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THE FII 20210G→A, FV LAIDEN AND MTHFR 677C→T POLYMORPHISMS AND THE RISK OF PREGNANCY LOSS, FETAL MALFORMATIONS AND CHROMOSOMAL ABNORMALITIES

FII 20210G→A, FV LAIDEN AND MTHFR 677C→T POLIMORFIZMI I RIZIK OD GUBITKA TRUDNOĆE, FETALNIH MALFORMACIJA I HROMOZOMSKIH ABERACIJA

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Abstract

Broad spectrum of undesirable events and severe pregnancy complications, from early fetal loss to fetal malformations and chromosomal abnormalities, are results of complex interaction of environmental and inherited factors. The etiology still remains unclear in more than half of such events, but strong genomic impact is increasingly recognized. Gene mutations leading to a hypercoagulable state, such as factor V Leiden, factor II prothrombin, methylenetetrahydrofolate reductase (MTHFR) mutation, are associated with pregnancy loss and diverse embrional dysmorphogenesis. ***Objective*** of our study was to explore association between prothrombin, factor V Laiden and MTHFR genes polymorphisms and pregnancy loss and diverse fetal dysmorphogenesis. ***Methods:*** Women with history of pregnancy loss and presence of fetal/child`s malformations and chromosomal abnormalities were enrolled in this study. Polymorphisms in genes for factor II prothrombin (20210G→A), factor V Laiden (1691 G→A) and MTHFR (677C→T), were determined in all subjects. ***Results***: A group of 248 women with recurrent pregnancy loss in 1st trimester, unexplained intrauterine fetal death in 2nd and 3rd trimester of pregnancy and fetal malformations and chromosomal abnormalities was investigated for factor II prothrombin, factor V Laiden and MTHFR polymorphisms. Mutations in investigated genes were found in 23% women of study group. Significantly higher presence of homozygous 677C→T mutation in MTHFR gene was found among women with different APO in our study (18%) than in control. The significantly highest prevalence of homozygous 677C→T mutation in MTHFR gene was found in women with fetal/child`s chromosomal abnormalities (29%). Mutation in II prothrombin (20210G→A), and factor V Laiden (1691 G→A) genes were found slightly higher than in control group, but without statistical significance. ***Conclusion***: Established association of homozygous 677C→T mutation in MTHFR gene to recurrent pregnancy loss, unexplained intrauterine fetal/child`s death, and fetal malformations and chromosomal abnormalities implies that this mutation might be a significant risk factor for a broad spectrum of APO, suggesting the need for further research and thrombophilia screening and providing genetic counseling with prophylaxis for women with increased APO risk.

Key words
FII 20210G→A, FV Laiden and MTHFR 677C→T polymorphisms, thrombophilia, pregnancy loss, fetal malformations and chromosomal abnormalities

Ključne reči
FII 20210G→A, FV Laiden i MTHFR 677C→T polimorfizmi, trombofilija, gubitak trudnoæe, fetalne malformacije i hromozomske aberacije fetusa

INTRODUCTION

Successful pregnancy outcome is determined by interaction of numerous hereditary and environmental influences, from preconception period to delivery. Numerous undesirable pregnancy events, such as recurrent pregnancy loss, unexplained intrauterine fetal death, intrauterine growth restriction, pre-eclampsia and eclampsia, abruptio placenta, premature labor[1], and even presence of fetal malformations and/or chromosomal abnormalities[2,3], or infertility and implantation failure[4], represent a wide spectrum of adverse pregnancy outcomes. An adverse pregnancy outcome (APO) is considered to be any event which reduces the likelihood of having healthy offspring[5]. The etiology of APO appears to be multifactorial, and although it is known such occurrences share a common origin with strong genomic impact, around 50% of those cases remain unexplained, despite constant research focus on the topic[6, 7].

While chromosomal abnormalities, uterine malformations, and hormonal and immune system disorders are common and well defined causes of APO, the latest studies mainly focus on the area of genetic polymorphisms, specifically inherited blood clotting factors. Thrombophilias are inherited or acquired conditions which predispose an individual to thromboembolism[8].

Pregnancy is a hypercoagulable state, with six-times greater risk for thromboembolysm then in non-pregnant women. The increased blood clothing tendency could lead to severe pregnancy complications resulting in APO. Women with inherited or acquired thrombophilia are at an increased risk for thrombosis during pregnancy which could result in APO[9,10]. A successful pregnancy outcome is dependent upon development of efficient uteroplacental circulation, thus many pregnancy-related disorders are interpreted as consequences of impaired microvascular function and might be viewed as a mild form of venous thromboembolic disease[4].

The risk of APO in each woman is determined by the interaction of many genetic influences and environmental factors, but only few of these have so far been identified. Recent studies of coagulation related genes showed that their single gene polymorphisms (SNPs) may be responsible for inherited thrombophilias and majority of thromboembolic events in patients with unrecognized risk for thrombosis. SNPs in thrombophilia associated genes: factor II (FII) 20210G→A, factor V (FV) Laiden 1691 G→A and MTHFR 677C→T, encoding methylene-tetrahydrofolate reductase enzyme, are most frequently investigated in patient with significant thrombotic events, including women with APO history[11].

Mutation in FV 1691G→A, found in about 5% of general population, has been linked with an increased risk for thromboembolism and it is found in 15-50% deep venous thrombosis (DVT) patients. Heterozygous mutation in FV gene increases risk for thrombosis 5 to 10 times, while homozygous mutation is rare, implicating a more severe, up to 80 times increased risk[8,12,]. FII mutation 20210 G→A present in 6-18% of DVT patients and only 2-4% of general population, is associated with higher plasma prothrombin concentrations and 2-4 fold increased risk for venous thromboembolism. Vast majority of these FII mutations are in a heterozygous state, while recessive homozygots are extremely rare and with more severe thrombotic consequences[8,12].

Homozygous mutation for the 677C→T in gene for MTHFR, results in decreased synthesis of 5-methyltetrahydrofolate, the primary methyl donor in the conversion of homocysteine to methionine. The resulting increase of homocysteine concentrations in plasma is a risk factor for thrombosis[8]. 677C→T mutation is responsible for reduced MTHFR activity, and it is found significantly influential only in recessive homozygous state[13]. Heterozygous form, which is not associated with increased risk for thromboembolism, is widely found in general population (30-50%), while prevalence of homozygous mutation varies from 5 up to 20%, depending on ethnicity of the population and study inclusion criteria[14,15].

Furthermore, 677C→T polymorphisms in gene for MTHFR (CT and TT genotypes), with inherent low folate levels, play a significant role as factors that compromise oocyte maturation before conception and successful DNA methylation and demethylation, causing point mutations, chromosome breakage, defective chromosome recombination, and may set the stage for a genetically conditioned high-risk conception, with an increased risk for maternal chromosomal nondisjunction, which is the main cause of fetal chromosomal aneuploidies[3,16]. Reduction of follicle growth and delayed ovulation, associated with MTHFR polymorphisms and/or folate deficiency, are markers for retardation of embryonic growth and a spectrum of developmental anomalies[2].

The aim of our study was to evaluate the association between wide spectrum of adverse pregnancy outcomes and polymorphisms in genes related to hereditary thrombophilic conditions.

PARTICIPANTS AND METHODS

The study was conducted in the Center for Medical Genetics and Immunology, Clinical Center of Montenegro, upon approval of Ethic Committee of Clinical Center of Montenegro.

Study population

The total number of 248 women (Caucasian, same ethnicity) with unexplained APO was enrolled in the study, after the initial examination of 346 couples with history of APO, who were referred to our Centre for Medical Genetics, from January 2012. to June 2013. Women with co-morbidity risk factors associated with APO (infection during pregnancy, uterine malformations, hormonal disturbances, chronic and auto-immune disorders, acquired thrombophilia, recognized exposure to teratogenenes, and balanced chromosomal state in either partner) had been excluded from the study.

Study group was divided in two groups.

- APO group I: 134 women with pregnancy loss and/or implantation failure. Pregnancy loss was considered as: two or more 1st trimester consecutive pregnancy losses, one or more 2nd and 3rd trimester pregnancy loss, and unexplained intrauterine fetal death. Implantation failure

was considered as three or more unsuccessful IVFs, or at least two unsuccessful IVFs in addition to another adverse pregnancy outcome.

- APO group II: 114 women whose fetus/child has severe malformations and chromosomal abnormalities, diagnosed prenatally or postnatnatally.

Data for Controls were used from a previously published study of a similar homogenous Caucasian population, with common ethnic and geographic origin (same country – Serbia and Montenegro, until year 2006), given that population studies of hereditary thrombophilia in Montenegro have not yet been performed. Control group consisted of 128 healthy women, with no history of adverse pregnancy outcomes or thrombotic events, and with at least one successful delivery [15].

Genotype analysis

Single nucleotide polymorphisms in genes for coagulation factors FV and FII prothrombin, and in gene for MTHFR were analyzed for total of 248 women with APO. Genetic testing was carried out at the Center for Medical Genetic and Immunology.

DNA was isolated from peripheral blood samples, collected in tubes with EDTA and stored at -20ºC. DNA was extracted by QIAamp DNA Blood Mini Kit (QIAGEN, Germany). Three single nucleotide polymorphisms were determined: 20210G→A in gene for factor II prothrombin, 1691G→A, in gene for factor V Laiden and 677C→T polymorphism in gene for MTHFR. DNA testing was performed by Attomol factor II-QT, factor V-QT and MTHFR-QT (KRISHGEN Biosystems). For DNA amplification (PCR Mastercycler gradient Eppendof), HotStar Taq DNA Polymerase (QIAGEN, Germany) was used. Analysis of PCR products was performed by agarose electroforesis (2,5%), stained with ethidium bromide, and visualized by UV light.

Hereditary thrombophilia as an increased risk for APO was considered as presence of at least one of following polymorphisms/mutations in genes related to hereditary thrombophilia:

- heterozygot (GA) or recessive homozygote (AA) in gene for FII prothrombin
- heterozygot (GA) or recessive homozygote (AA) in gene for FV genes
- recessive homozygot (TT) in gene for MTHFR.

Statistical data analyses were obtained with GraphPad prism statistical software. Fisher's exact test was used for comparison of prevalence of examined genotypes between study group and controls(17). All p values <0.05, were considered to be statistically significant.

RESULTS

The average age of women in study group was 34.2 (range: 19 - 47 years). Group I, consisted of 134 women, from age 19 to 46 (average 35,5). The average age of 114 women in the Group II was 32,7 (range of 21 - 47 years). Average age in study and control group(15) were similar, with no significant difference (p>0.05).

Presence of hereditary thrombophilia (at least one aforementioned mutation in investigated genes) was found in 23,4% of women in study group, with statistically significant difference when compared with control group[15], and with the highest mutation frequency among women with fetal chromosomal abnormalities (table1).

Combination of two mutations was found in 8 women, all in group I, women with pregnancy loss and implantation failure (6%). One woman had two homozygote mutations (FV Laiden and MTHFR), one had factor FII heretozygous and MTHFR homozygous mutation. The others had combination of two heterozygotes for factor FV Laiden and MTHFR (4 women), and for F II and MTHFR (2 women).

***Table 1**: Prevalence of hereditary thrombophilia among women in study and control groups[15]*

		Hereditary thrombophilia				
		Study group N=248		Control[15] N=128		Significance §
		No	%	No	%	P values
Study group	N=248	58	23,4	11	8.6	0.0004***
APO group I	N=134	32	23,9	11	8.6	0.0008***
APO group II	N=114	25	21,9	11	8.6	0.0039**
Women with FCA	N=52	15	28,8	11	8.6	0.0009***

§Fisher's exact test, FCA: fetal chromosomal abnormalities, *** p<0.001, ** p<0.01

Recessive homozygote TT in MTHFR gene (677C→T) was found as the most frequent mutation among women in our study. The prevalence of this mutation was 17,7% in women with APO history. Compared with control group[14], prevalence of this mutation was significantly higher (table 2).

Observing the presence of recessive homozygote in MTHFR gene (677C→T) in different groups of our study population (APO I group, APO II group, women with fetal /child`s congenital malformations and women with fetal/child`s chromosomal abnormalities), we have found significant difference in prevalence within all groups in comparison to the control group[15]. The highest prevalence was found in group of women with fetal/child`s chromosomal abnormalities (28,8%), with a strongest statistical significance (table 2). There were no statistical differences in recessive homozygote prevalence between groups of study population, except between APO group I and group of women with fetal/child`s chromosomal abnormalities (table 2). The prevalence of recessive homozygote in MTHFR gene (677C→T) in all groups of study population and control group is shown in graph 1.

Mutations in factor FII prothrombin gene (20210 G→A) and in FV Laiden gene (1691G→A), were rare in our study group (2,8% and 2,4%, respectively), all found in women with pregnancy loss and implantation failure (APO group I: 5,2% and 4,2%, respectively), but with no significant difference when compared with control[15].

***Table 2**: Prevalence of mutations in genes related to thrombophilia among women in study group and control group*[15]

	Prevalence of gene mutations				
	No	%	No	%	P values §
	Study group N=248		**Control [15] N=128**		
F II Protrombin 20210$_{G\ A}$	7	2,8	3	2,3	NS
F V Laiden 1691 $_{G\ A}$	6	2,4	2	1,6	NS
MTHFR 677$_{C\ T}$	44	17,7	6	4,7	0.0003***
	APO I group N=134		**Control[15] N=128**		
F II Protrombin 20210$_{G\ A}$	7	5,2	3	2,3	NS
F V Laiden 1691 $_{G\ A}$	6	4,5	2	1,6	NS
MTHFR 677$_{C\ T}$	19	14,2	6	4,7	0,0109*
	APO II group N=114		**Control[15] N=128**		
F II Protrombin 20210$_{G\ A}$	0	0	3	2,3	NS
F V Laiden 1691 $_{G\ A}$	0	0	2	1,6	NS
MTHFR 677$_{C\ T}$	25	21,9	6	4,7	0,0001***
	Women with FCA N=52		**Control[15] N=128**		
F II Protrombin 20210$_{G\ A}$	0	0	3	2,3	NS
F V Laiden 1691 $_{G\ A}$	0	0	2	1,6	NS
MTHFR 677$_{C\ T}$	15	28,8	6	4,7	0,0001***
	Women with FCM N=62		**Control[15] N=128**		
F II Protrombin 20210$_{G\ A}$	0	0	3	2,3	NS
F V Laiden 1691 $_{G\ A}$	0	0	2	1,6	NS
MTHFR 677$_{C\ T}$	10	16,1	6	4,7	0,0116*
	APO I group N=134		**APO II group N=114**		
F II Protrombin 20210$_{G\ A}$	7	5,2	0	0	0,0164*
F V Laiden 1691 $_{G\ A}$	6	4,5	0	0	0,0324*
MTHFR 677$_{C\ T}$	19	14,2	25	21,9	NS
	APO II group N=134		**Women with FCA N=52**		
F II Protrombin 20210$_{G\ A}$	7	5,2	0	0	NS
F V Laiden 1691 $_{G\ A}$	6	4,5	0	0	NS
MTHFR 677$_{C\ T}$	19	14,2	15	28,8	0,0329*

§Fisher's exact test, FCA: fetal/child`s chromosomal abnormalities, FCM: fetal/child`s malformations *p<0.05; **p<0.01; *** p<0.001

DISCUSSION

Pregnancy is a state of increased risk for thrombosis. In the cases when women have mutations in genes involved in blood coagulation (FII, FV and MTHFR genes), that are strongly associated with susceptibility to thrombosis, the risk for adverse pregnancy outcome is greatly increased[9, 10,17].

In our study, we have analyzed the association between different APO and mutations in genes associated with thrombophilia (FII and FV heterozygotes and recessive homozygotes, and MTHFR recessive homozygotes). Hereditary thrombophilia, presenting an increased risk for APO, was considered as presence of at least one of investigating polymorphisms/mutations in genes associated with blood clothing. Obtained results were also compared with geographically and ethnically similar control group of women with no APO history and at least one successful pregnancy. (the same county until 2006)[15].

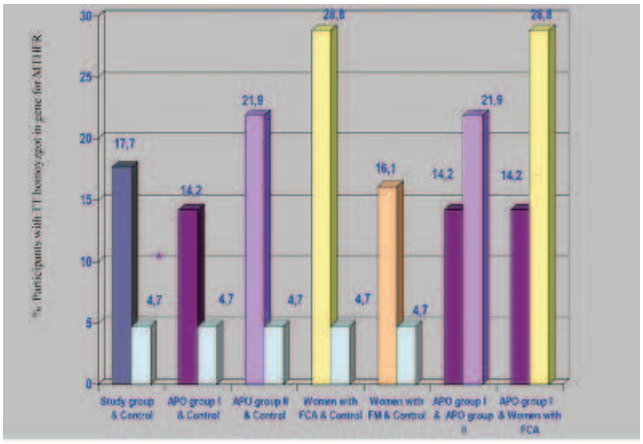
Many studies and meta-analyses in the last decade were focused on investigation of the hereditary thrombophilia and APO, suggesting that prothrombotic genotypes in thrombophilia related genes are associated with various APO[18,19], but not rarely with very dissimilar, even contradictory results, depending on study design, inclusive criteria and geographic population[20]. A significantly higher prevalence of hereditary thrombophilia (at least one mutation) was found in women with different APO in many studies, with prevalence that varies: 52 - 56% in Kupferminc et al. studies[8,19] in women with preeclampsia/eclampsia and intrauterine growth retardation, 45% in women with venous thromboembolism in pregnancy and fetal loss[15], 21 - 66% in recurrent pregnancy loss[21]. There is also a number of studies that report no significantly higher prevalence in women with APO, compared with control population [20,22,23].

Presence of hereditary thrombophilia in our study was significantly higher than in control group[15], found within an expected range, as reported in other studies: 18% in women with any APO, with the highest prevalence within women with fetal/child`s chromosomal abnormalities (29%).

Many studies showed higher prevalence of FV Leiden mutation (18-26%) and FII mutation [18,19,24-27] in women with preeclampsia/eclampsia and intrauterine growth retardation, than in control (3-4%). Despite the established association between APO and mutation in FII and FV genes, numerous studies report controversial results. Studies investigating the association of FV mutation and RPL, showed broad variation of prevalence within study groups (3 - 46%)[7,28]. There are also numerous studies that report no difference in prevalence of FV mutation[20,29-31], or in prevalence of FII mutation[18-20,23,32] between study groups and controls. Similarly to later, we have not found significantly different prevalence of FII and FV mutation between study group and control[15].

Similarly to the aforementioned controversies, studies of recurrent pregnancy loss, preeclampsia, intrauterine growth retardation, 2nd and 3rd trimester fetal death, also lead to

***Graph 1**: The prevalence of recessive homozygote in MTHFR gene (677C→T) in all groups of study population and control*



contradictory reports, regarding relation to the mutation in MTHFR gene. Recessive homozygote TT (677C→T mutation) in MTHFR gene is recognized as a risk factor for thrombosis, but the exact role of this mutation still remains unclear [2,33].

Many studies confirmed strong correlation between and presence of 677C→T mutation in MTFHR gene and preeclampsia, intrauterine growth retardation, as well as recurrent pregnancy loss or 2nd and 3rd trimester fetal death[18,19,34,35,38]. Our results with significantly higher prevalence of 677C→T mutation in MTHFR gene among women with pregnancy loss and implantation failure, support the findings of these studies. On the contrary, numerous studies have failed to show the association between mutation in MHFR gene and APO[23,24,30,31,36,39,40].

We have shown that the highest prevalence of 677C→T mutation in MTHFR gene is found among women with fetal/child`s dismorphogenesis, especially in those with fetal chromosomal anomalies (29%). A broad spectrum of congenital malformation, such as neural tube defect (NTD)[2,41], congenital heart anomalies[42-45] and chromosomal abnormalities, ie Down Syndrome[3,16,46,47] are reported to be associated with maternal MTHFR polymorphism. Underlying mechanisms by which recessive homozygote 677C→T in MTHFR gene is associated with congenital malformations and chromosomal abnormalities are not fully recognized, but it seems that TT homozygote is associated with insufficient remethylation of homocystein, essential for DNA repair, DNA synthesis and DNA imprinting processes, as well as for oocyte maturation and epigenetic reprogramming in oocyte and embrio[2,48].

CONCLUSION

Our study showed an association between hereditary thrombophilia and adverse pregnancy outcome, especially regarding recessive homozygot 677C→T in MTHFR gene, as the most prevalent. Significantly higher presence of 677C→T mutation in MTHFR gene among women with different APO, compared with women with no history of APO and successful reproductive history, showed that mutation in MTHFR gene might be a risk factor for adverse pregnancy outcome. The significantly highest prevalence of 677C→T mutation in MTHFR gene in women with fetal/child`s congenital malformations and chromosomal abnormalities implies a necessity of further research of genes related to thrombophilia and genetic counseling with providing prophylaxis for the following pregnancies, to assure healthy outcomes.

Further research should include investigation of polymorphisms in genes related to thrombophilia in general Montenegrin population, wider research in various groups of women with adverse pregnancy outcomes, and broadening the research on to other polymorphisms associated with thrombophilia, such as PAI-1, as well as different polymorphisms in genes for factor II prothrombin and MTHFR.

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Sažetak

Široki spektar nepovoljnih ishoda i ozbiljnih komplikacija tokom trudnoće, od ranih gubitaka trudnoće do različitih anomalija i hromozomskih aberacija fetusa, rezultat je kompleksne interkcije sredinskih i nasljednih faktora. Etiologija više od polovine ovakvih događaja i dalje ostaje nejasna, mada je prepoznat snažan uticaj genomskih faktora. Mutacije u genima kakvi su faktor II protrombin, faktor V Leiden, i u MTHFR genu, odgovorne za stanje hiperkoagulabilnosti, dovode se u vezu sa gubicima trudnoće i pojavom dismorfogeneze fetusa. ***Cilj*** naše studije je da se ispita povezanost polimofrizama u genima za faktor II protrombin, faktor V Leiden, i u MTHFR genu sa neobjašnjenim gubicima trudnoće i fetalnom dismorfogenezom. ***Metod:*** U ovu studiju uključene su žene sa gubicima trudnoća i dijagnostikovanim anomalijama i hromozomskim aberacijama kod fetusa/djeteta. Ispitivani su polimorfizmi u genima za faktor II protrombin (20210G→A), faktor V Laiden (1691 G→A) i u MTHFR genu (677C→T). ***Rezultati***: Ispitivanje polimofrizama u genima za faktor II protrombin, faktor V Leiden, i u MTHFR genu sprovedeno je u grupi od 248 žena sa ponavljanim spontanim pobačajima u prvom trimestru, neobjašnjenom intrauterinom smrti ploda u drugom i trećem trimestru trudnoće, i sa prisutnim malformacijama i hromozomskim aberacijama kod fetusa/djeteta. Mutacije u ispitivanim genima nađene su kod 23% žena u studijskoj grupi. Značajno veće prisustvo homozigotne 677C→T mutacije u MTHFR genu pronađena je među ženama sa različitim nepovoljnim ishodima trudnoće, u poređenju sa onima iz kontrolne grupe. Statistički najznačajnija razlika u prevalenci homozigotne 677C→T mutacije u MTHFR genu nađena je u grupi žena sa hromozomskim aberacijama fetusa/djeteta (29%). Prisustvo mutacija u genima za faktor II protrombin (20210G→A) i faktor V Laiden (1691 G→A) nađeno je u nešto većem procentu nego u kontrolnoj grupi, ali bez statistički značajne razlike. ***Zaključak:*** Utvrđena povezanost prisustva homozigotne 677C→T mutacije u MTHFR genu sa ponavljanim spontanim pobačajima, neobjašnjenom intrauterinom smrti fetusa i prisustvom malformacija i hromozomskih aberacija fetusa/djeteta , upućuje da ova mutacija može predstavljati značajan faktor rizika za nepovoljan ishod trudnoće, ukazujući na potrebu za daljim istraživanjima, ali i za skriningom na urođene trombofilije i genetskim savjetovanjem, sa ciljem obezbjeđivanja profilakse za one žene koje su u povišenom riziku od nepovoljnih ishoda trudnoće.

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