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

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ABSTRACT

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

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

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

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Materials

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

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

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

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

Methods

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

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

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

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

Table 1. Nucleotide sequences of PCR primers

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

Table 2. Single base extension primers data

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

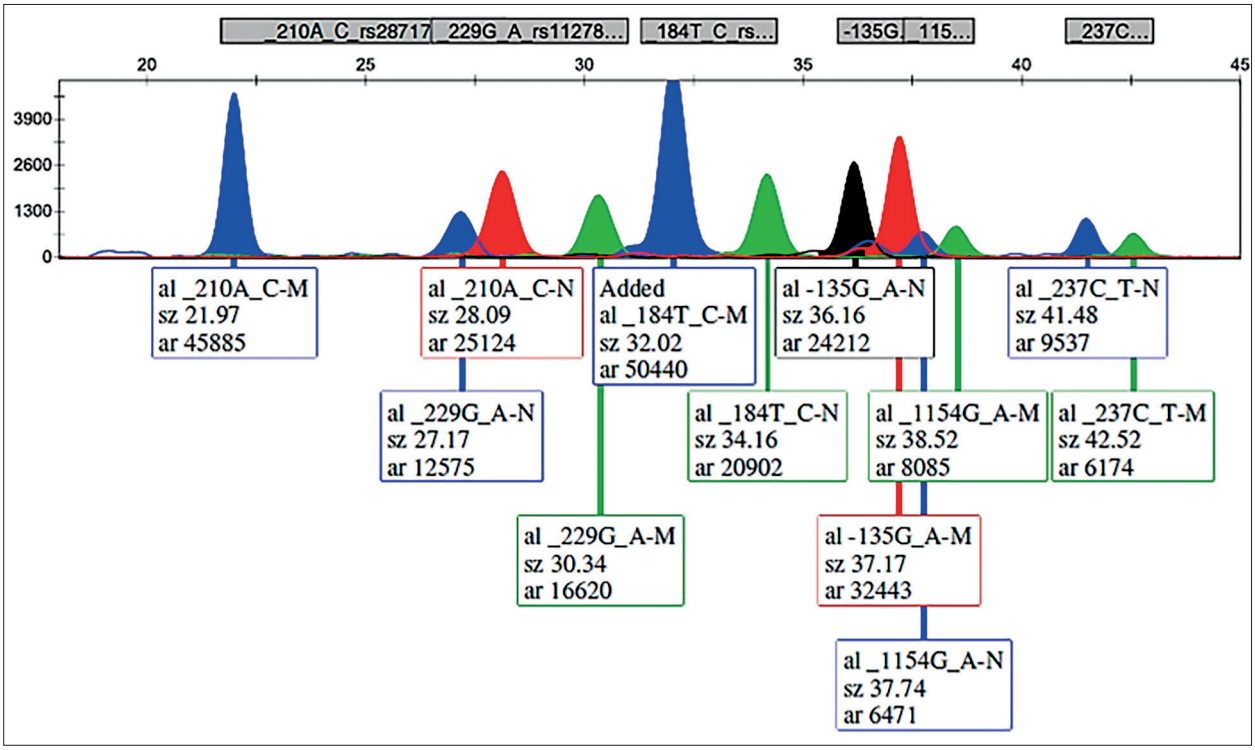

Figure 1. Electrophoretogram of an individual heterozygous for all ANXA5 and VEGFA examined variants.

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

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

RESULTS

ANXA5 variants

Haplotype-based classification

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

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

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

Comparison based on history of pregnancy loss and live births

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

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

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

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

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

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

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

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

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

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

VEGFA gene

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

Comparison based on history of pregnancy loss and previous live birth

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

CONCLUSION

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

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CONFLICT OF INTEREST
Not declared.

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