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Association between the thrombophilic polymorphisms *MTHFR* C677T, Factor V Leiden, and *prothrombin* G20210A and recurrent miscarriage in Brazilian women

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ABSTRACT. Some cases of recurrent first trimester miscarriage have a thrombotic etiology. The aim of this study was to investigate the prevalence of the most common thrombophilic mutations - factor

V (*FV*) Leiden G1691A (*FVL*), prothrombin (*FII*) G20210A, and methylenetetrahydrofolate reductase (*MTHFR*) C677T - in women with recurrent miscarriages. In this case-control study, we included 137 women with two or more consecutive first-trimester miscarriages (£12 weeks of gestation) and 100 healthy women with no history of pregnancy loss, and with at least one living child. DNA was extracted from the patient samples, and the relevant genes (*FVL*, *FII*, and *MTHFR*) were amplified by PCR, followed by restriction fragment length polymorphism, to assess the polymorphisms in these genes. The allelic frequencies of polymorphisms were not significantly different between the case and control groups. Polymorphisms in the *MTHFR*, *FVL*, and *FII* genes were not associated with recurrent miscarriage during the first trimester of pregnancy in Brazilian women (P = 0.479; P = 0.491 and P = 0.107, respectively). However, the etiologic identification of genetic factors is important for genetic counseling.

Key words: Recurrent miscarriage; First trimester miscarriage; Factor V Leiden; Methylenetetrahydrofolate reductase C677T; *FII* G20210A

INTRODUCTION

Recurrent miscarriage (RM) is a heterogeneous disorder, affecting women of reproductive age: an estimated 5% of all women of reproductive age undergo two consecutive miscarriages; whereas 1% are subject to 3 or more consecutive miscarriages (Nair et al., 2012). RM has been attributed to several factors, including genetic, infective, anatomical, endocrine, and immune factors; however, over 50% of RM cases remain unexplained (Hatasaka, 1994; Nair et al., 2012).

Recent studies have identified thrombophilia as a possible cause of RM. The FV Leiden (*FVL*) and G20210A mutations in the *FV* and *FII* (prothrombin) genes are believed to lead to enhanced blood coagulation, while mutations in the methylene tetrahydrofolate reductase (*MTHFR*) gene results in an elevation in the homocysteine levels; both sets of mutations have been identified as risk factors for thrombosis (Cao et al., 2013; Creus et al., 2013; Cao et al., 2014). Thrombophilia is a major cause of spontaneous loss of the fetus during the early stages of pregnancy, and is associated with complications such as preeclampsia, intrauterine growth restriction, placental abruption, and stillbirth, during the late stages of pregnancy (Frosst et al., 1995; Kovalevsky et al., 2004; Kujovich, 2011). The aim of this study was to assess the association between the *FVL*, *FII* G20210A, and *MTHFR* C677T variants and idiopathic recurrent miscarriage.

MATERIAL AND METHODS

Study subjects

This case-control study was designed to investigate the association between idiopathic recurrent miscarriages and three thrombophilic mutations: *MTHFR* C677T, *FVL*, and *FII*

G20210A. The case group was comprised 137 women with an obstetrical history of two or more consecutive first-trimester abortions (£12 weeks gestation). Inclusion and exclusion criteria were consistent with those defined in a previous study conducted by our group (Gonçalves et al., 2014). The control group consisted of 100 healthy women with no history of pregnancy loss, with at least one living child, and ≤40 years of age. Signed informed consent forms were obtained from all patients and subjects prior to the study.

Ethical standards

This study was approved by the Research Ethics Committee of the Maternity Climério of Oliveira, under resolution 010/2010. The protocol and procedures were in accordance with the ethical standards of the committee on human subjects and the Helsinki Declaration of 1964 (as revised in 2008).

Thrombophilic mutation and DNA extraction

Genetic testing for three thrombophilic mutations - *MTHFR* C677T, *FVL*, and *FII* G20210A - was performed on genomic DNA extracted from leukocytes isolated from whole blood samples obtained from all patients and controls, using a standard kit (Qiagen, Venlo, Netherlands).

PCR amplification was performed using a standard protocol, with the following primer sequences: *MTHFR* C677T: F-5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and R-5'-AGG ACG GTG CGG TGA GAG TG-3'; *FVL*: F-5'-TGC CCA AGT GCT TAA CAA GAC CA-3' and R-5'-CTT GAA GGA AAT GCC CCA TTA-3'; *FII* G20210A: F-5'-TCT AGA AAC AGT TGC CTG GC-3' and R-5'-ATA GCA CTG GGA GCA TTG AAG C-3'. The PCR products of *MTHFR* C677T, *FVL*, and *FII* G20210A were digested overnight at 37°C with the restriction endonucleases *Hin*fI, *Mnl*I, and *Hin*dIII (New England BioLabs, Ipswich, MA, USA), respectively. The digested DNA fragments were separated by electrophoresis in a 7% polyacrylamide gel stained with SyBR Green (Molecular Probes, Inc., Madison, WI, USA). The bands were then examined under a UV light.

Statistical analysis

Statistical analyses were performed using the SPSS 20.0 software package (IBM, Armonk, NY, USA). Parametric variables were compared by the Student *t*-test and nonparametric variables were analyzed by the Mann-Whitney tests. The correlations between variable pairs were determined using the Pearson and Spearman tests. Differences with P values <0.05 were considered to be statistically significant.

RESULTS

The patients had undergone 2-7 abortions (mean: 2.8 ± 1.0), with 47.6 and 37.7% of all included women having undergone two and three abortions, respectively. Epidemiological data was obtained from 46 of the 137 patients. Regular coffee and alcohol (consumption once or twice every month) consumption was recorded by 76% (35/46) and 54.3% (25/46) of the patients and 86.4% (83/96) and 43.7% (42/96) of the control subjects. However, a greater

number of control subjects [11.4% (11/96)] smoked (1 or 2 cigarettes every day), compared to the patients [4.3% (2/46)]. However, these differences between patients and controls were not statistically significant (Table 1).

Table 1. Epidemiological data of women with recurrent miscarriage and control subjects.

Data	Cases	Controls	P value
Age (mean)	32.1	25.8	<0.001
Mean number of abortions	2.8	1.8*	
Smoking status	11.4%	4.3%	>0.05
Alcohol consumption status	54.3%	43.7%	>0.05
Coffee	76.0%	86.4%	>0.05
Mean number of meals/day	4.0	4.2	>0.05
Thrombosis	0	0	
Use of medication (Yes)	4.3%	3%	>0.05
College	30.4%	9%	>0.05
High School	69.6	91%	>0.05
Caucasian**	17.3%	16%	>0.05
Black**	11.0%	27.0%	>0.05
African descent**	71.7%	57.0%	>0.05

*Live children, **according to Krieger et al. (1965).

The genotype and allele frequencies of *MTHFR* C677T, *FVL*, and *FII* G20210A were similar between the cases and controls (Table 2). In addition, we observed no differences between the number of abortions and the gene variants (data not shown). In order to evaluate the distribution of these variants in the different age groups, women with recurrent miscarriage were categorized into two age groups: £30 years and ³31 years. The frequency of the mutant genotype of the *MTHFR* C677T, *FVL*, and *FII* G20210A polymorphisms was higher in the women aged over 31 years (27.3, 3.0, and 2.2%, respectively). However, we observed no significant differences (P = 0.62, 0.48, and 0.30, respectively) between these frequencies and the mutant genotype frequencies in women aged £30 years (14.7, 0, and 0.8%, respectively).

Table 2. Allelic and genotypic frequencies of the *MTHFR* C677T, *FVL*, and *FII* G20210A polymorphisms in RM patients and control subjects, determined using 1000 Genomes and HapMap.

Mutation	Case [N (%)]		Control [N (%)]		P	Allele frequency 1000 Genomes*		Genome frequency HapMap	
						EUR	AFR	EUR	AFR
MTHFR	CC	80 (58.4)	CC	59 (59)	>0.05	35%	11%	31%	11%
	CT	51 (37.2)	CT	37 (37)					
	TT	6 (4.4)	TT	4 (4)					
	T	63 (23)	T	45 (22.5)					
FVL	GG	132 (97)	GG	98 (98)	>0.05	1%	0%	2%	NA
	GA	4 (2.9)	GA	2 (2)					
	AA	0 (-)	AA	0 -					
	A	4 (1.5)	A	2 (1)					
FII	GG	134 (97)	GG	100 (100)	>0.05	1%	0%	1.5%	1.6%
	GA	4 (2.9)	GA	0 (-)					
	AA	0 (-)	AA	0 (-)					
	A	4 (1.4)	A	0 (-)					

*Data from European/Euro-descendants and African/Afro-descendants. NA = not available.

Women with more than three abortions showed a higher frequency of *MTHFR* C677T and *FVL* mutations compared to those with less than three abortions (57.2 and 9.5% *vs* 39.1

and 1.8%, respectively). Alternately, women with less than three abortions showed a higher frequency of the *FII* G20210A mutation (3.5%) compared to women with more than three abortions (0%). However, we observed no significant association between the frequency of mutant genotypes of the *MTHFR*, *FVL*, and *FII* G20210A polymorphisms and the number of abortions (£3 or >3) (P = 0.098, 0.504, and 0.111, respectively).

DISCUSSION

We found no difference in the frequency of genotypic and allelic variants of *MTHFR* C677T, *FVL*, and *FII* G20210A between women with RM and controls. Previous studies evaluating these polymorphisms in RM have shown contradicting results. This could be attributed to the small sample size, which is inadequate for evaluating the *FVL* and *FII* mutations. Dutra et al. (2014), Baumann et al. (2013), and Serrano et al. (2011) have reported results similar to ours. However, the results reported by Govindaiah et al. (2009) and Settin et al. (2011) were contradictory to those reported herein. The study quality may have been affected by differences in the methodological aspects, such as the inclusion of participants with other potential underlying causes of RM or the lack of stratification based on the ethnicity and gestational age of loss of patients (Vettriselvi et al., 2008; Ayadurai et al., 2009). RM is a multifactorial entity; therefore, the variations in the strength of the association between various polymorphisms and RM seen in different studies may be indicative of additional risk factors (Jivraj et al., 2006; Hussein et al., 2010). Therefore, we attempted to diminish these potential biases in this study by selectively including patients with RM that was unexplained during the first trimester.

The gestational age of pregnancy loss may also influence the strength of this association. Miscarriage is a clinical condition that covers a period extending from the biochemical identification of pregnancy up to the 22nd week of pregnancy, and can be attributed to several biological mechanisms (Mierla et al., 2012). Considering this, several researchers have attempted to analyze the impact of hereditary thrombophilia on each trimester of pregnancy. Their research has indicated a high prevalence of *FVL* in women with recurrent loss, especially in the second trimester of pregnancy; additionally, *FII* G20210A has been identified as a risk factor for recurrent loss in the first trimester (Martinelli et al., 2000; Kujovich, 2011).

We observed no association between the maternal age and the studied variants, which was in agreement with the results obtained by Govindaiah et al. (2009). Males usually present higher mutation rates because of elevated rates of male germ cell division. However, somatic mutations occur with approximately equal frequency both two sexes, and are not significantly impacted by the age of the patients (Crow, 2006).

We also observed no association between the number of abortions and the *MTHFR* C677T, *FVL* G1691A, and *FII* G20210A mutations, which was in agreement with the results reported by Jaslow et al. (2010), who found equal frequencies of the *MTHFR* C677T, *FVL*, and *FII* G20210A polymorphisms in 1020 women from Tennessee with two, three, or four or more recurrent pregnancy losses.

The association between the *FVL* and *FII* G20210A polymorphism and recurrent miscarriages remains a controversial topic. Factors such as the sample size and ethnicity are important, as the rate of these mutations could differ in various populations. People of European descent express a high frequency of heterozygous genotypes (5-7%), while these heterozygotes are almost absent (<1.0%) in Asians and among African descendants (Cleary-

Goldman et al., 2003; Parveen et al., 2013). These results are reinforced by data obtained from 1000 Genomes and HapMap (Table 2). In Brazil, the two mutations are present in about 2% of the population with European ancestry (Rosendaal et al., 1998).

The frequency of the *MTHFR* C677T mutation has high geographic and ethnic variability worldwide. The prevalence of the homozygous mutant genotype (677TT) in Brazil ranges from 2.7 to 17.5%. In contrast, the prevalence of this genotype in people of African descent in the United States and South America, and in Hispanic Americans and Colombians, is about 1 and 20%, respectively (Sharp and Little, 2004; Ferreira-Fernandes et al., 2013). These results agree with data obtained from 1000 Genomes and HapMap (Table 2).

Our results showed the increased prevalence of *MTHFR* C677T, *FVL* G1691A, and *FII* G20210A polymorphisms in women with RM. However, its low frequency in the Brazilian population and the small sample size (because of the selection of patients with recurrent abortions during the first trimester) does not allow for the establishment of an association between recurrent miscarriage and the thrombophilia-related variants. Considering the low frequency of the mutated alleles of the *FII* and *FVL* mutations in the Brazilian population (82.7% African descent, Table 1), at least 262 cases and 262 controls would be required to detect an OR > 3 for polymorphisms in *MTHFR*, and at least 804 cases and 804 controls would be needed to detect an OR >3 for the *FII* and *FVL* mutations, according to sample size estimation.

This is the first study conducted in Salvador, Brazil, that has analyzed abortion at £12 weeks of gestation. The impact of thrombophilic mutations on RM remains a controversial issue (Serrano et al., 2011). The results obtained in this study are in accordance with the results of previous research, and indicate that the *MTHFR* C677T, *FVL*, and *FII* G20210A polymorphisms are not associated with recurrent miscarriage during the first trimester of gestation in the Brazilian population. Additional, larger-scale studies are required to clarify the association between these variants and RM, and their role in this condition.

Conflicts of interest

The authors declare no conflict of interest.

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