a common framework would be helpful, some variations and freedom should be allowed between faculties due to specificities in medical genetics practice in each country and curricula of other subjects. The representative of Croatia emphasises that this curriculum should not be provisory but should also be aligned with the already existing document Core Competences in Genetics for Health Professionals in Europe published by the European Society of Human Genetics specifically for physicians who are not specialists in medical genetics (ESHG).

The representatives demonstrated more variation in their answers to the question on whether a there should be a single, uniform curriculum for all compulsory courses in medical genetics internationally. For example, the representatives of Croatia, Montenegro and Serbia think that a common framework for the Balkan area would be more appropriate due to the local specificities and different level of genetic services. On the contrary, the representatives of North Macedonia and Slovenia believe that there should be a common framework, although adapted to national health systems, which would enable common standards of knowledge for the European Union health systems, whereas the representative of Bosnia and Herzegovina thinks that a single uniform curriculum for all compulsory courses internationally would lead to better optimization of the scientific plan. Finally, all representatives agree that variations and freedom should be allowed to each course coordinator.

Opportunities for Training in Medical and Laboratory Genetics in Balkan Countries

Medical Genetics as a Medical Specialty

Of the six included countries, medical genetics is offered as a medical specialty only in North Macedonia (from 2015) and Slovenia (from 2002). In Montenegro and Serbia, clinical genetics is offered as a sub-specialist education after a previously completed specialty (e.g. in pediatrics, internal medicine, gynaecology, etc.). Neither of the previously mentioned opportunities are offered in Bosnia and Herzegovina and Croatia.

Laboratory Genetics as a Medical Specialty

Similar to the opportunities for medical genetics training, laboratory genetics is available as a medical specialty in North Macedonia and Slovenia. In the case of North Macedonia,

training in medical genetics was previously available only for biologists at the Medical faculty, University in Skopje; however, a new specialty—Clinical laboratory genetics, was introduced in 2012, which is open to health professionals, including medical doctors. In Montenegro, training in laboratory genetics is recognized in terms of the necessary conditions for work in genetic laboratories but residents need to perform their training in other countries considering that it is not available in their country. Neither of the previously mentioned opportunities are offered in Bosnia and Herzegovina and Croatia.

Obstacles for Optimization of Clinical Courses in Medical Genetics in Balkan Countries

In the final question, the representatives were asked to share their opinion on the main obstacles for optimization of the courses in their respective countries.

The representative of Bosnia and Herzegovina shared a detailed evaluation on the current situation in their country, including that knowledge of medical genetics among teaching staff is very limited considering that there are no specialists in medical and laboratory genetics. In addition, financial challenges are obvious, especially in organizing laboratory work, such as demonstrations. Finally, the representative emphasises the inconsistencies of the entire education system as a separate issue.

The representative of Croatia believes that the fact that mandatory clinical courses in medical genetics are even offered in Croatia is a success of its own considering there is no training in medical or laboratory genetics. The biggest issue for their optimization is the lack of sufficient awareness of clinical decision makers about the importance of medical genetics and its place in modern medicine, which contrasts with great agility among medical faculty teachers towards the introduction of medical genetics in clinical practice, especially at the Faculty of Medicine, University of Rijeka. The fact that clinicians underestimate the necessity that medical students learn about medical genetics and do not integrate genetic contents or discuss patients with genetic disorders with their students represents the greatest obstacle for proper implementation of medical genetics in clinical practice in Croatia. One of the possible reasons for this is the low level of genetic literacy among different specialists. The representative of Montenegro, who believes that the small population of the country does not enable the sustainability of all types of education and that there is insufficient awareness of decision makers about the importance of medical genetics and its place in modern medicine, shared a similar opinion. In addition, the representative of Serbia thinks that better synchronization is needed between basic, laboratory and clinical aspects of medical genetics, both in education and in practice in their country. Finally, the Slovenian representative believes that there is a disconnection between medical faculties, which are dominated by non-medical scientists involved in teaching and decision making, and clinical centres, which are the seats of actual genetic medical practice.

DISCUSSION

In the present study, we evaluated the current state of compulsory basic and clinical courses in genetics for medical students offered at medical faculties in six countries associated by Slavic languages, including Bosnia and Herzegovina, Croatia, Montenegro, North Macedonia, Serbia, and Slovenia. With the help of representative authorities in both human and medical genetics from each country, we performed the first such study in the Balkan peninsula, which was of the utmost importance for gaining insight into the present situation, as well as planning for future directions in mandatory genetics education at medical faculties for medical students in this area. A detailed analysis of each country revealed that Bosnia and Herzegovina and Serbia precede in the number of medical faculties (six in each country), and are followed by Croatia (4), North Macedonia (3), Slovenia (2), and Montenegro (1). Except for Slovenia, all other countries offer some sort of compulsory courses in genetics for medical students: either courses in basic education in human genetics (Bosnia and Herzegovina, North Macedonia, Serbia) or both basic education in human genetics and clinical education in medical genetics (Croatia and Montenegro). However, in the case of Croatia, basic education in human genetics is offered at just one medical faculty, whereas clinical education in medical genetics is offered at three different medical faculties. Therefore, currently the best example for an integrative approach to medical students' comprehensive education in genetics is represented by the Faculty of Medicine, University of Podgorica in Montenegro, which offers basic education in human genetics in the first year of study and clinical education in medical genetics at the fifth year of study.

Basic Courses in Human Genetics

Compulsory basic courses in human genetics are offered at 17 medical faculties in five countries (Bosnia and Herzegovina 6, Croatia 1, Montenegro 1, North Macedonia 3, Serbia 6). Interestingly, except for Croatia, which represents a special case, and Slovenia, which does not offer any type of basic education in human genetics, this result indicates that mandatory education in human genetics is offered at every medical faculty in Bosnia and Herzegovina, Montenegro, North Macedonia, and Serbia. Most of the courses (15) are offered in the first year of study, with highly similar aims, learning outcomes and course content. Although the mandatory literature is also similar, commendably, certain course coordinators also have their own accredited handbooks, emphasising and encouraging the importance of allowing freedom to each course coordinator. All these results indicate high awareness of the importance of basic sciences in modern medicine in these countries and represents an excellent basis for the introduction of clinical courses in medical genetics in the later years of study, like in Montenegro.

As indicated, Croatia represents a special case because although a compulsory course "Medical biology" is offered at all four medical faculties in the country, covering certain topics of the basics of human genetics, this is not reflected in the title of the course and was therefore excluded from further analysis.

However, an initiative might be launched at the national level to rename the courses to reflect their contents in a more accurate manner (e.g. Medical biology with human genetics). We also encountered certain illogicality at the Faculty of Medicine, University of Split, where the title of the basic course "Immunology and Medical Genetics" does not reflect the content and should therefore be renamed and separated from immunology. In addition, after the modification of the course aims, learning outcomes and contents, the course should be moved to a higher year of study, as is the case with the remainder of medical faculties in the country. It is unclear how this artificial merging of two highly diverse courses occurred considering that this not in line with the Croatian national curriculum.

Although the representatives of Bosnia and Herzegovina, Montenegro and North Macedonia believe that the position of the courses are too low in the study year, the representative of Serbia considers that the position is appropriate and that an additional clinical course should be introduced at the higher years of study.

Clinical Courses in Medical Genetics

The current situation regarding compulsory clinical courses in medical genetics is completely different than for basic courses in human genetics. Generally, clinical courses in medical genetics are highly underrepresented in Balkan countries. Specifically, only four compulsory clinical courses are offered in just two countries—at three medical faculties in Croatia and one in Montenegro. Interestingly, neither country offers medical or laboratory genetics as a medical specialty. In addition, although these courses are similar with regards to study year position (fifth or sixth year), number of contact hours (45–60), ECTS (3–4) and contents, they vary considerably with respect to the aims, learning outcomes, types of classes, ratio of types of classes, teaching methods and methods for student evaluation. Not only do the courses vary between Croatia and Montenegro, but they also vary substantially between the medical faculties in Croatia. For example, students attend practicals only at the pediatrics departments at the Faculty of medicine, University in Zagreb, whereas at the Faculty of medicine, University of Osijek, students do not have practicals at all. On the other hand, at the Faculty of medicine, University of Rijeka, the course is based on clinical reasoning and is aligned with key competencies according to Core Competences in Genetics for Health Professionals in Europe published by the European Society of Human Genetics specifically for physicians who are not specialists in medical genetics (ESHG European Society of Human Genetics, 2008; Robinson and Fong, 2008). The course content, teaching methods (primarily case-based reasoning) and methods of evaluation were analysed in detail on two generations of medical students and the results, which were previously published (Čargonja et al., 2021), confirmed that needs-based education not only increases the knowledge of medical students, but also helps develop positive attitudes and self-confidence, which is crucial for proper patient care. It is noteworthy to emphasise that the same course at the same medical faculty was among the most problematic in the entire medical study several years ago and received constant negative feedback from students. The main reason for this criticism from students was highly justified since the course contained mostly basic topics in human and laboratory genetics, such as detailed descriptions of methodology and even performance of molecular-genetic methods of genetic testing, which is not relevant for future physicians. All of this is in line with the adult learning theory, in which motivation and purposefulness of content is crucial (Thammasitboon and Brand, 2021). However, the course was completely altered with the new course coordinator and is now in tune with the actual requirements of medical professionals at the end of their integrated undergraduate and graduate education.

Obstacles for Optimization of Clinical Courses in Medical Genetics in Balkan Countries

The reasons for such low integration of compulsory clinical courses in medical genetics at the medical faculties for medical students in Balkan countries are numerous. The Balkan area is a highly specific geographic area in Southeast Europe and is sometimes associated with different cultural and historical explanations. First, this is an area which is synonymous with conflict and violent confrontation, which undoubtedly slowed down the progress and development of certain Balkan countries. The best evidence for this is Slovenia, which is the only country that did not suffer substantial war consequences and was the first of the Balkan countries included in this study to introduce both medical and laboratory genetics specialties and experience profound progress in the application of the most modern technologies in genetic testing to everyday clinical practice. In fact, specialists in medical and laboratory geneticists from Slovenia are the ones who are nowadays helping professionals in other Balkan countries develop medical and laboratory genetics with their knowledge, experience, and clinical services. Second, a direct consequence of the afore-mentioned concerns are economic issues of the Balkan countries, which are still evident in the present time (emphasised by the representatives of Bosnia and Herzegovina and Croatia) and does not allow for the same opportunities for the procurement of expensive modern genomic technologies as in the Central and West European countries. Third and final, considering the substantial delay in medical genetics in comparison with West European countries, most diagnostic genetic laboratories were led by non-medical professionals, especially biologists and molecular biologists, who were consequently also the first course coordinators of clinical courses in medical genetics (especially in Croatia). Considering that non-medical professionals did not associate the contents in their courses with clinical practice, future physicians did not see the benefits of medical genetics in clinical practice. When these students became physicians, they could not integrate medical genetics into their clinical teachings, leading to a consequently huge gap and a vicious circle between basic scientists and clinicians, which is still ongoing.

In this study, the representative of each country shared their opinion on this topic for their country and these are in line with the afore-mentioned issues. With certain specificities in their

answers, all representatives agree that the biggest issue in each country is insufficient awareness of decision makers (be they clinical or basic professionals) about the importance of medical genetics and its place in modern medicine.

Directions for the Future

In terms of the basic courses in human genetics, although they are highly similar on paper (with respect to biggest differences in the methods for student evaluation, as expected), further research would require the analysis of course performance. Therefore, future research would require peer-review and attendance of all courses to evaluate the transfer of content to students, especially in the context of analysing the achievement of course aims and learning outcomes, as well as applied teaching and learning methods (e.g. the application of active learning methods and better horizontal integration with clinical courses). Future studies should also analyse vertical integration with clinical courses to allow for updates in the curricula. Also, feedback from student evaluation of the courses must be considered because student opinion is crucial for advancing any curriculum or syllabus.

For the clinical courses, Balkan countries are in desperate need of introducing these to higher years of study consequent to the rapid development of medical genetics and its integration into all fields of modern medicine. However, course coordinators should bear in mind that it is crucial that their courses are aligned with the minimum core competencies for future physicians and that the education is needs-based. Otherwise, if medical students do not see usefulness, purposefulness, and application of the course contents in their future clinical practice, opposite, unwanted effects might be achieved. Therefore, it would be important to follow the rules of adult-learning theory and apply active learning methods (e.g. clinical reasoning) and critical thinking to the maximum extent (Wolyniak et al., 2015; Čargonja et al., 2021). Although representatives of all six countries agree that a consensus in the form of a national and/or regional Balkan curriculum might benefit medical faculties, it is important to allow freedom to each course coordinator to align the course with national and local specificities.

Additionally, vertical and horizontal integration of medical genetics with other clinical courses would be of the utmost importance and continuous emphasis on the importance of genetics through other medical specialties to medical students is indispensable for their understanding of the importance genetics has in modern medicine. Thus, genetic education of clinicians of other specialties might help prevail this obstacle.

Finally, only two countries offer medical and laboratory genetics as a medical specialty (North Macedonia and Slovenia), and in addition to introducing mandatory genetic education for medical students and clinicians, the remaining countries should focus on the introduction of both specialties for postgraduate students.

CONCLUSION

In the present study, we performed the first research on the current state of basic and clinical courses in genetics for medical students offered at medical faculties in six Balkan countries with Slavic languages (Bosnia and Herzegovina, Croatia, Montenegro, North Macedonia, Serbia, and Slovenia). Except for Slovenia, all other countries offer some sort of compulsory courses in genetics for medical students at a total of 20 medical faculties: either courses in basic education in human genetics (Bosnia and Herzegovina, North Macedonia, Serbia) or both basic education in human genetics and clinical education in medical genetics (Croatia and Montenegro). Most of the basic courses in human genetics are similar concerning their aims, learning outcomes and course content. On the other hand, clinical courses in medical genetics are offered only at three medical faculties in Croatia and one in Montenegro. In addition, although these courses are similar with regards to study year position, number of contact hours, ECTS and contents, they vary considerably with respect to the aims, learning outcomes, ratio of types of classes, teaching methods and student evaluation. Further research warrants the analysis of performance of basic courses, as well as introducing clinical courses in medical genetics to higher years of study across Balkan countries. Increasing genetic literacy in medical genetics in clinicians of other medical specialties is also crucial. Finally, this study emphasises the need for collaboration and is the first step towards breaking the years-long barriers that have prevented the consensus on medical genetics education in Balkan countries with Slavic languages, all for the benefit of future physicians and their patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NP—study design, questionnaire development, data collection and analysis, manuscript writing and revision. RT—data collection and analysis, manuscript revision. DP-K data collection and analysis, manuscript revision. OM—data collection and analysis, manuscript revision. IN—data collection and analysis, manuscript revision. ŽP—data analysis, manuscript revision. SO—study design, questionnaire development, data collection and analysis, manuscript revision. BP—study design, questionnaire development, data collection and analysis, manuscript revision.

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Association of *ATG16L1* rs2241880 and *TP53* rs1042522 with characteristics and course of diffuse large B-cell lymphoma

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ABSTRACT

Background: Diffuse large B-cell lymphoma (DLBCL) represents the most frequent lymphoma in adults. Prognosis for DLBCL patients may be evaluated through the most prominent clinical/laboratory parameters or pattern of gene expression. In order to improve prognostic/prediction scores or provide new therapeutic targets, novel genetic markers are needed. This study evaluates the association of *ATG16L1* rs2241880 and *TP53* rs1042522 with clinical characteristics and course of DLBCL.

Methods: The study included 108 DLCBL patients treated with R-CHOP. Of these, 44 patients were subjected to stem cell transplantation and 55 to radiotherapy. Genotyping was performed by TaqMan genotyping assays.

Results: Amongst analyzed characteristics and prognostic scores, genotypes were associated with clinical stage (*TP53* CG+CC vs GG $p = 0.06$), extranodal disease (*ATG16L1* AG vs AA $p = 0.07$; AG vs GG $p = 0.04$), lymphocyte-to-monocyte ratio (LMR) (*ATG16L1* AA vs AG+GG, $p = 0.052$; AA vs GG, $p = 0.054$) and neutrophils-to-lymphocytes ratio (NLR) (*ATG16L1* AA vs AG+GG, $p = 0.033$; AA vs GG, $p = 0.003$). Analyzed genotypes didn't impact response to therapy, relapse and therapy-related complications. Considering outcome, patients with *ATG16L1* AA had higher survival rate than GG carriers ($p = 0.04$). In all patients, duration of overall survival (OS) and relapse free survival (RFS) was not affected by analyzed genotypes. When subjected to radiotherapy, patients with *ATG16L1* A allele ($p = 0.05$) or AA genotype ($p = 0.03$) had superior OS.

Conclusion: Our results demonstrated the association of *TP53* rs1042522 with clinical stage and *ATG16L1* rs2241880 with extranodal disease, LMR and NLR. The impact of *ATG16L1* genotypes on OS in patients subjected to radiotherapy, indicates significance of individual single nucleotide polymorphisms (SNPs) in particular subgroups of DLBCL.

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent lymphoma in adults, accounting for approximately up to 50 % of all non-Hodgkin lymphomas, and characterized by biological and clinical heterogeneity [1,2]. About 60 % of patients with DLBCL can be cured with the standard R-CHOP regimen (rituximab, cyclophosphamide, doxorubicin, vinkristin and prednisone), while 30–40 % of patients failed R-CHOP due to refractoriness or relapse [3]. The most prominent clinical and laboratory parameters that predict progression-free survival (PFS) and overall survival (OS) are included in the International Prognostic Index (IPI) score; for better risk stratification in the rituximab era,

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Revised IPI (R-IPI), National Comprehensive Cancer Network-IPI (NCCN-IPI) and age-adjusted IPI (aaIPI) were developed [4,5]. The pattern of gene expression classifies DLBCL into two major subtypes with prognostic implications; patients presenting germinal center B-cell (GCB) subtype can be cured by R-CHOP, whereas the majority of patients with activated B-cell (ABC) subtype relapse and die from DLBCL [6]. Furthermore, the presence of MYC rearrangements, alone or in the co-occurrence with BCL2 and/or BCL6 translocations, is associated with an aggressive clinical phenotype and poor response to both up-front and salvage therapy [7]. However, there is still a need for novel genetic markers that will expand our understanding of DLBCL, improve prognostic/prediction scores or hopefully, provide new therapeutic targets.

Previous reports highlighted an important role of autophagy in cancer, and therefore implied the possible benefits of autophagy-based treatment strategies, in particular for relapsed or refractory disease [8,9]. Autophagy is one of the most studied cellular processes that enables the degradation and elimination of unwanted or dysfunctional intracellular components. In such a way, autophagy counteracts different types of cellular stress, e.g. induced by cancer treatment. However, recycling of some receptors by autophagy may reduce the efficacy of targeted therapies [10]. The process of autophagy is driven by a number of autophagy-related genes (*ATG*s), among which the autophagy-related 16 like 1 (*ATG16L1*) plays one of the key roles [11]. *ATG16L1* gene is located on the long arm of chromosome 2 (2q37.1). The single nucleotide polymorphism (SNP) at position 898 [c.898 A>G(p.Thr300Ala); rs2241880] is associated with defective autophagy, increased secretion of TNF-α and IL1-β, and thus enhanced inflammatory response [12].

Autophagy can be modulated by tumor suppressor protein TP53 which plays a dual role; nuclear TP53 induces autophagy, while cytoplasmic TP53 acts as its repressor [13]. However, the exact mechanism of TP53-mediated autophagy is not completely clear. The „gatekeeper" *TP53* is one of the most frequently studied genes in human malignancies. A number of studies have reported that 20–25 % of DLBCL cases harbour *TP53* mutations, whereby the incidence of mutations is similar between GCB and ABC subtype and usually correlates with poor prognosis [14]. In addition, the polymorphic nature of the *TP53* has been frequently studied in the context of cancer susceptibility. One of the most studied SNPs is rs1042522 [c 0.251C>G(p.Pro72Arg)], encoding two alleles with different capacities to induce transcription, target proteasome and modulate apoptosis, respectively [15].

The present study attempts to evaluate the association of *ATG16L1* rs2241880 and *TP53* rs1042522 with relevant clinical characteristics and survival of patients with DLBCL.

2. Methods

2.1. Ethics

The study design was approved by the ethics committee of Military Medical Academy (MMA) Belgrade, Serbia, in accordance with principles outlined in the Declaration of Helsinki.

2.2. Patients

The study included 108 patients (61 men and 47 women) with DLBCL, aged 17–78 years (average 47.78, median 51 years). Diagnosis was based on histopathology and immunohistochemistry according to the World Health Organisation classification [1]. Clinical examination, laboratory testing, bone marrow biopsy and standard radiological investigations were undertaken in all patients. Excluding criteria for participation in the study was the previous history of malignant disease or immunosuppression-related DLBCL. All patients received 6–8 cycles of R-CHOP; 44 patients were subjected to stem cell transplantation (SCT). Radiotherapy was applied in 55 patients with bulky disease or residual masses.

Diagnostic procedures, treatment and follow-up were conducted at the Clinic of hematology, MMA. The follow-up period ranged from one to 219 (median 92.5) months; patients who achieved complete clinical remission (CCR) were followed at least three years.

2.3. Genotyping

Blood was taken in EDTA tubes and stored at −40 ºC until DNA isolation (PureLinkTM Genomic DNA MiniKit, Invitrogen, CA, USA). *TP53* rs1042522 and *ATG16L1* rs2241880 genotyping was performed on 7500 Real-Time PCR System (Applied Biosystems™, CA, USA) using the TaqMan Genotyping assays C__2403545_10 and C__9095577_20 (Applied Biosystems, CA, USA).

2.4. Statistical analysis

The association between genotypes and clinical chatacteristics, response to therapy, incidence of relapse and presence of post-therapeutic complications was analyzed using the Pearson chi-squre test or two-tailed Fisher exact test. In addition, odds ratio (OR) and 95 % confidence interval (CI) were calculated. The information of survival outcome was used for determination of cut-off values of pretreatment lymphocyte-to-monocyte ratio (LMR), neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR). The correlation between genotypes and markers of inflammation and LMR, NLR and PLR was analyzed by two-tailed Mann Whitney test.

Progression free survival (PFS) was defined as the time from the start of treatment to relapse/progression of DLBCL. Overall survival (OS) was calculated as the time from the start of the treatment to death for any reason or to the last follow-up time-point. Survival curves were generated using the method of Kaplan and Meier and compared by the log rank test. The most prominent factors for outcome and OS were determined by logistic regression and Cox proportional hazard regression (Wald test).

The p value <0.05 was considered statistically significant. Calculations were performed by SPSS for Windows Statistics 20.0 (SPSS, Inc., Chicago, IL, USA) and EZR 1.36 (Saitama Medical Center Jichi Medical University, Saitama, Japan).

3. Results

The *ATG16L1* rs2241880 genotyping was conclusive in 108 patients and in 100 patients for *TP53* rs1042522. Obtained frequencies of genotypes and alleles are presented in Fig. 1.

3.1. Clinical characteristics

Frequencies of *ATG16L1* rs2241880 and *TP53* rs1042522 genotypes in patients with different baseline characteristics are summarized in Table 1.

The association between genotypes and clinical characteristics (gender, age, performance status, B-symptoms, clinical stage, extranodal disease, number of extranodal sites, bulky disease) or prognostic scores (IPI, NCCN IPI and aaIPI) was evaluated in codominant, dominant and recessive genetic model, respectively. Patients with *ATG16L1* AG genotype more frequently presented extranodal disease compared to patients with AA ($p = 0.07$) and GG genotype ($p = 0.04$; OR 2.88, 95 % CI 1–8.22). In addition, majority of patients with more than one extranodal site had AG genotype (63.6 %), but obtained p values were of marginal statistical significance (AA vs AG $p = 0.09$; GG vs AG $p = 0.08$). Considering *TP53*, carriers of C allele (CG/CC genotypes) more frequently presented DLBCL of unfavorable clinical stage III/IV than patients with GG genotype ($p = 0.06$).

Patients with *ATG16L1* AA genotype had lower LMR than carriers of G allele (AA vs AG+GG, $p = 0.052$;) and GG genotype (AA vs GG, $p = 0.054$). NLR values were significantly higher in carriers of *ATG16L1* A allele (AA+AG vs GG, $p = 0.004$) and AA genotype (AA vs AG+GG,

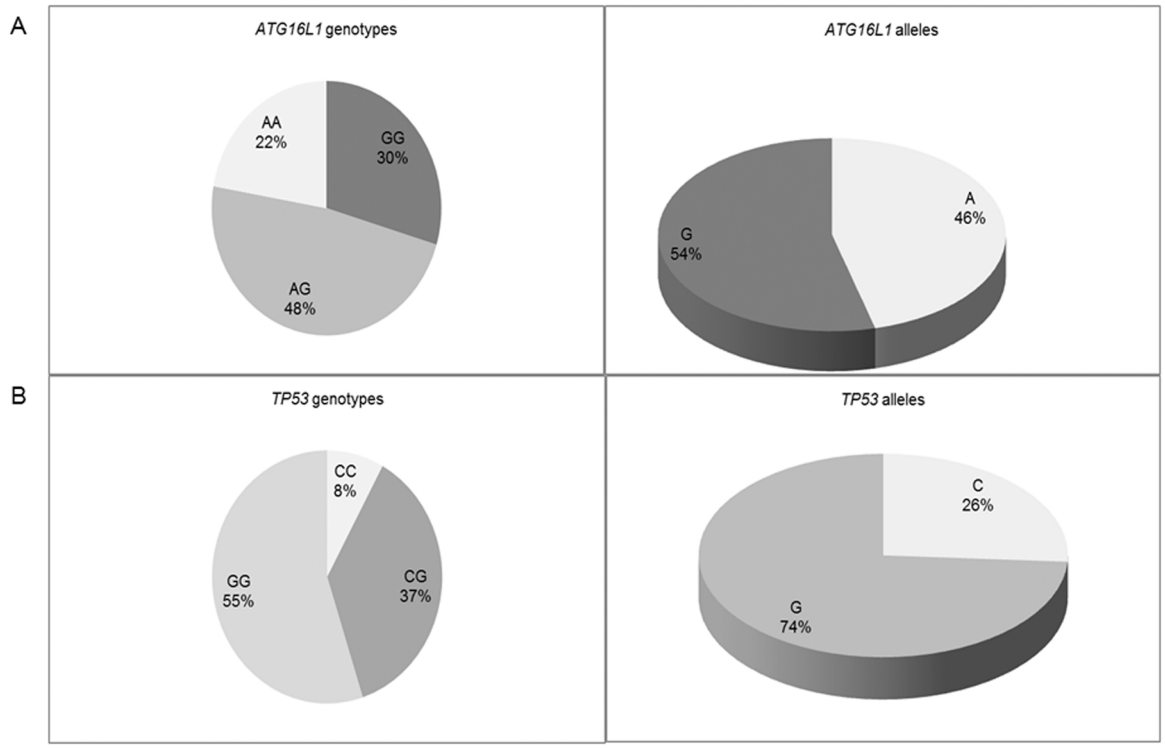

Fig. 1. Frequencies of *ATG16L1* rs2241880 and *TP53* rs1042522 genotypes and alleles in DLBCL patients**,** *ATG16L1* rs2241880 genotyping was conclusive in 108 patients. Obtained frequencies of genotypes were as follows: 30 % of GG (32/108), 48 % of GA (52/108) and 22 % of AA (24/108). Frequencies of A and G alleles were 46 % and 54 %, respectively (A).*TP53* rs1042522 genotyping was conclusive in 100 patients. GG, GC and CC genotypes were detected in 55 % (55/100), 37 % (37/100) and 8 % (8/100) of patients, respectively. Frequencies of G and C alleles were 74 % and 26 % (B).

p = 0.033; AA vs GG, p = 0.003) (Fig. 2). *ATG16L1* genotypes were not significantly associated with PLR values. *TP53* genotypes were not associated with neither LMR, NLR and PLR values. ROC curve analysis established the following cut-off values for survival: 3.67 (AUC 0.67, 95 %CI, 0.46–0.75) for LMR, 1.54 (AUC 0.57, 95 %CI, 0.44–0.7) for NLR, and 1.28 (AUC 0.56, 95 %CI, 0.42–0.71) for PLR. The number of patients with LMR value below the cut-off was significantly higher among the carriers of A allele (AA+AG vs GG, p = 0.03; OR 2.93, 95 %CI 1.09–7.87) and AA genotype (AA vs AG+GG, p = 0.005; OR 11.93, 95 % CI 1.51–94.12 and AA vs GG, p = 0.002, OR 17.77, 95 %CI 2.05–154.21). NLR values above cut-off were more frequent in patients with *ATG16L1* A allele (AA+AG vs GG, p = 0.02; OR 4.19, 95 %CI 1.24–14.12). The proportion of patients with PLR values below/above cut-off was similar in carriers of different *ATG16L1* genotypes. In addition, the proportion of patients with LMR, NLR and PLR values below/above cut-off was similar in carriers of different *TP53* genotypes.

The association between *ATG16L1/TP53* genotypes and gender, age, performance status, B-symptoms, and bulky disease was not statistically significant in any of analyzed genetic models. Also, there was no significant association between analyzed genotypes and prognostic scores (IPI, NCCN IPI, aaIPI).

3.2. Response to therapy and the course of disease

In the analyzed group of 108 patients, the overall response rate (ORR) following R-CHOP was 92.6 %. CCR was achieved in 59 patients (54.6 %), while partial response (PR) was observed in 41 patients (38 %). Initially resistant disease (IRD) was present in eight patients (7.4 %). In the group of 100 patients with conclusive *TP53* genotyping, 94 (94%) responded to R-CHOP. CCR was achieved in 52 patients (52 %), 40 patients (40 %) had PR and eight (8 %) of them presented IRD. *ATG16L1* and *TP53* genotypes were not associated with response to therapy.

Early- and late-therapy-related complications were observed in 17/108 patients (15.7 %) and 21/108 patients (19.4 %), respectively. Infections during the therapy were present in 16 patients (14.8 %), while nine patients (8.3 %) experienced therapy-related toxicity. In the group of 100 patients with conclusive *TP53* genotyping, early- and late-therapy-related complications were present in 16 % and 20 % of patients, respectively. Therapy-related toxicity appeared in nine patients (9 %), while infections were observed in 15 patients (15 %). Therapy-related complications, toxicity and infections were not associated with *ATG16L1* and *TP53* genotypes.

In the study group, 30/108 (27.8 %), that is 27/100 (27 %) patients relapsed. Considering patients who achieved CCR, relapse occurred in 19/59 (32.2 %) patients with conclusive *ATG16L1* genotyping and 16/52 (30.8 %) patients with conclusive *TP53* genotyping. *ATG16L1* and *TP53* genotypes did not impact occurrence of relapse.

3.3. Survival analyses and outcome

During the follow-up, 38/108 (35.2 %) patients died: 5/38 (13.2 %) had *ATG16L1* AA and 15/38 (39.5 %) had GG genotype (AA vs GG, p = 0.04; OR 0.3, 95 % CI 0.09–0.99). In order to identify the most prominent factors for survival, logistic regression was used. High or high/intermediate IPI, high or high/intermediate NCCN IPI, the presence of B-symptoms and bulky disease, male gender, LMR below cut-off, NLR and PLR above cut-off value and *ATG16L1* GG genotypes were entered in logistic regression as unfavorable prognostic factors. Among all, only high or high/intermediate NCCN IPI was associated with poor outcome [death caused by DLBCL (n = 32), therapy-related complications (n = 3) or secondary malignancies (n = 3)]; OR 4.34, 95 % CI 1.24–15.1; p = 0.02. *TP53* genotypes were not associated with outcome.

Log rank test demonstrated that *ATG16L1* and *TP53* genotypes didn't affect OS and RFS (recessive, dominant and co-dominant genetic models were employed). Analysis of OS and RFS in patients with different prognostic characteristics (clinical stage, IPI, NCCN IPI) and therapy

Table 1
Frequencies of *ATG16L1* (n = 108) and *TP53* (n = 100) genotypes in DLBCL patients with different clinical and prognotic characteristics.

Characteristics	*ATG16L1* n (%)			*TP53* n (%)		
	GG	GA	AA	GG	GC	CC
Gender						
Female	14	23	10	22	15	4
Male	(29.8)	(48.9)	(21.3)	(53.7)	(36.6)	(9.7)
	18	29	14	33	22	4
	(29.5)	(47.5)	(23)	(55.9)	(37.3)	(6.8)
Age						
<60 years	21	36	18	39	27	5 (7)
≥60 years	(28)	(48)	(24)	(54.9)	(38)	3
	11	16	6	16	10	(10.3)
	(33.3)	(48.5)	(18.2)	(55.2)	(34.5)	
Performance Status						
0, 1	27	42	22	45	32	8
2	(29.7)	(46.1)	(24.2)	(52.9)	(37.7)	(9.4)
	5	10	2	10	5	0 (0)
	(29.4)	(58.8)	(11.8)	(66.7)	(33.3)	
B-Symptoms						
absent	15	20	11	25	14	3
present	(32.6)	(43.5)	(23.9)	(59.5)	(33.3)	(7.1)
	17	32	13	30	23	5
	(27.4)	(51.6)	(21)	(51.7)	(39.7)	(8.6)
Clinical Stage						
I, II	17	25	10	31	14	3
Clinical stage III,	(32.7)	(48.1)	(19.2)	(64.6)	(29.2)	(6.2)
IV	15	27	14	24	23	5
	(26.8)	(48.2)	(25)	(46.2)	(44.2)	(9.6)
Extranodal Disease[a]						
absent	11	8	8	16	6	1
present	(40.7)	(29.6)	(29.6)	(69.6)	(26.1)	(4.3)
	21	44	16	39	31	7
	(25.9)	(54.3)	(19.8)	(50.6)	(40.3)	(9.1)
Number of						
Extranodal Sites	25	31	19	40	22	7
0,1	(33.3)	(41.3)	(25.3)	(58)	(31.9)	(10.1)
>1	7	21	5	15	15	1
	(21.2)	(63.6)	(15.2)	(48.4)	(48.4)	(3.2)
Bulky Disease[b]						
absent	17	31	14	34	18	5
present	(27.4)	(50)	(22.6)	(59.6)	(31.6)	(8.8)
	15	21	10	21	19	3 (7)
	(32.6)	(45.7)	(21.7)	(48.8)	(44.2)	
LDH Serum Level						
normal	17	20	8	22	15	3
elevated	(37.8)	(44.4)	(17.8)	(55)	(37.5)	(7.5)
	15	32	16	33	22	5
	(23.8)	(50.8)	(25.4)	(55)	(36.7)	(8.3)
Absolute						
lymphocytes/	11	14	1 (3.9)	17	8	1
absolute monocyte	(42.3)	(53.8)	21	(65.4)	(30.8)	(3.8)
ratio[3]	13	31	(32.3)	30	23	5
≥3.67	(20)	(47.7)		(51.7)	(39.7)	(8.6)
<3.67						
Absolute						
neutrophils/	7	4	2	9	3	1
absolute	(53.8)	(30.8)	(15.4)	(69.2)	(23.1)	(7.7)
lymphocytes ratio[4]	17	41	20	38	28	5 (7)
<1.54	(21.8)	(52.6)	(25.6)	(53.5)	(39.4)	
≥1.54						
Absolute						
thrombocytes/	5	9	3	10	6	1
absolute	(29.4)	(52.9)	(17.7)	(58.8)	(35.3)	(5.9)
lymphocytes ratio[5]	19	36	19	37	25	5
<1.28	(25.7)	(48.6)	(25.7)	(55.2)	(37.3)	(7.5)
≥1.28						
Prognostic Score						
IPI						
low/low-	23	31	18	38	23	7
intermediate	(31.9)	(43.1)	(25)	(55.9)	(33.8)	(10.3)
intermediate-	9 (25)	21	6	17	14	1
high/high		(58.3)	(16.7)	(53.1)	(43.8)	(3.1)
NCCN IPI						
low/low-	12	23	10	26	12	4
intermediate	(26.7)	(51.1)	(22.2)	(61.9)	(28.6)	(9.5)
intermediate-	20	29	14	29	25	4
high/high	(31.8)	(46)	(22.2)	(50)	(43.1)	(6.9)
AaIPI[6]						
low/low-	9	22	9	24	12	2
intermediate	(22.5)	(55)	(22.5)	(63.1)	(31.6)	(5.3)
intermediate-	12	14	9	14	15	3
high/high	(34.3)	(40)	(25.7)	(43.7)	(46.9)	(9.4)

[3,4,5]Data were available for 91 patients with conclusive *ATG16L1* genotyping and for 84 patient with conclusive *TP53* genotyping; cutt of walue were obtained by ROC analysis. [6]Analyses included 75 patients younger than 60 years.
[a] Extranodal disease was represented with at least one extranodal site.
[b] Bulky disease was represented with tumor mass ≥7 cm.

approach (stem cell transplantation or radiotherapy), showed that carriers of *ATG16L1* A allele (AA+AG vs GG) had superior OS when treated with R-CHOP and radiotherapy (p = 0.05); in addition, patients with *ATG16L1* AA had better OS than GG carriers (p = 0.03) (Fig. 3). Cox proportional hazard regression (included high or high/intermediate IPI, high or high/intermediate NCCN IPI, the presence of B-symptoms, bulky disease, male gender, LMR below cut-off, NLR and PLR above cut-off value and ATG16L1 GG genotypes as unfavorable prognostic factors) identified only high or high/intermediate NCCN IPI [hazard ratio (HR) 3.35, 95 % CI 1.24–9.06; p = 0.02] and high PLR (HR 0.29, 95 % CI 0.09–0.95; p = 0.04) as independent factors for poor OS. When Cox regression included only patients with *ATG16L1* AA and GG genotypes, high or high/intermediate NCCN IPI and high PLR remain significantly associated with OS, while impact of *ATG16L1* GG genotype was noted as a trend (p = 0.06) (Table 2). However, Cox proportional hazard regression didn't identify independent prognostic factors for OS in DLBCL patients subjected to R-CHOP and radiotherapy.

4. Discussion

The present study analyzed the impact of *ATG16L1* rs2241880 and *TP53* rs1042522 genotypes on the characteristics and course of DLBCL. Obtained frequencies of both *ATG16L1* rs2241880 and *TP53* rs1042522 genotypes/alleles in the study participants were similar as those reported for other European populations [16,17].

The rs2241880 on *ATG16L1* is perhaps the most studied SNP on autophagy-related genes. The majority of previous reports analyzed its association with Crohn's disease, but a number of studies demonstrated the link with different cancers types (colorectal, gastric and prostate cancer, melanoma, head and neck squamous cell carcinoma) [18]. In melanoma patients, rs2241880 GG genotype has been shown to influence a decrease in Breslow thickness and earlier stage at diagnosis, while AG genotype was associated with younger age at diagnosis [19]. In colorectal cancer, patients with rs2241880 GG genotype had better long-term OS and decreased metastasis [20]. In this study, DLBCL patients with AG genotype more frequently presented extranodal disease. Furthermore, the rs2241880 AA genotype/A allele was associated with lower LMR and higher NLR. A number of recent studies have demonstrated that the ratio of different cell populations in peripheral blood, such as LMR, NLR or PLR, can be used as prognostic marker in lymphoma and other malignancies [21,22]. It has been hypothesized that the pretreatment absolute number of neutrophils, lymphocytes and monocytes reflects systemic inflamatory response to malignancy, host immunity to tumor/tumor-infiltrating lymphocytes and tumor-associated macrophages, respectively [23]. As previously reported, low LMR at diagnosis correlates with poor OS and PFS, while LMR recovery is associated with improved clinical outcomes [24]. In contrast, poor survival was found in DLBCL patients with higher pretreatment NLR [25,26]. In the present study, NLR was significantly higher in the carriers of rs2241880 AA genotype/A allele than in G allele

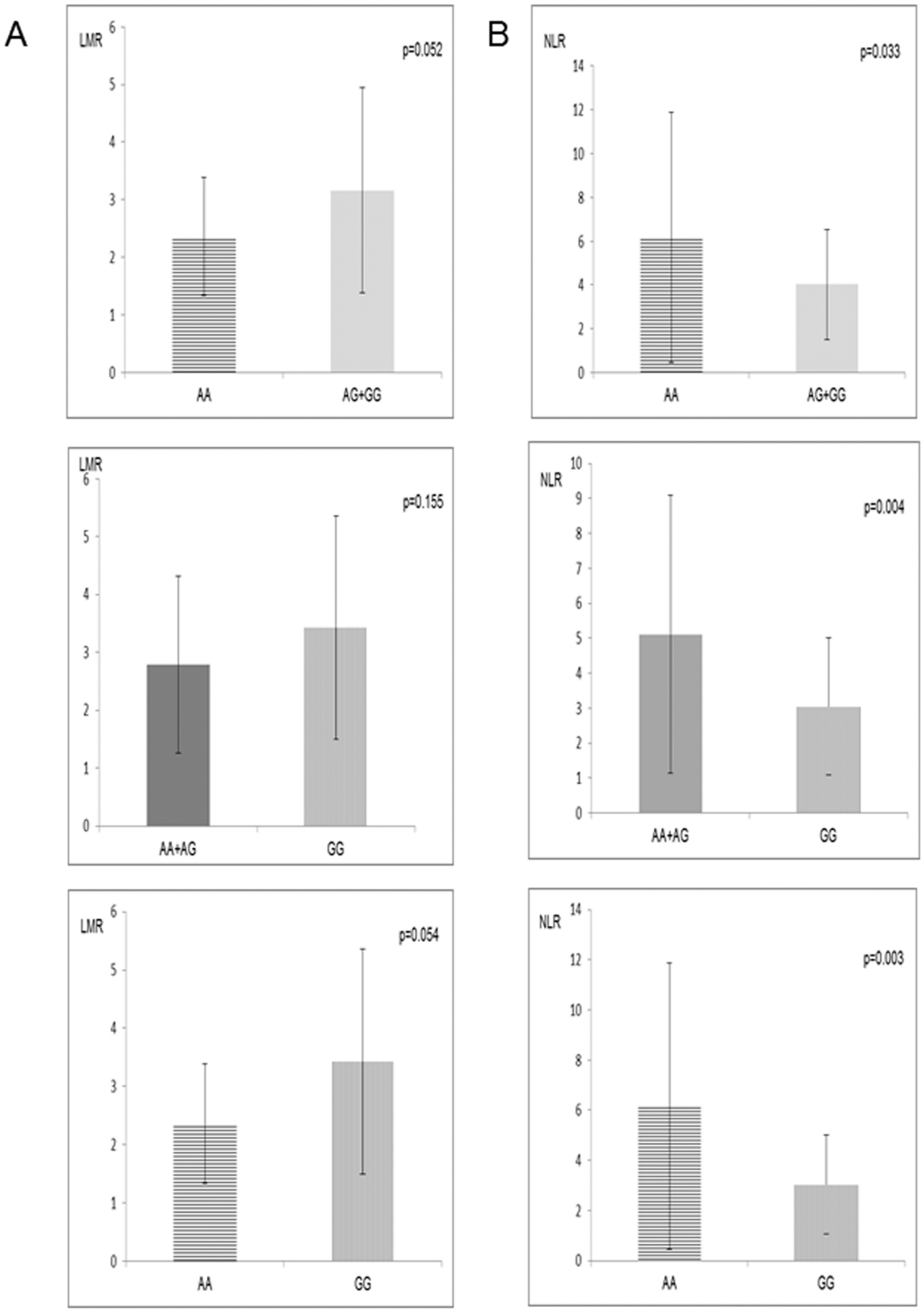

Fig. 2. Lymphocyte-to-monocyte ratio (LMR) and neutrophil-to-lymphocyte ratio (NLR) in DLBCL patients with different *ATG16L1* rs2241880 genotypes. Patients with *ATG16L1* AA genotype had lower LMR than carriers of AG/GG genotype (2.36 ± 1.03 vs 3.16 ± 1.78, p = 0.052;) and GG genotype (2.36 ± 1.03 vs 3.43 ± 1.93, p = 0.054). LMR values were not significantly different between carriers of AA/AG and GG genotype (2.79 ± 1.53 vs 3.43 ± 1.93, p = 0.155) (A).NLR values were significantly higher in patients with *ATG16L1* AA genotype than in AG/GG (6.17 ± 5.7 vs 4.02 ± 2.51, p = 0.033) and GG carriers (6.17 ± 5.7 vs 3.04 ± 1.96, p = 0.004). NLR values were also significantly different between carriers of AA/AG and GG genotype (5.11 ± 3.97 vs 3.04 ± 1.96, p = 0.003) (B).

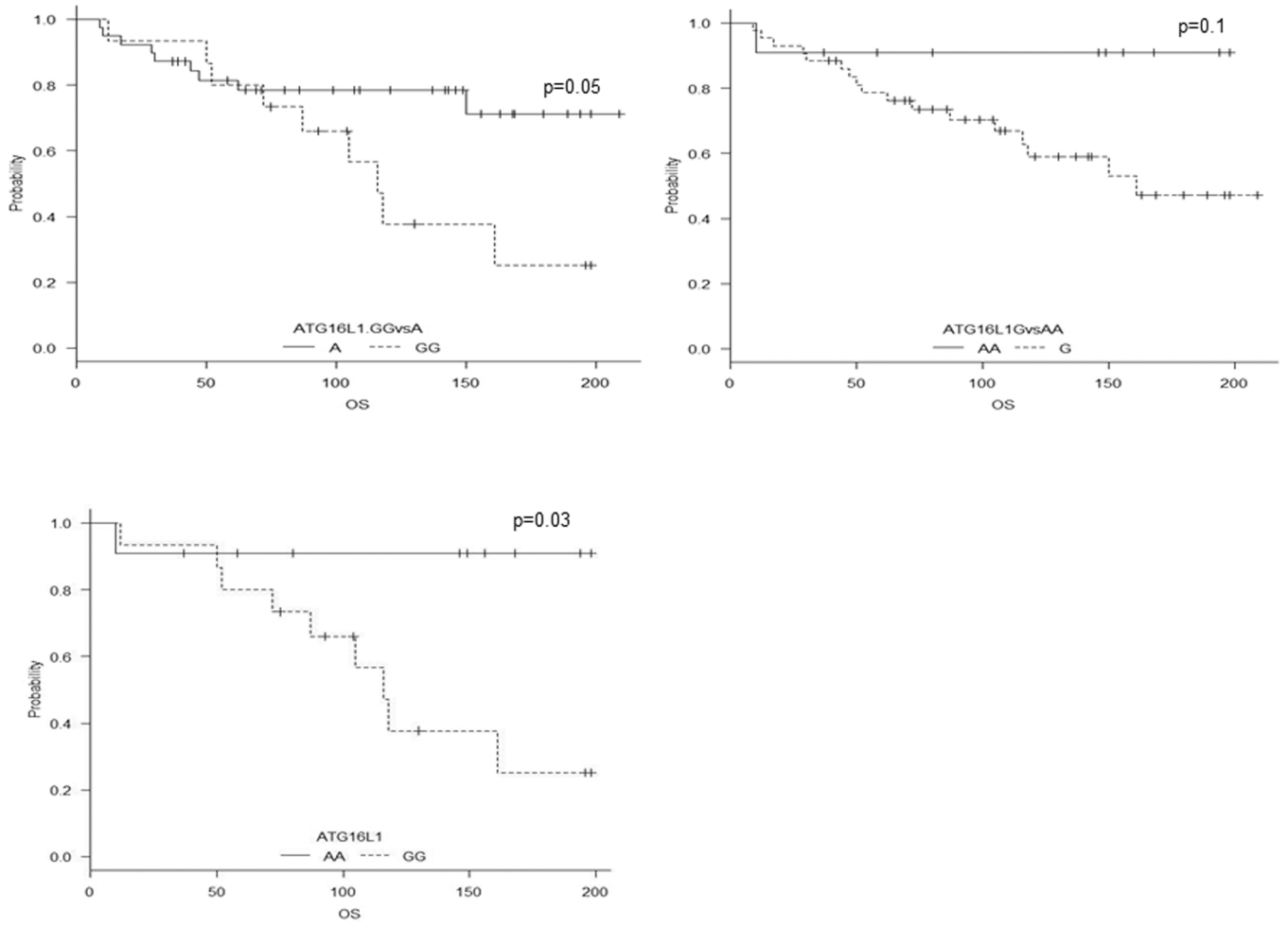

Fig. 3. Overall survival in DLBCL patients treated with R-CHOP and radiotherapy. Superior overall survival (months) was observed in carriers of *ATG16L1* A allele (AA/AG vs GG, p = 0.05; AG/GG vs AA, p = 0.102). Comparison between only AA and GG carriers demonstrated superior overall survival in DLBCL patients with *ATG16L1* AA genotype (p = 0.03). Survival curves were generated using the method of Kaplan and Meier and compared by the log rank test.

Table 2
Cox proportional hazard ratio for identification of the most prominent prognostic factors for overall survival in *ATG16L1* AA and GG carriers treated with R-CHOP and radiotherapy.

Factor	Hazard Ratio	95 % CI	p (Wald test)
ATG16L1 GG	3.75	0.93–15.06	**0.06**
B-symptoms	0.62	0.18–2.17	0.46
Bulky disease	0.68	0.17–2.68	0.58
Gender M	0.62	0.17–2.23	0.46
IPI high/intermediate-high	1.4	0.33–5.9	0.65
NCCN IPI high/intermediate-high	8	1.59–40.15	**0.01**
LMR below cut-of	0.57	0.1–3.29	0.53
NLR above cut-of	2.99	0.5–17.96	0.23
PLR above cut-of	0.06	0.01–0.68	**0.02**

carriers; LMR values were significantly lower in AA than in GG carriers. The increasing knowledge regarding (neutrophil) autophagy in recent years pointed out its important role in both the innate and adaptive immunity. Neutrophil autophagy, similar to neutrophils, displays pro-tumorigenic or anti-tumorigenic activities depending on the tumor type and tumor microenvironment. As previously reported, the upregulation of autophagy promotes progression of hepatocellular carcinoma and some other solid tumors; in hematological malignancies (particularly in acute myeloid leukemia), it may be essential for blockade of disease progression [27,28]. Considering published studies, induction/suppression of neutrophil autophagy seems attractive as therapeutic intervention. However, one should keep in mind that all mechanisms of neutrophil autophagy, including explicit function of ATG proteins, are not completely explored. In addition, in previous reports on the tumor microenvironment, autophagy has been signified as an important regulator of homeostasis, activation and biological functions of immune cells [28]. The role of autophagy in the immune surveillance of cancer is controversial. Autophagy may enhance antigen presentation, trigger CD8+ T cell cytotoxic activity and consequently reduce tumor growth; on the opposite, it may upregulate the expression of PD-1 and CTLA-4 or degrade granzyme B and thus protect malignant cells [29]. It has been underlined that obtained results differ due to experimental context/models [30]. In our study LMR values (reflecting host immunity to tumor/tumor-infiltrating lymphocytes) were lower in patients with rs2241880 AA genotype, i.e. in those with functional ATG16L1. We can only speculate and make parallel with previous findings on enhanced autophagy in patients with advanced lung cancer and low-levels of tumor-infiltrating T-cells [31], or association between rs2241880 G allele and reduced risk of brain metastasis in patients with non-small cell lung cancer [32], and prolonged OS in patients with colorectal cancer [20]. However, despite the association with extranodal disease, NLR and LMR, rs2241880 genotypes didn't impact response to therapy, the incidence of relapse, outcome, as well as RFS and OS of our patients. The only exception was found in patients subjected to radiotherapy; in this group, carriers of *ATG16L1* rs2241880 A allele/AA genotype had superior OS than GG carriers. The link between autophagy and radiotherapy has been extensively studied, but available data remain

conflicting and inconsistent. Some authors emphasized the radioprotective role of autophagy and suggested that the radiosensitivity of tumor cells may be increased upon autophagy inhibition. From a different point of view, induction of autophagy can restrict proliferation of tumor cells and contribute via different mechanisms to radiosensitization, resulting in autophagic cell death [33,34]. In addition, variants of *ATG*s (particularly *ATG10* and *ATG16L2*) have been shown to impact the efficacy and toxicity of radiotherapy [35]. Despite significantly accumulated knowledge in the field, more extensive research is needed to translate *ATG*s variations into routine pharmaco/radiogenetic testing and to enable improvement of treatment protocols by modification of autophagy.

The tp53 has been described as a tumor suppressor since it can arrest the cell cycle and induce apoptosis under the various conditions of genotoxic stress. Loss of tp53 function is common in human cancers, including DLBCL. However, the prognostic significance of mutations and common polymorphisms in *TP53* has been inconsistent across tumors. Considering previous reports, dysregulation of *TP53* contributes to chemoresistance and indicates the poor outcome of DLBCL patients treated with R-CHOP [3]. Polymorphism rs1042522 (c 0.251C>G) results in a change of Pro to Arg at codon 72 of exon 4, whereby the Arg variant is a more powerful inducer of apoptosis. In the Serbian population, the Pro variant (C allele) has been described as a risk factor for breast cancer [36] while the Arg variant (G allele) was likely protective against lung adenocarcinoma [37]. In the present study, carriers of C allele (CG/CC genotypes) more frequently presented DLBCL of unfavorable clinical stage III/IV than patients with GG genotype. We can assume that advanced DLBCL in the C allele carriers may be associated with the reduced ability of tp53 Pro variant to induce apoptosis of lymphoma cells. However, in this study *TP53* rs1042522 genotypes didn't impact the course of DLBCL.

5. Conclusion

The association of chronic inflammation with malignant transformation and onset of DLBCL has been recognized already. In the available literature, the rs2241880 polymorphism of *ATG16L1* gene is commonly discussed as a risk factor of Crohn's disease due to its role in the regulation of autophagy, intestinal epithelial homeostasis and inflammatory immune response. Our results demonstrated the association of *ATG16L1* rs2241880 with NLR and LMR that represent a systemic inflammatory response to malignancy and tumor infiltrate. In addition, the impact of analyzed polymorphisms on the presence of extranodal disease (*ATG16L1* rs2241880) and clinical stage (*TP53* rs1042522) was observed. In this study, the most prominent prognostic factor for survival was NCCN IPI, confirming the importance of this score in DLBCL patients treated with R-CHOP. However, the significance of *ATG16L1* genotypes for OS in patients subjected to radiotherapy indicates that analyses of individual SNPs may be of importance in different subgroups of DLBCL patients. Findings of the present study imply that *ATG16L1* rs2241880 and *TP53* rs1042522 should be considered as a candidates for panel of prognostic/predictive markers in DLBCL. Hopefuly, the subsequent studies will bring simmilar results and enable the usage of these analyses in routine clinical practice.

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Ethic approval

The study design was approved by the ethics committee of Military Medical Academy (MMA) Belgrade, Serbia, in accordance with principles outlined in the Declaration of Helsinki (May 23, 2013).

CRediT authorship contribution statement

Bojana Cikota-Aleksić and Anđelina Živanović designed the research; Anđelina Živanović, Dragana Stamatović, Olivera Tarabar and Miroslav Mišović performed clinical management of the patients and data collection; Bojana Cikota-Aleksić and Nataša Strelić performed DNA extraction and genotyping; Bojana Cikota-Aleksić, Zvonko Magić and Olivera Miljanović performed statistical calculations and analyzed data through different genetic models; Anđelina Živanović, Dragana Stamatović, Olivera Tarabar and Svetlana Đukić analyzed obtained results through clinical settings; Bojana Cikota-Aleksić and Anđelina Živanović wrote the paper; all authors revised and approved the final version of the paper.

Consent

All patients provided signed consent to participate in the study after being informed about its design and aims.

Declaration of Competing Interest

The authors declare no relevant financial or non-financial interests to disclose.

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Podgorica, 11. april 2022.

Na osnovu člana 18 stav 1 Zakona o upravnom postupku („Službeni list RCG", br. 56/14, 20/15, 40/16 i 37/17), Sporazuma između Vlade Crne Gore i Vlade Republike Slovenije o naučnoj i tehnološkoj saradnji, potpisanog 02. jula 2008. godine, Konkursa za sufinansiranje naučne i tehnološke saradnje između Crne Gore i Republike Slovenije za 2021/2022. godinu, objavljenog 13. septembra 2019. godine i Protokola sa šestog zasijedanja Mješovite komisije o naučnoj i tehnološkoj saradnji između Crne Gore i Republike Slovenije, potpisanog elektronskim putem 05. novembra 2020. godine u Podgorici/Ljubljani, Ministarstvo prosvjete, nauke, kulture i sporta Crne Gore donosi

R J E Š E N J E

I. PRIHVATA SE sufinansiranje mobilnosti istraživačkih timova u 2021. i 2022. godini, angažovanih u naučnoistraživačkom projektu:

Nosioci istraživanja: **Univerzitet Crne Gore – Medicinski fakultet i Univerzitetski klinički centar – klinički centar za medicinsku genetiku, Ljubljana**

1. Naziv projekta: GENETIČKA EPIDEMIOLOGIJA KONGENITALNIH ANOMALIJA U SLOVENIJI I CRNOJ GORI

Rukovodioci istraživanja: **DR OLIVERA MILJANOVIĆ**
DR BORUT PETERLIN

Sufinansiranje mobilnosti istraživača na projektu u 2021. i 2022. godini izvršiće Ministarstvo prosvjete, nauke, kulture i sporta, po osnovu boravka slovenačkih istraživača u Crnoj Gori i po osnovu putnih troškova crnogorskih istraživača u Republiku Sloveniju, u ukupnom iznosu od 1.000,00 € godišnje.

Sufinansiranje projekta od strane Ministarstva prosvjete, nauke, kulture i sporta:

- u periodu od 01. januara 2022. do 31. decembra 2022. godini iznosi 1.000,00 €.

SVEGA ZA UPLATU:

u periodu od 01. januara 2022. do 31. decembra 2022. – 1.000,00 €.

II. Realizacija sredstava odobrenih u tački I. ovog Rješenja, izvršiće se uplatom na žiro - račun Nosioca istraživanja iz Crne Gore.

III. Obavezuje se Nosilac istraživanja iz Crne Gore, da do 31. marta 2023. godine, dostavi Ministarstvu prosvjete, nauke kulture i sporta izvještaje o realizovanoj mobilnosti istraživačkih timova sa dokazima o putnim i troškovima boravka za svakog istraživača pojedinačno, za navedene godine.

IV. Ovo Rješenje realizovaće Direkcija za finansije i računovodstvo Ministarstva prosvjete, nauke, kulture i sporta.

V. Ovo Rješenje je konačno u upravnom postupku.

O b r a z l o ž e nj e

Na Konkurs za sufinansiranje naučne i tehnološke saradnje između Crne Gore i Republike Slovenije za period 2021 – 2022. godina, objavljen 13. septembra 2019. godine, prijavio se nosilac istraživanja sa prijavom projekta iz dispozitiva ovog Rješenja.

S obzirom da je predmetni projekat pozitivno ocijenjen od strane eksperata i prihvaćen u Protokolu o naučnoj i tehnološkoj saradnji između Crne Gore i Republike Slovenije za 2021/2022. godinu, Ministarstvo prosvjete, nauke, kulture i sporta Crne Gore prihvatilo je njegovo sufinansiranje, kao u dispozitivu ovog Rješenja.

Sufinansiranje projekta iz tačke I. ovog Rješenja, od strane Ministarstva prosvjete, nauke, kulture i sporta u 2022. godini, iznosi 1.000,00 € godišnje.

Realizacija odobrenih sredstava izvršiće se uplatom na žiro - račun Nosioca istraživanja iz Crne Gore.

Obavezuje se Nosilac istraživanja iz Crne Gore, da do 31. marta 2023. godine, dostavi Ministarstvu prosvjete, nauke, kulture i sporta izvještaje o realizovanoj mobilnosti istraživačkih timova sa dokazima o putnim i troškovima boravka za svakog istraživača pojedinačno, za navedene godine.

Na osnovu iznijetog riješeno je kao u dispozitivu Rješenja.

PRAVNA POUKA: Protiv ovog Rješenja može se pokrenuti upravni spor tužbom pred Upravnim sudom Crne Gore u roku od 30 dana od dana dobijanja rješenja.

MINISTARKA

Prof. dr Vesna BRATIĆ

Dostavljeno: Medicinski fakultet-UCG, Podgorica
Dosije projekta,
Direkcija za financije i računovodstvo i
A/A

Kontakt osoba: mr Martina Lukić, samostalna savjetnica
Načelnica: mr Smilja Kažić Vujačić
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Odobrio: Goran Drobnjak, generalni direktor
Direktorat za evropske integracije, programiranje i implementaciju EU fondova i međunarodnu saradnju