Research Article



ISSN: 2398-5399

Comparison of the thrombophilic gene polymorphisms and recurrent pregnancy loss: Results on combined gene effect of *FV* Leiden, *FVR2*, FXIII, *MTHFR*(A1298C and C677T), PAI-1 4G/5G and ACE I/D genes in RPL Women from Minsk/Belarus and Canakkale - Sivas/Turkey

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Abstract

Aim: Recurrent pregnancy loss (RPL) is a serious fertility problem with polygenic multifactorial etiology. Thrombophilia plays an important role in RPL. We compare the combined effect of the thrombophilic gene mutations as: *FVL*, *FactorVR2*, *MTHFR*C677T, *MTHFR*A1298C, *PAI*-15G/4G, *ACEI*/D and *FXIII*103G>T on RPL women from two different Caucasian populations (Minsk/Belarus and Canakkale - Sivas/Turkey) was compared. This is the first report of thrombophilic mutations from South Slavic population of Belarusian.

Methods: 1590 RPL women (830 Turkish and 760 Belarussian) were evaluated and compared in the current results. Buccal smears (for Belarussian) and peripheral blood-EDTA (for Turkish) samples were used for DNA isolation. Reverse hybridization method (for Turkish) and Real time PCR (for Turkish and Belarusian populations) were used for genotyping.

Results: *FVL*, *FVR*2G/A, *MTHFRA*1298C are significantly frequent in Turkish RPL population than Belarusian cohort (p<0.001, p:0.002, p:0.0009 respectively) and *PAI*-1 5G/4G, *ACE* I/D, *FXIII*103G/T mutations are significantly frequent in Belarusian RPL population than Turkish cohort (p:0.03, p:0.001, p<0.001 respectively). *MTHFR* 677T allele is frequent in both populations (0.32, 0.30, p:0.2) but homozygosity is more frequent in Turkish cohort significantly, this is probably because of the frequent consanguineous marriage. *ACE+PAI*-1 combination is the most frequent double mutations in both cohort. The triple *FVR2+ACEI*/D+*FXIII*103G>T mutations were same in both populations.

Conclusion: Our results showed similar germ-line prothrombotic gene variations in RPL women in both populations, but the mutated *FVL*, *FVR*2G/A and *MTHFRA*1298G SNPs are significantly high in Turkish RPL women when compare to the Belarusian RPL women.

Introduction

Recurrent pregnancy loss (RPL) is defined if a couple having consecutive loss of two or more pregnancies with the same partner before 20 weeks of gestation and not more than one living child [1-4]. The RPL is a significant clinical problem with many etiologies. RPL is a complex process that a lots of molecular ethilogical factors may cause this dilemma but still large amount of cases are unidentified. Inherited and/or acquired thrombophilia is an important contributing factor in unexplained RPL [5-9]. RPL women are basicly classified into three groups as: women without previous 2 live birth, live birth after pregnancy losses, and pregnancy losses followed by a live birth [10]. Some RPL couples have an additional parental associated genetic risk factors but some have non-genetic histories [11,12]. In general, the RPL risk is enhanced by a variety of etiological factors such as; chromosomal abnormalities, uterine abnormalities, endocrinological defects, infections and environmental factors. Maternal, fetal, environmental and paternal based ethiological risk factors were also reported in RPL pathogenesis [13-15]. Thrombophilias have been postulated as a cause of RPL complications such as stillbirth, fetal growth restriction, severe preeclampsia, and placental abruption. Inherited and/or acquired thrombophilic gene polymorphisms are the basic debated problems that may common acceptable in women with unexplained RPL [14-16]. Recently, large amount of literature findings from different populations showed the incidence of specific thrombophilic gene polymorphisms in RPL women. Numereous studies have reported an association but some others have a lack of any association between thrombophilic gene mutations and RPL.

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Key words: single nucleotide polymorphisms, factor V leiden, genetic testing and counseling, population screening

Received: September 01, 2016; Accepted: September 26, 2016; Published: September 30, 2016

Silan F (2016) Comparison of the thrombophilic gene polymorphisms and recurrent pregnancy loss: Results on combined gene effect of FV Leiden, FVR2, FXIII, MTHFR(A1298C and C677T), PAI-1 4G/5G and ACE I/D genes in RPL Women from Minsk/Belarus and Canakkale - Sivas/Turkey

In the current retrospective study we basically aimed trombophilic polymorphisms to compare the gene of FVLeiden(rs6025), FactorVR2(rs1800595), MTHFRC677T(rs1801133), MTHFRA1298C(rs1801131), PAI-15G/4G (rs1799889), ACEI/ D(rs4646994) and FXIII103G>T(rs5985) genes in RPL women from Minsk/Belarus and Canakkale - Sivas/Turkish populations.

Materials and methods

Patients, clinical diagnosis and laboratory assessment

Thrombophilic gene polymorphisms were correlated in two Caucasian populations in the current results (Minsk/Belarus and Canakkale - Sivas/Turkey). In a total of 1590 RPL women were enrolled between May 2007 - July 2015 retrospectively and eavluated. Results of the allelle frequencies and combined gene efficiency of target genes in RPL women were discussed. Eight hundred and thirty RPL women the mean age-min-max; $28.1 \pm 1.80(17-45)$ from Turkish and 760 RPL women the mean age-min-max; $30 \pm 4.76(19-46)$ from Belarusian populations were evaluated. Peripheral blood-EDTA samples from each patient were used for germ-line target genes profiling. The results belong to the germ-line polymorphisms for target genes were compared for both populations.

Genotyping

The total genomic DNA was extracted by the MagnaPure Compact (Roche) and Invitek kit extraction techniques (Invitek^{*}; Invisorb spin blood, Berlin, Germany). Target genes were simultaneously amplified in a biotin-labelled single multiplex amplification reaction (Viennalab*; PGX-HIV StripAssay, Vienna, Austria) which is based on the reverse-hybridization principle automatically and by Real Time PCR, LightCycler 2.0 methods (Roche). The multiple polymerase chain reaction (PCR) was performed in a Perkin Elmer 9600 and the profile consisted of an initial melting step of 2 min at 94°C; followed by 35 cycles of 30s at 94°C, 30s at 61°C, and 30s at 72°C; and a final elongation step of 7 min at 72°C for stripAssay genotyping. Some suspicious samples were clarified by real-time PCR technique (LightCycler 2.0, Roche).

All samples were genotyped for target genes in Turkish women with RPL history. Briefly, LightCycler FastStart DNA Master HybProbes, master mix and DNA template were used for real-time amplification. The amplification conditions for 45 cycles were; denaturation in 95°C for 10 seconds, annealing for 5-20 seconds, extension in 72°C, melting curve step with denaturation in 95°C, annealing for 30 seconds, melting in 95°C and cooling step in 40°C for 30 seconds. Software programme (LightCycler 2.0, Roche) was used for detection of the mutated and normal genotype profiles of target genes in the current RPL women (Figures 1 and 2).

The total genomic DNA was extracted from buccal swabs (nucleosorb A extraction kit, Primetech, Minsk, Belarusian) for the current RPL cohort from Belarusian population. The quantities of all DNA samples were measured by using dsDNA Assay Kit, BroadRange for Qubit[®] Fluorometer from Invitrogen and multiplex Real-time PCR was performed in a CFX 96 (Bio-rad, US) using 96-well plates for target genes genotyping.



Figure 1. Shows the Real Time-PCR melting peaks profiles for *PAI-1* genotypes that evaluated for the current RPL women. A. Homozygous 5G/5G genotype (Tm: 61°C), B. Heterozygous 5G/4G genotype (Tm: 61°C, C. Homozygous 4G/4G genotype (Tm: 54°C).



Figure 2. Frequencies of being dual homozygous and heterozygous combined mutations for some target genes in RPL women from Turkish and Belarussion populations. No significant difference was noted in thrombophilic gene mutations in the current Caucasian population cohorts.

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The PCR reactions were performed in a total volume of $10 \,\mu$ L with a reaction mixture containing 300 nM of each primer, 2XiTaq Universal probes supermix (Bio-rad, US), 50 nM probes, and 15 ng of template DNA samples. The taqman probes, master mix that contains forward and reverse primer sequences for target genes and DNA template were used for Real-Time amplification. Briefly, the amplification conditions for 40 cycles were; denaturation in 95°C for 5 min, annealing for 5-20 seconds, extension in 60°C for 5-20 seconds.

Statistical analysis

Alternative genotype frequencies for mutated target gene markers in RPL women from both populations were compared using Pearson's chi-square test and multiple logistic regression analysis. Statistical analysis was performed using SPSS version 15 (SPSS, Chicago, IL, USA). Values of P<0.05 were considered statistically significant and mutated allele frequencies were discussed in the current report.

Results

Results from peripheral blood-EDTA and buccal smear samples from Turkish and Belarussian RPL women that were genotyped for thrombophila genes were enrolled and compared for the current study retrospectively. Eight hundred and thirty RPL women the mean agemin-max; $28.1 \pm 1.80(17-45)$ from Turkish and 760 RPL women the mean age-min-max; $30 \pm 4.76(19-46)$ from Belarusian populations were evaluated. The average pregnancy losses were 2.7(1-10) for Turkish and 2.0(1-7) for the Belarusian RPL women cohorts (Table 1). The estimate risk was examined by odds ratio. Retrospectively evaluated results showed similar germ-line variations for target studied genes in RPL women in both populations.

The distribution of genotype frequencies of the studied polymorphisms between the current RPL women form both populations were in Hardy–Weinberg equilibrium and no statistically significant differences was found in terms of the age and gender. The both germ-line T and C alleles frequencies of *MTHFR* 677 C>T and 1298 A>C SNPs have been shown to be a risk factor for the current cohort RPL women for both populations (Table 2). The prevalence of genotypes of *MTHFR* gene C677T SNP in Turkish RPL women (48% for CC, 40% for CT and 12% for TT respectively) were the similar as Belarusian RPL cohort (48.29% for CC, 43.16% for CT and 8.55% for TT) respectively, (Table 2). The T allele frequency were 0.320 for the Turkish and 0.301 for the Belarusian RPL women. No statistical significance detected between allel frequencies (Table 2), (OR: 1.0936, CI: 0.9409-1.2711), p<0.2437) but the *MTHFR* 677TT (homozygous) genotype were significantly higher in Turkish cohort (12.0%) when

 Table 1. Some clinical characteristics for studied cohort of RPL women from Turkish and Belarussian populations.

Clinical Characteristics	Population		
	Turkish	Belarussian	
Patients (n)	RPL Women n:830	RPL Women n:760	
Biological Material(n/%)	Peripheral Blood- EDTA (830/100)	Buccal Smear (760/100)	
Age(year), Median ± S. D. (range)	28.1±1.80(17-45)	30 ± 4.76(19-46)	
No of pregnancy losses Median (range)	2.7 (1-10)	2.0(1-7)	
No of live births Median (range)	0	0	

RPL: Recurrent pregnancy loss

 Table 2. The Genotype and Allele Frequency of MTHFR C677T and MTHFR A1298C

 SNPs in RPL women for both populations.

Patient Group (n)	atient Group (n) Population				
		Turkish	Turkish		
		RPL Women	RPL Women		
		(n:830)	(n:830)		
Gene/SNP		MTHFR C677T		MTHFR C677T	
Genotypes (n/%)					
CC		398/48.0	367/48.29		
СТ		332/40.0	328/43.16		
TT		100/12.0	65/8.55		
Alleles					
С		1128/0.680	1062/0	0.699	
Т		532/0.320	458/0	0.301	
Statistically Analy	sis				
	P Value	P=0.0230	P=0.0230		
Recessive Model	OR	1.4647	1.4647		
	CI(95%)	1.0540 - 2.0355	1.0540 - 2.0355		
	P Value	P=0.8929	P=0.8929		
Dominant Model	OR	1.0136	1.0136		
	CI(95%)	0.8324 - 1.2343			
	P Value	P=0.2437	P=0.2437		
Allel Frequencies	OR	1.0936			
	CI(95%)	0.9409 - 1.2711	0.9409 - 1.2711		
Gene/SNP		MTHFR A1298C	MTHI	FR A1298C	
		(n:772)	(n:760)	
Genotypes (n/%)					
AA		289/37.44 355/46.72		5.72	
AC		376/48.70	323/42	2.50	
CC		107/13.86	82/10.	78	
Alleles					
Α		972/0.618	1033/0	0.680	
С		590/0.382	487/0.	320	
Statistically Analy	sis				
	P Value	P=0.0683			
Recessive Model	OR	1.3304	1.3304		
	CI(95%)	0.9788 - 1.8083			
	P Value	P=0.0002	P=0.0002		
Dominant Model OR CI(95%)		1.4649	1.4649		
		1.1948 -1.7962			
P Value P=0.0		P=0.0009			
Allele Frequency	OR	1.2875	1.2875		
	CI(95%)	1.1098 - 1.4937	1.1098 - 1.4937		

compare to the Belarusian cohort (8.55%) (p:0.023).

Significant difference was detected in the second SNP marker (A1298C) of *MTHFR* gene in Turkish (37.44% for AA, 48.70% for AC and 13.86% for CC respectively) and Belarusian RPL women (46.72% for AA, 42.5% for AC and 10.78% for CC) respectively, (Table 2). The C allele frequency was 0.382 for Turkish and 0.320 for Belarusian RPL women. The *MTHFR* 1298 A>C SNP (homozygous) genotype was found 13.86% for Turkish and 10.78% for the Belarusian cohorts (Table 2), (OR: 1.2875, CI: 1.1098-1.4937), p<0.0009.

The prevalence of genotypes of *PAI-15G/4G* gene in Turkish RPL women (20.63% for 5G/5G, 56.33% for 5G/4G and 23.04% for 4G/4G), respectively was the similar as in Belarusian group (20.0% for 5G/5G, 50.0% for 5G/4G and 30.0% for 4G/4G), (Table 3). The 4G allele frequency was 0.513 for the Turkish and 0.550 for the Belarusian cohort. The *PAI-14G/4G* gene (homozygous) genotype was found 23.04% in Turkish and 30.0% in Belarusian RPL women and difference

was statistically significant (Table 3), (OR: 1.1648, CI: 1.0114-1.3415), p<0.0342).

The prevalence of genotypes of *ACE* I/D in Turkish RPL group (35.47% for D/D 48.26% for I/D and 16.27% for I/I) and (23.94% for D/D 50.14% for I/D and 25.92% for I/I) in Belarusian group respectively (Table 4). The I allele frequency was 0.404 for Turkish and 0.510 for the Belarusian groups. The *ACE* I/I (homozygous) genotype was found 16.27% and 25.92% in Turkish and Belarusian RPL women respectively. The mutated I allele frequency was different and that difference was statistically significant (Table 4), (OR: 1.5342, CI: 1.3075-1.8003), p<0.0001.

The prevalence for genotypes of FVLG/A gene were (87.23% for G/G 12.41% for G/A and 0.36% for A/A) and (97.89% for G/G

Table 3. The Genotype and Allele Frequency of PAI-1 5G/4G gene polymorphisms in studied RPL women for the current results.

Patient Group (n)		Population		
		Turkish	Belarussian	
		RPL Women (n:790)	RPL Women (n:760)	
Gene/SNP		PAI-1 5G/4G	PAI-1 5G/4G	
Genotypes (n/%)				
5G/5G		163/20.63	152/20.00	
5G/4G		445/56.33	380/50.00	
4G/4G		182/23.04	228/30.00	
Alleles				
5G		771/0.487	684/0.450	
4G		809/0.513	836/0.550	
Recessive Model	P Value	P=0.0019		
	OR	1.4317		
	CI(95%)	1.1410 - 1.7964		
Dominant Model	P Value	P=0.7569		
	OR	0.9617		
	CI(95%)	0.7508 -1.2318		
Allele Frequency	P Value	P=0.0342		
	OR	1.1648		
	CI(95%)	1.0114 -1.3415	1.0114 -1.3415	

 Table 4. The Genotype and Allele Frequency of ACE I/D gene polymorphisms in RPL and control individuals from both populations.

Patient Group (n)		Population		
		Turkish	Belarussian	
		RPL Women (n:516)	RPL Women (n:760)	
Gene/SNP		ACE I/D	ACE I/D	
Genotypes (n/%)				
D/D		183/35.47	182/23.94	
D/I		249/48.26	381/50.14	
I/I		84/16.27	197/25.92	
Alleles				
D		615/0.596	745/0.490	
Ι		417/0.404	775/0.510	
Recessive Model	P Value	P=0.0001*		
	OR	1.7995		
	CI(95%)	1.3539 - 2.3918		
Dominant Model	P Value	P<0.0001*		
	OR	1.7453		
	CI(95%)	1.3653-2.2310		
Allel Frequencies	P Value	P<0.0001*		
	OR	1.5342		
	CI(95%)	1.3075-1.8003		

*: Significant

Biomed Genet Genomics, 2016 doi: 10.15761/BGG.1000117

2.11% for G/A and 0.0% for A/A) for Turkish and Belarussian groups respectively (Table 5). The A allele frequency was 0.066 for Turkish and 0.011 for the Belarusian groups. The FVLA/A (homozygous) genotype was found 0.36% and 0.00% in Turkish and Belarusian RPL women respectivelly. Mutated A allele frequency was lower in Belarusian patients and that difference was statistically significant (Table 5), (OR: 6.6061, CI: 3.8903-11.2177), p<0.0001. Statistically significant difference was also detected in FVR2 G/A SNP in the studied populations in the current results. The prevalence of genotypes of FVR2G/A in Turkish RPL group (94.82% for G/G 4.94% for G/A and 0.24% for A/A) and (98.29% for G/G 1.71% for G/A and 0.0% for A/A) in Belarusian group respectively (Table 6). The A allele frequency was 0.027 for Turkish and 0.009 for the Belarusian groups. The FVR2A/A (homozygous) genotype was found 0.24% and 0.00% in Turkish and Belarusian RPL women respectively. Mutated A allele frequency was lower in Belarusian patients and that difference was statistically significant (Table 6), (OR: 3.2301, CI: 1.7356-6.0112), p<0.0002.

Increased frequency of mutated Factor *FXIII*103G>T gene was detected in Belarusian RPL cohort in the current results. That difference was also statistically significant when comapred to Turkish RPL cohort. The prevalence of genotypes for *FXIII*103G>T in Turkish RPL group (59.89% for G/G, 38.37% for G/T and 1.74% for T/T) and (45.00% for G/G, 45.79% for G/T and 9.21% for T/T) in Belarusian group respectively (Table 7). The Leucine aminoasid frequency was 0.209 for Turkish and 0.321 for the Belarusian groups. The *FXIII*103G>T (homozygous) genotype was found 1.74% and 9.21% in Turkish and Belarusian RPL women respectively. Lecuine frequency was 5.29-fold increased in the Belarusian patients, difference was statistically significant (Table 7), (OR: 1.7864, CI: 1.4852-2.1486), p<0.0001.

The double and triple combined gene mutations profiles were also compared in the current RPL cohorts. Bar graphs in Figure 2 and 3 shows the frequencies of being dual and triple homozygous and/or heterozygous combined mutations for the target thrombophilic genes in RPL women from Turkish and Belarusian populations. *ACE+MTHFR*

 Table 5. The Genotype and Allele Frequency of FVL G/A gene polymorphisms in RPL women from both populations

Patient Group (n)		Population		
		Turkish	Belarussian	
		RPL Women	RPL Women	
		(n:830)	(n:760)	
Gene/SNP		FVLG/A	FVLG/A	
Genotypes (n/%)				
G/G		724/87.23	744/97.89	
G/A		103/12.41	16/2.11	
A/A		3/0.36	0/0.00	
Alleles				
G		1551/0.934	1504/0.989	
A		109/0.066	16/0.011	
Recessive Model	P Value	P=0.2185*		
	OR	6.4332		
	CI(95%)	0.3317 to 124.7553		
Dominant Model	P Value	P<0.0001*		
	OR	6.8080		
	CI(95%)	3.9851 -11.6307		
Allel Frequencies	P Value	P<0.0001*		
	OR	6.6061		
	CI(95%)	3.8903 -11.2177		

*: Significant

Table 6. The Genotype and Allele Frequency of *FVR2G/A* gene polymorphisms in RPL women from both populations.

		Population		
Patient Group (n)		Turkish	Belarussian	
		RPL Women	RPL Women	
		(n:830)	(n:760)	
Gene/SNP		FVR2G/A	FVR2G/A	
Genotypes (n/%)				
G/G		787/94.82	747/98.29	
G/A		41/4.94	13/1.71	
A/A		2/0.24	0/0.00	
Alleles				
G		1615/0.973	1507/0.991	
A		45/0.027	13/0.009	
	P Value	P=0.3256*		
Recessive Model	OR	4.5896		
	CI(95%)	0.2200 - 95.7567		
	P Value	P=0.0004*		
Dominant Model	OR	3.1396		
	CI(95%)	1.6748 - 5.8854		
Allel Frequencies	P Value	P=0.0002*		
	OR	3.2301		
	CI(95%)	1.7356- 6.0112		

*:Significant

Table 7. The Genotype and Allele Frequency of *F XIII 103G>T* gene polymorphisms in RPL women from both populations

		Population	
		Turkish	Belarussian
Patient Group (n)		RPL Women	RPL Women
		(n:516)	(n:760)
Gene/SNP		FXIII103G>T	FXIII103G>T
Genotypes (n/%)			
G/G		309/59.89	342/45.00
G/T		198/38.37	348/45.79
T/T		9/1.74	70/9.21
Alleles			
G		816/0.791	1032/0.679
Т		216/0.209	488/0.321
	P Value	P<0.0001*	
Recessive Model	OR	5.7150	
	CI(95%)	2.8281 - 11.5485	
	P Value	P<0.0001*	
Dominant Model	OR	1.8245	
	CI(95%)	1.4543 -2.2888	
	P Value	P<0.0001*	
Allel Frequencies	OR	1.7864	
	CI(95%)	1 4852 -2 1486	

*:Significant

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A1298C, *PAI-1+MTHFR*A1298C, *MTHFR*C677T+*MTHFR*A1298C, *FXIII+MTHFR*C677T and *FXIII+PAI-1* double combined mutations are common in both populations. The higest double combined mutations were detected in ACE+PAI-1 genes in both RPL cohort (Figure 2). The triple *FVR2+ACEI/D+FXIII* mutations were totaly the same in both populations (Figure 3). The higest triple combined mutations were detected in *FVL+MTHFR*A1298C+*PAI-1*, *FVL+MTHFR*A1298C+*ACE*, *FVR2+ ACE+PAI-1* and *FXIII+FVR2+PAI-1* genes respectivelly. The lowest triple combined mutations was detected in *FXIII+FVL* gene combinations (Figure 3).

Discussion

Two Caucasian populations were basically compared for thrombophila genes in RPL dilemma in the current results. Here is the first report for the thrombophilic gene mutations in South Slavic Belarusian population. The common polymorphic genes of FVLeiden, *FVR2, FXIII, MTHFR*(A1298C and C677T), *PAI-1*4G/5G and *ACEI/D* were compared for two populations. The presented retrospective results from peripheral blood-EDTA and buccal smear samples from Turkish and Belarussian RPL women showed the similar/strong association between RPL and thrombophilia.

The distribution of genotype frequencies of the studied polymorphisms between the current RPL women form both populations were in Hardy–Weinberg equilibrium and no statistically significant differences was found in terms of the age and gender. The studied *FVL*, *FVR2*, *MTHFR* mutations were frequently observed in Turkish cohort and *ACE*, PAI1 and *FXIII* were frequent mutations in Belarusian cohort.

Trombophilic factors may have an important role in influencing the pregnancy outcome. Incompatible literature findings showed the possible relation between RPL and thrombophilia genes. Some showed significant association [15-19] and some other showed no significance [20,21]. Pregnancy losses due to de novo chromosomal aneuploidies occur at similar frequencies in some populations. Some RPL couples have additional associated genetic factors such as thrombophilic mutated genes and some have non-genetic etiologies. Qiao et al. have claimed that the compound heterozygous deleterious mutations affecting DYNC2H1 and ALOX15 genes may contribute to the pathogenesis of RPL after whole exome sequencing in couples with pregnancy loss [22]. A single nucleotide polymorphism (SNP) in codons C677T and A1298C of the *MTHFR* gene causes a thermolabile enzyme with reduced function and defects in DNA hypomethylation.



Figure 3. Shows the frequencies of being triple homozygous and heterozygous combined mutations for some target genes in RPL women from Turkish and Belarussion populations. No significant difference was noted in thrombophilic gene mutations in the current Caucasian population cohorts.

Naushad et al have claimed that the *MTHFR* C677T polymorphism influences the RPL complications such as; hyperhomocysteinemia and its associated complications of RPL, NTDs, DVT, MI and POF [23,24].

The both germ-line T and C alleles frequencies of MTHFR 677 C>T and 1298 A>C SNPs have been shown to be a risk factors for the current cohort RPL women from Turkish and Belarusian populations. The mutated T allele frequencies were similar as 0.382 for the Turkish and 0.320 for the Belarusian RPL women cohorts but homozygosity is frequent in Turkish population, possibly because of high rate of consanguineus marriages. The MTHFR 1298 A> C SNP (homozygous) genotype was found 13.86% for Turkish and 10.78% for the Belarusian cohorts. Statistical significance was detected in C677T at recessive model and A1298C both recessive and dominant models. As claimed by Jeon et. al. the abberant PAI-1 gene expression may contribute to thrombosis and inflammation, leading to the RPL. The prevalence of genotypes for PAI-I gene were also similar but frequent at Belarusian women; the 4G allele frequency was 0.513 for the Turkish and 0.550 for the Belarusian cohort (p:0.03) and difference is more significant for recessive model (p=0.0019). Mutated I allele frequency in ACE I/D gene was different in the comparison of both populations and that difference was also statistically significant. Subrt et al. Were also reported the homozygous PAI-1 (-675) 4G/4G genotype that associated with increased risk of RPL [25].

The *FVL* mutation profiles from Southern Slavic population were reported by Clark *et al.* [26]. The current results also show the prevalence of genotypes of *FVL G/A* in Turkish and Belarusian RPL groups. The mutated A allele frequency in *FVL* gene was 0.066 for Turkish and 0.011 for the Belarusian groups in the current results. The *FVLA/A* (homozygous) genotype was found 0.36% and 0.00% in Turkish and Belarussian RPL women respectivelly. Mutated A allele frequency was different in the comparison of the both populations and that difference was statistically significant.

Two other thrombophilic factors of FVR2 and FXIII genes were also compared in the presented results. An association with RPL has also been reported for the codon 103G>T polymorphism of the FXIII gene by leading to fibrin degradation [27]. Statistically significant difference was also detected in FVR2 G/A SNP in the studied populations in the current results. The prevalence of genotypes of FVR2G/A in Turkish RPL group (94.82% for G/G 4.94% for G/A and 0.24% for A/A) and (98.29% for G/G 1.71% for G/A and 0.0% for A/A) in Belarusian group respectively (Table 6). The A allele frequency was 0.027 for Turkish and 0.009 for the Belarusian groups. The FVR2A/A (homozygous) genotype was found 0.24% and 0.00% in Turkish and Belarusian RPL women respectivelly. Mutated A allele frequency was different in the comparison of the both populations and that difference was statistically significant. Increased frequency of mutated Factor XIII103G>T was also detected in Belarusian RPL cohort in the current results. That difference was also statistically significant when comapred to the Turkish RPL cohort. The prevalence of genotypes for FXIII 103G>T in Turkish RPL group (59.89% for G/G, 38.37% for G/T and 1.74% for T/T) and (45.00% for G/G, 45.79% for G/T and 9.21% for T/T) in Belarusian group respectively (Table 7). The Leucine aminoasid frequency was 0.209 for Turkish and 0.321 for the Belarusian groups. The Factor XIII103G>T (homozygous) genotype was found 1.74% and 9.21% in Turkish and Belarussian RPL women respectivelly. Missense mutated Lecuine frequency was significantly frequent (5.29-fold increased) in the Belarusian cohort. The double and triple combined gene mutations profiles were also compared in the current RPL women from Turkish and Belarusian populations. The higest double combined

mutations were detected in ACE+PAI-1 genes in both RPL cohort. The triple FVR2+ACEI/D+FXIII mutations were totaly the same in both populations. The higest triple combined mutations were detected in FVL+MTHFRA1298C+PAI-1, FVL+MTHFRA1298C+ACE, FVR2+ ACE+PAI-1 and FXIII+ FVR2+PAI-1 genes respectivelly. The lowest triple combined mutations was detected in FXIII+FII+FVL gene combinations. There are lots of literature findings show mutation frequencies for the thrombophilic genes in health controls in Turkish population [28-30]. The current presented results from Turkish RPL women were remarkable different when compare to the health control individuals that has been published in some reports [28-30]. Current results are the first report on mutation frequencies for the thrombophilic genes in RPL women in Belarusian population. Preliminary ongoing results on health controls from the Belarusian group show remarkable difference (dato not shown) when compare to the presented RPL women from the same population. With an overview to the current study, the thrombophilic gene mutations have a combined risk effect on RPL. The karyotype analyses and other molecular genetic testing of the inherited thrombophilic genes in RPL women with two or more losses may help to understand of risk factors beyond the etiology and may direct interventions, prenatal diagnosis for the couples with pregnancy losses.

In conclusion, retrospectively evaluated results showed similar germ-line prothrombotic gene variations in RPL women in both populations. The *FVL* and *FVR2G/A* mutations frequencies were significantly less than Turkish RPL women when compare to the Belarus RPL women. Results also suggest that thrombophilic polymorphisms, either alone or in combination, are major determinants of the development of RPL in both studied Caucasian populations. Based on the current results, evaluation of multiple polymorphisms linked with inherited thrombophilia should be recommended in couples with two or more pregnancy losses.

Authors' contributions

F.S., IM and A.V.K; acquisition of data, performed the bioinformatical work, analyzed the clinical data and designed the clinical experiments, O.O., F.S, and A.G.; designed the experiments, performed PCR, analyzed the data, N.S., B.K. and A.G.; interpretation of data, performed the statistical analysis, O.O.; supervised the project, wrote and F.S., and I.M.; made the fine redaction of article

All authors read and approved the final manuscript.

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