ORIGINAL ARTICLE

ASSOCIATION BETWEEN INHERITED THROMBOPHILIA IN PREGNANCY AND MICRONUCLEUS FREQUENCY IN PERIPHERAL BLOOD LYMPHOCYTES

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ABSTRACT

The aim of this study was to determine possible predictors of an increased frequency of micronucleus (MN) and the impact of thrombophilia on the chromosomal instability in peripheral blood lymphocytes (PBL) of pregnant women in their first trimester. This study was designed as a case-control study on 74 pregnant women. It was performed in the gestational age of 11 to 14 weeks, when blood samples were collected and incubated for 72 hours. The individual MN frequency in PBL was measured by cytokinesis-block micronucleus (CBMN) assay. Women were grouped in control group [≤4 MN/1000 binucleated (BN) cells] and case group (>4 MN/1000 BN cells). Potential mutagenic effects of exogenous/endogenous factors in pregnant women were analyzed. By analyzing the given results, it can be concluded that pregnant women with thrombophilia have 26.69-times more chance of having a frequency of >4 MN/1000 BN than pregnant women with no thrombophilia. Our research was primarily aimed at showing that the presence of thrombophilia was a statistically important predictor of an increased MN frequency in pregnant women and it can predict about one-third of the total variance in MN frequency in the studied population.

Keywords: Lymphocytes; Micronuclei; Pregnant women; Thrombophilia.

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INTRODUCTION

During their lifetime, humans are exposed to exogenous and endogenous agents who may react with cellular biomolecules, especially with the DNA molecule and induce changes in the genetic material that can lead to genomic instability [1]. Micronucleus (MN) is a cytoplasmic small nucleus containing particles of chromatin material that is not incorporated into the nucleus of one of the daughter cells during division. Under the influence of clastogenic and aneugenic agents, micronuclei (MNi) are produced and they may come from acentric chromatid/ chromosome fragments or whole chromatids/chromosomes [1,2]. In health biomonitoring, cytokinesis-block MN (CBMN) assay is used to measure basal and induced chromosomal damage in the peripheral blood lymphocytes (PBL) [2-3] and MN frequency represents a quantitative indicator of structural and/or numerical chromosomal aberrations [4-6].

The basal (spontaneous) MN frequency in PBL is a measure of accumulated chromosomal aberrations occurring during the lifetime of circulating lymphocytes [7]. The level of basal MN frequency is determined by the contribution of genetic damage to the DNA that comes from environmental factors and endogenous factors (genetic and non genetic determinants) and the elimination of DNA damage (that is determined by individual variations in genes involved in DNA repair) [8]. The induced MN frequency is the result of exposure to possible different chemical, physical and biological mutagens and carcinogens of natural or artificial origin [2-4]. Gender, age, body weight, diet, life habits, presence of inflammation, ionizing radiation and the use of certain medicines, affect the increase of the MN frequency [9,10]. Higher MN frequency in PBL is seen in persons with carcinoma [11], neurodegenerative diseases [12], cardiovascular diseases [13,14], autoimmune diseases [15], diabetes mellitus type

2 (T2DM) [16] and in persons with Down syndrome and their parents [17]. For example, elevated MN frequency is positively correlated with the occurrence and the severity of coronary artery disease (CAD) [16,18]. It has been reported that the TT genotype in the methylenetetrahydrofolate reductase (MTHFR) *C677T* gene variant is significantly associated with the higher MN in CAD patients [5]. Genomic stability is essential for normal fetal growth and development [19] and previous studies have indicated the potential role of elevated MN in predicting pregnancy complications in humans [20].

Thrombophilia presents a hereditary and acquired hemostatic system disorder in which there is a tendency towards thrombosis. During pregnancy, hypercoagulability and hypofibrinolysis are present, which, together with inherited and acquired thrombophilia conditions, can lead to pregnancy complications [21]. A previous study has shown the effectiveness of the treatment with low-molecular-weight heparin (LMWH) on pregnancy outcomes in women with thrombophilia [22].

There have been no clinical studies conducted to assess the mutagenic potential of thrombophilia and complications associated with this condition during pregnancy. The aim of this study was to evaluate known risk factors, to determine possible predictors of an increased frequency of MN in PBL and the impact of thrombophilia on the chromosomal instability in pregnant women in the first trimester.

MATERIALS AND METHODS

Experimental Design and Subjects. The study was designed as a case-control study involving pregnant women admitted to the Department of Obstetrics and Gynecology of our Clinic in 2015. Having been informed, the pregnant women signed the agreement to take part in the study approved by the local Ethics Council (No. 01-12294) and filled in the questionnaire containing basic medical history necessary for research in the field of cytogenetics as well as for evaluations of exposure history. The study included 74 pregnant women of gestational age 11 to 14 weeks. The excluding criteria were the following: exposure to environmental and professional mutagens, exposure to X-ray medical procedures, using oral hormonal contraceptives in the previous year, the presence of other chronic diseases (except thrombophilia), the intake of antibiotics and anti-epileptics during pregnancy and using narcotics. Blood samples from the pregnant women with thrombophilia were taken before starting anticoagulant therapy with LMWH. The pregnant women were grouped

according to the value of the MN in the control group [≤4MN/ 1000 binucleated (BN) cells] and the group of cases (>4MN/ 1000BN cells).

Blood samples were drawn following the usual procedure and they were kept refrigerated for 24 hours. All the heparinized blood (0.5 mL) was cultured in duplicate in 5 mL complete medium (Gibco® PB-MAX™ Karyotyping Medium; Invitrogen, Carlsbad, CA, USA) and incubated at 37 °C.

Cytokinesis-Block Micronucleus Assay. The CBMN assay was performed 44 hours after the cultivation began by adding Cytohalazin B™ (Sigma-Aldrich, St. Louis, MO, USA) to the cultures in final concentration of 4 µg/ mL. After continual incubation of cell cultures for an additional 28 hours, the cells were treated with cold (4 °C) hypotonic (0.56% KC1) solution and fixed three times with fresh Carnoy's fixative composed of methanol and glacial acetic acid (ZORKA Pharma-HEMIJA d.o.o, Šabac, Serbia) in a ratio of 3:1. The cell material was dripped onto dry and cold microscope slides and the dried slides were colored by 2.0% Giemsa stain solution (BioGnost® d.o.o, Zagreb, Croatia). Micronucleus frequencies were determined by scoring 1000 BN cells per person, according to the criteria previously defined by Fenech *et al.* [23].

Statistical Data Processing. The entire statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 22.0 for Windows software (IBM, Armonk, NY, USA). The results are shown in the tables. To present the results of the categorical variables, absolute values and their percentage distribution were used. The experimental results of continuous numerical values were presented as mean ± standard deviation (SD) values. The χ^2 test was applied for determining the differences in the frequency of categorical variables. The significance of the differences between the means of studied variables was tested by the Mann-Whitney and Kruskal-Wallis tests. Binary logistic regression analysis was applied to identify risk factors and to assess the impact of independent variables on the case and control groups. The results are presented as odds ratio (OR) with 95% confidence interval (95% CI) and the *p* value. Univariate analysis was applied to all the parameters, while multivariate analysis was used for statistically significant parameters. A *p* value of <0.05 was considered significant.

RESULTS

The examined women included in the study ($n = 74$) were 21 to 39 years old, median 30 years old. The average age of women included in this study was 29.93 ± 4.51. Within this group, the first trimester combined aneuploidy screening test was applied to 62 women below the age

of 35. Pregnancy associated plasma protein-A (PAPP-A), free β human chorionic gonadotropin (fbHCG) and fetal nuchal translucency (NT) were compared between healthy pregnant women ($n = 33$) and pregnant women with thrombophilia ($n = 29$). The PAPP-A (mLU/L) values were significantly higher ($p = 0.028$), whereas fbHCG (IU/L) levels were lower and fetal NT (mm) measurements were higher in women with thrombophilia, but these differences were not statistically significant.

The average MN frequency in the examined population of pregnant women was 6.09 ± 4.78 MN/1000BN. The obtained median of 4.50 MN/1000BN for the whole population was used as a limit for forming examination case and control groups ($\leq$4MN/1000BN cells and >4MN/1000 BN cells). In this model, we investigated the influence of the predictor variables on the outcome.

The average age in the control group was not significantly different from the one in the case group (29.49 ± 4.58 *vs.* 30.38 ± 4.46; $t = 0.848$, $p = 0.399$). In the case group, there were more cases of thrombophilia ($p = 0.000$) and miscarriages ($p = 0.002$), an increased number of miscarriages ($p = 0.010$) and of pregnancies ($p = 0.023$) (Table 1).

Table 2 shows the exogenous risk factors and risk factors related to family anamnesis. It can be observed that most of the pregnant women did not smoke (81.1%), or use alcohol (90.5%) during pregnancy. Pregnant women who consumed alcohol did it irregularly. It was determined that most of them did not have relatives with malignant diseases, venous thromboembolism and CADs or T2DM (Table 2). In the group of pregnant women with frequency >4MN/1000BN, there were more women who used alcohol compared to the control group (18.9 *vs.* 0.0%; $p = 0.017$). In the group with higher MN frequency, we also noticed statistically significant increased alcohol consumption compared to the control group with lower MN frequency ($p = 0.021$) (Table 2).

The prediction of all the studied risk factors in our model for the percentage of pregnant women with frequency >4MN/1000BN was determined by univariate binary logistic regression analysis (Table 3). Using the goodness-of-fit test, it showed how well our model (a set of predictor variables shown in Tables 1 and 2) predicted results. It was shown that with a given set of predictor variables our model anticipated the results of the univariate binary logistic regression analysis (p <0.005, with an

Table 1. Examined risk factors related to personal anamnesis (age, thrombophilia, previous miscarriages and body mass index.)

Examined Risk Factors		Total (%)	Control ≤4MN/1000BN		Cases >4MN/1000BN		χ^2 (*p* value)
			n	%	*n*	%	
Thrombophilia presence	no	50.0	31	83.8	6	16.2	0.000[a]
	yes	50.0	6	16.2	31	83.8	
Year categories	20-29	50.0	19	51.4	18	48.6	1.000
	30-39	50.0	18	48.6	19	51.4	
Previous miscarriages	no	51.4	26	70.3	12	32.4	0.002[a]
	yes	48.6	11	29.7	25	67.6	
Miscarriage categories	0+1	86.5	34	91.9	30	81.1	0.308
	≥2	13.5	3	8.1	7	18.9	
Number of miscarriages	0	51.4	26	70.3	12	32.4	
	1	33.8	8	21.6	17	45.9	
	2	12.2	3	8.1	6	16.2	0.010[a]
	≥3	2.7	0	0.0	2	5.4	
FMU	no	94.6	37	100.0	33	89.2	0.123
	yes	5.4	0	0.0	4	10.8	
Number of FMU	0	94.6	37	100.0	33	89.2	0.123
	1	5.4	0	0.0	4	10.8	
Pregnancy order	1	31.1	17	45.9	6	16.2	
	2	29.7	10	21.6	12	32.4	
	3	25.7	8	5.4	11	29.7	
	≥4	13.5	2	20.0	8	21.6	0.023[a]
BMI	<24.99 kg/m²	74.3	29	78.4	26	70.3	0.595
	≥24.99 kg/m²	25.7	8	8 (21.6)	11	11 (29.7)	

FMU: *fetus mortus in utero*; BMI: body mass index.

[a] Statistically significant (p <0.05).

Table 2. Examined risk factors related to exogenous agents exposure during the present pregnancy as well as a potential risk factors in the families.

Examined Risk Factors		Total (%)	Control ≤4MN/1000BN		Cases >4MN/1000BN		χ^2 (*p* value)
			n	%	*n*	%	
Smoking	no	81.1	33	89.2	27	73.0	0.138
	yes	18.9	4	10.8	10	27.0	
Number of cigarettes	0	81.1	33	89.2	27	73.0	
	1-9	16.2	4	10.8	8	21.6	0.140
	10-19	2.7	0	0.0	2	5.4	
Alcohol use	no	90.5	37	100.0	30	81.1	0.017[a]
	yes	9.5	0	0.0	7	18.9	
Amount of alcohol	0 mL	90.5	37	100.0	30	81.1	
	1-100 mL	8.1	0	0.0	6	16.2	0.021[a]
	>100 mL	1.4	0	0.0	1	2.7	
Carcinoma in family	no	79.7	32	86.5	27	73.0	0.247
	yes	20.3	5	13.5	10	10.0	
CAD in family	no	74.3	27	73.0	28	75.7	1.000
	yes	25.7	10	27.0	9	24.3	
Thrombosis in family	no	79.7	32	86.5	27	73.0	0.247
	yes	20.3	5	13.5	10	27.0	
T2DM in family	no	87.8	33	89.2	32	86.5	1.000
	yes	12.2	4	10.8	5	13.5	

CAD: coronary artery disease; T2DM: diabetes mellitus type 2.

[a] Statistically significant (*p* <0.05).

indicator χ^2 from 64,356,589 for 19 degrees of freedom). The Hosmer and Lemeshow test supported the claim that the model was good (*p* = 0.980>0.05). The given set of variables in our models explained between 58.1% (Cox & Snell R Square) and 77.5% (Nagelkerke R Square) of variance in MN frequency.

Significant risk factors in the univariate model were thrombophilia (*p* = 0.000), miscarriages (*p* = 0.002), the number of miscarriages (*p* = 0.004) and the number of pregnancies (*p* = 0.005). The significant risk factors from the univariate model were included in the multivariate logistic regression analysis, which showed that hereditary or combined thrombophilia during pregnancy (OR = 76.06; 95% CI = 7.97-724.39; *p* = 0.000) had a significant partial influence on the frequency of >4MN/1000BN occurrence (Table 3).

In the examined group, the pregnant women who consumed alcohol had frequencies of >4MN/1000BN. The analysis showed that there was a lack of statistically significant difference in the mean values of the MN frequency between the groups of women who did not use alcohol and those who consumed alcohol during pregnancy (5.87 ± 4.18 *vs.* 7.36 ± 7.50; *p* = 0.939). There was also no statistically significant difference between the mean MN frequency in the groups concerning an increased intake of alcohol (*p* = 0.246). These results are consistent with the univariate analysis in which alcohol consumption and an increased alcohol intake do not represent a significant risk factor for the increased frequency of MN.

By analyzing the given results it can be concluded that pregnant women with thrombophilia are 26.69-times more likely to have the frequency of >4MN/1000BN than pregnant women without thrombophilia. Pregnant women who had previous miscarriages or recurrent miscarriages and a higher number of pregnancies, are 4.92, 2.85 and 2.05 more likely to have the frequency of >4MN/1000BN than pregnant women with no present risk factors (Table 3).

DISCUSSION

Micronucleus presence is an indicator of genomic instability and accumulated damages that appeared during the lymphocyte's life cycle and can be detected *in vitro* [3]. Every process that affects the DNA damage and/or chromosomes and spindle apparatus directly or indirectly increases the MN frequency. The most important processes are oxidative processes in the cell [2], gene polymorphisms and mutations that affect genomic instability [3].

Micronuclei are present in humans, as well as spontaneously formed in the range of 0-12 MN/1000BN cells [7]. In our study, the individual variation of the MN frequencies in the group of pregnant women was within a wide range of MN frequency variations (1-25 MN/1000BN). Wide varia-

Table 3. Univariate and multivariate logistic regression analyses of examined factors influence on frequency presence >4MN/1000BN in pregnant women.

Examined Risk Factors	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	*p* Value	OR (95% CI)	*p* Value
Thrombophilia	26.694 (7.754-91.901)	0.000[a]	76.058 (7.986-724.391)	0.000[a]
Age	1.046 (0.944-1.159)	0.394	–	–
Year categories	1.114 (0.448-2.773)	0.816	–	–
Previous miscarriages	4.924 (1.838-13.190)	0.002[a]	0.256 (0.014-4600)	0.355
Miscarriage categories	2.644 (0.627-11.149)	0.184	–	–
Number of miscarriages	2.852 (1.400-5.815)	0.004[a]	0.486 (0.080-2.941)	0.432
FMU	1811289975.000 (0.00-/)	0.999	–	–
Number of FMU	1811289975.000 (0.00-/)	0.999	–	–
Pregnancy order	2.049 (1.244-3.376)	0.005[a]	2.355 (0.792-7.008)	0.124
BMI	1.534 (0.535-4.398)	0.426	–	–
Smoking	3.056 (0.861-10.839)	0.084	–	–
Number of cigarettes per day	2.999 (0.940-9.569)	0.063	–	–
Alcohol	1,239 (0.343-4.480)	0.744	–	–
Alcohol amount	79899842.300 (0.00-/)	0.998	–	–
Relatives with carcinoma	2.370 (0.722-7.787)	0.155	–	–
Relatives with CAD	0.868 (0.305-2.466)	0.790	–	–
Relatives with thrombosis	0.422 (0.128-1.386)	0.155	–	–
Relatives with diabetes	1.289 (0.317-5.237)	0.723	–	–

OR: odds ratio; 95% CI: 95% confidence interval; FMU: *fetus mortus in utero*; BMI: body mass index; CAD: coronary artery disease.

[a] Statistically significant.

tions of the MN frequency in the same group may be the result of different factors that influence chromosomal damage.

Kopjar *et al.* [3] reported mean frequency of 6.9 ± 3.32 MN/1000BN cells in their study performed on healthy male and female subjects aged 20 to 61 in Croatia. The authors concluded that due to the differences in the organization of the research and the size of the population examined, the mean MN frequency was often higher or lower than the one determined in their research [3]. Our research has shown that the mean MN frequency in the studied population of pregnant women aged 20 to 33 is 6.09 ± 4.78 MN/1000BN cells.

The results of the previous studies have shown that chromosomal aberrations could be found in the karyotype of 3.0-6.0% of couples with recurrent miscarriages [24]. Fenech [20] wrote about MN frequency increase as one of the factors that could be connected with recurrent miscarriages, and Furness *et al.* [19] showed that in the high risk group [patients who had recurrent pregnancy loss (RPL), preeclamsia/eclampsia, intrauterine growth retardation (IUGR), placental abruption or preterm delivery] there was a statistically higher MN frequency comparing to low risk group. Toljic *et al.* [25] showed that in gestational diabetes

(GDM) and pregnancy-induced hypertension (PIH), there were elevated levels of oxidative stress, as well as damage to DNA and chromosomal aberration, which could be detected owing to elevated values of MNi in PBL of pregnant women.

Upon the performed examination, we deduced that the case group (>4MN/1000BN) contained more statistically significant previous miscarriages and a higher number of miscarriages. The results presented in this study show that previous miscarriages and number of miscarriages are separate independent variables and represent significant predictors in the case group (>4MN/1000BN), while the multivariate analysis allocated only thrombophilia as an important predictor of an increased MN frequency in the case group (>4MN/1000BN).

Studies analyzing the effects of smoking on MN frequency have shown contradictory findings. Studies performed by Kopjar *et al.* [3] as well as the one conducted by Nefic *et al.* [26] showed that smokers have statistically significant higher MN frequency than non smokers. In most of the research studies, the link between smoking and MN frequency was not found. Fenech *et al.* [27] concluded that radical MN frequency increase can be found only in

smokers who smoke more than 30 cigarettes a day and who are not professionally exposed to genotoxic agents. In our study, the maximal number of cigarettes was between 1 and 9 and this study was in accordance with some of the previous findings that showed no link between smoking status and an increased MN frequency.

Alcohol is a proven teratogenic agent that influences normal embryo and fetal development and it is implicated in the pathogenesis of the fetal alcohol syndrome. It is not known if any amount of alcohol is safe in pregnancy, but there are speculations that even a small amount of alcohol may harm the fetus. It is well-known that ethanol can easily cross the feto-placental barrier in both directions and concentrations in fetal and maternal circulation thus equalize [28]. Studies on animal models have shown that ethanol may cause disorders on epigenetic level and that it may also disturb the coordinated process of cellular differentiation [29].

Alcohol toxicity can be seen not only as a direct effect of the ethanol, but also as an indirect effect through its metabolic products and reactive oxygen species (ROS) that appear during alcohol biotransformation [30]. Studies on animal models have shown that alcohol may induce higher ROS production, cause oxidative stress and react with proteins, lipid and DNA causing their damage or complete degradation [31]. Different studies have confirmed the genotoxic effect of ethanol by various cytogenetic and molecular tests [32,33]. In the study by Santovito *et al.* [34], significant differences in MN frequency between alcoholics and controls were not found, but they did find significant differences in frequency of sister chromatid exchanges (SCEs) and chromosome aberrations (CAs). The results of the studies by Benassi-Evans and Fenech [35] support the hypothesis that chronic exposure to alcohol induces formation of MN in two human B lymphoblastoid cell lines: WIL2-NS and GM13705.

We have shown that in the group of pregnant women with frequency of >4 MN/1000BN there were significantly more women who consumed alcohol and the amount of alcohol consumed by the pregnant women was higher, but we did not find that these variables represented significant predictors of an increased MN frequency in the group of cases (>4MN/1000BN). A statistically significant difference in the mean values of the MN frequency in women who consumed and did not consume alcohol during pregnancy was not found. This can be explained by the fact that the minority of women in our study consumed alcohol (<10.0%) and no pregnant women chronically consumed alcohol.

During pregnancy hypercoagulability and hypofibrinolysis are present and together with inherited and acquired thrombophilia disorders they can lead to early complications of pregnancy (RPL) and late complications of pregnancy [PE, IUGR, placental abruption, premature birth and intrauterine fetal death (IUFD)] [24]. Prothrombophilic genetic variants Factor V Leiden and Prothrombin A20210G were significantly associated with a higher prevalence of RPL [24,36]. Šošić *et al.* [37] found that in the group of women with inherited thrombophilia from Šumadija District, Serbia, previous miscarriages were most commonly noticed in double owners of gene variants plasminogen activator inhibitor-1 (PAI-1) 5G/4G and MTHFR, C677T.

Karsli *et al.* [38] found that there was an impact of inherited thrombophilia on the first trimester combined aneuploidy screening test parameters. They determined that PAPP-A levels were significantly higher, whereas fbHCG levels and fetal NT measurements were lower in women with inherited thrombophilia. In a similar study, carried out by Cıkman *et al.* [39], the effect of inherited thrombophilia on second trimester combined aneuploidy screening test markers was investigated. They found that unconjugated estriol multiple of median (MoM) levels were significantly lower in women with inherited thrombophilia [39]. In our study, the results of the first trimester combined aneuploidy screening test were consistent with previous research of the PAPP-A levels.

Micronucleus forming in human cells is connected to many medical conditions. Pristov *et al.* [21] came up with the result that in pregnant women with thrombophilia, placental tissue is exposed to H_2O_2-mediated oxidative stress, which can originate from the mother's blood and the endothelium. The occurrence of H_2O_2 can be initiated by prothrombotic state present in mother, indicating a potential relation between a pregnancy complicated with thrombophilia and oxidative stress [21].

In our study, thrombophilia in pregnancy has a significant partial contribution to the occurrence of frequencies >4MN/1000BN. Pregnant women with thrombophilia are 26.69-times more likely to have a frequency of >4MN/1000BN compared to pregnant women without thrombophilia. In the population of pregnant women, thrombophilia accounts for around one-third of variance in MN frequency.

CONCLUSIONS

It can be concluded that the frequency of micronuclei >4MN/1000BN depends on the presence of thrombophilia, previous miscarriages, the number of miscarriages and the number of pregnancies. The presence of thrombophilia in pregnancy in the study group is the most important pre-

dictor variable of increased MN frequency. Based on the current knowledge, we hypothesize that the DNA damage, measured by the increase in frequency of MN, probably occurs as a result of oxidative stress initiated by prothrombotic condition in the mother's blood.

ACKNOWLEDGMENTS

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