

DNMT3B rs1569686 and rs2424913 gene polymorphisms are associated with positive family history of preterm birth and smoking status

Aim To evaluate the association between spontaneous preterm birth (SPTB) and DNA methyltransferase (*DNMT*)1, 3A, 3B, and 3L gene polymorphisms, and their contribution to the clinical characteristics of women with SPTB and their newborns.

Methods This case-control study, conducted in 2018, enrolled 162 women with SPTB and 162 women with term delivery. *DNMT1* rs2228611, *DNMT3A* rs1550117, *DNMT3B* rs1569686, *DNMT3B* rs2424913, and *DNMT3L* rs2070565 single nucleotide polymorphisms were genotyped using polymerase chain reaction and restriction fragment length polymorphism methods. The clinical characteristics included in the analysis were family history of preterm birth, maternal smoking, maternal age, gestational week at delivery, and fetal birth weight.

Results *DNMT* gene polymorphisms were not significantly associated with SPTB. *DNMT3B* rs1569686 and rs2424913 minor alleles (T) were significantly more frequent in women with familial PTB than in women with non-familial PTB, increasing the odds for familial PTB 3.30 and 3.54 times under dominant genetic models. They were also significantly more frequent in women with SPTB who smoked before pregnancy, reaching the most significant association under additive genetic models (odds ratio 6.86, 95% confidence interval 2.25-20.86, $P < 0.001$; odds ratio 3.77, 95% confidence interval 1.36-10.52, $P = 0.011$, respectively).

Conclusions *DNMT3B* rs1569686 and rs2424913 gene polymorphisms might be associated with positive family history of PTB and smoking status.

Anita Barišić¹, Maja Kolak², Ana Peterlin³, Nataša Tul⁴, Milena Gašparović Krpina⁵, Saša Ostojčić¹, Borut Peterlin⁵, Nina Perez⁷

¹Department of Medical Biology and Genetics, Faculty of Medicine, University of Rijeka, Rijeka, Croatia

²Department of Oncology, Clinical Hospital Center Rijeka, Rijeka, Croatia

³Clinical Institute of Medical Genetics, University Medical Center Ljubljana, Ljubljana, Slovenia

⁴Department of Obstetrics and Gynecology, University Medical Center Ljubljana, Ljubljana, Slovenia

⁵Department of Obstetrics and Gynecology, Clinical Hospital Center Rijeka, Rijeka, Croatia

⁶Division of Obstetrics and Gynecology, Clinical Institute of Medical Genetics, University Medical Center Ljubljana, Ljubljana, Slovenia

⁷Department of Biology, Faculty of Medicine, University of Rijeka, Rijeka, Croatia

Received: August 14, 2019

Accepted: December 20, 2019

Correspondence to:

Nina Perez
Department of Medical Biology and Genetics
Faculty of Medicine, University of Rijeka
Rijeka
B. Branchetta 20
51000 Rijeka, Croatia
nina.perez@medri.uniri.hr

Preterm birth (PTB), defined as birth before the 37th completed week of gestation, is the leading cause of neonatal mortality and morbidity (1). It also significantly increases the risk of long-term health complications compared with term birth (2). Up to 25% of PTBs are medically induced and 50% are initiated spontaneously with intact fetal membranes (SPTB or idiopathic PTB) (3,4). Due to its heterogeneous etiology, SPTB is considered a clinical syndrome (5). A recognized risk factor for SPTB is maternal and/or fetal (epi)genetic predisposition, which has been confirmed in many epidemiological studies (6-8).

DNA methylation patterns guide temporal and tissue-specific gene expression and ensure genome stability. These patterns are extensively modified during gametogenesis and prenatal development (8-10), which makes DNA methylation a good predictor of gestational age at or near birth and a source of information related to the developmental stage (11). Epigenetic alterations were associated with PTB, and global and site-specific DNA methylation patterns were changed in maternal blood, placenta, and cord blood of preterm newborns (12-17). DNA methylation among preterm infants is influenced by both prenatal and postnatal environmental factors, such as maternal stress, social deprivation, and smoking (18-22).

During methylation process, methyl groups are transferred to cytosines by DNA methyltransferases (DNMT), among which DNMT1, DNMT3A, and DNMT3B are the major catalytically active enzymes (23,24). DNMT1 binds to hemi-methylated DNA and is responsible for the maintenance of established patterns, whereas DNMT3A and DNMT3B guide *de novo* methylation. Unlike the other DNMTs, DNMT3L is an enzymatically inactive regulatory factor that binds to DNMT3A and DNMT3B and increases their activity (23).

Considering that single nucleotide polymorphisms (SNP) in *DNMT* genes might affect the genes' expression and consequently methylation, several studies assessed the association of these SNPs with different human reproductive disorders. Polymorphisms of *DNMT1* and *DNMT3A* genes were found to be associated with male infertility and spontaneous abortion after assisted reproduction or natural conception, respectively (25,26). *DNMT3L* gene variants affected birth-weight and were associated with male infertility and ovarian endometriosis (27-29), while maternal *DNMT3B* SNPs increased the risk for PTB and Down syndrome (27,30,31).

The present study examines the potential association between maternal *DNMT1*, *DNMT3A*, *DNMT3B*, and *DNMT3L* gene polymorphisms and SPTB. To identify the factors that cause epigenetic modifications related to SPTB, we also evaluated the association between *DNMT* gene polymorphisms and various clinical characteristics of women with SPTB and their newborns (family history of PTB, maternal smoking before pregnancy, maternal age and gestational week at delivery, and fetal birth weight).

PATIENTS AND METHODS

Patients

This case-control study, conducted in 2018, enrolled Slovenian and Croatian women who gave birth at the Division of Perinatology, Department of Obstetrics and Gynecology, University Medical Center in Ljubljana, Slovenia and Department of Obstetrics and Gynecology, Clinical Hospital Centre of Rijeka, Croatia. All participants gave written informed consent. The samples collected in Rijeka are part of the TransMedri Biobank – a bank of biosamples for the investigation of preterm birth (EU-FP7 Regpot-2010-5, Faculty of Medicine, University of Rijeka). The study was approved by the Slovenian National Medical Ethics Committee (98/12/10, 2010) and the Ethics Committee for Biomedical Research of the Faculty of Medicine, University of Rijeka (2170-29-02/15-17-2, 2017).

The patient group included 162 women with SPTB (113 Slovenian and 49 Croatian). Demographic and clinical data of women with SPTB and their newborns were collected in accordance with the guidelines for genetic epidemiology studies on PTB (2) by means of a self-developed interviewer-administered questionnaire. As described in more detail in our previous study (32), all women with SPTB had singleton pregnancies following natural conception and spontaneous initiation of PTB before the 37th week of gestation. Gestational age was estimated from the last menstrual period and confirmed by ultrasound in the first trimester. When the difference between the two estimates exceeded seven days, gestational age was revised according to the ultrasound measurement. The exclusion criteria for patients were the known risk factors for PTB, including diabetes, hypertension, kidney disease, autoimmune conditions, allergic diseases, birth canal infections, *in vitro* fertilization, and pregnancy complications. None of the live-born children had congenital anomalies or evidence of infection. Additional maternal and newborn characteristics are shown in Table 1. The control group enrolled 162 age- and parity-

matched women (119 Slovenian and 43 Croatian) who had a term singleton birth after an uncomplicated pregnancy.

DNA isolation and genotyping

Genomic DNA was isolated from peripheral blood leukocytes by standard procedure with a commercially available kit (Qiagen FlexiGene DNA kit, Qiagen GmbH, Hilden, Germany) and stored at -20°C .

DNMT1 rs2228611, *DNMT3A* rs1550117, *DNMT3B* rs1569686, *DNMT3B* rs2424913, and *DNMT3L* rs2070565 SNPs were genotyped using a combination of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Primers, PCR and RFLP conditions were modified from the previously published literature (Supplementary material 1) (33-36). Polymerase chain reaction was carried out in thermal cyclers (Mastercycle personal, Eppendorf, Hamburg, Germany and 2720 Thermal Cycler, Applied Biosystems, Carlsbad, CA, USA). All restriction enzymes were obtained from New

England Biolabs (Ipswich, MA, USA), and reactions were performed in accordance with the manufacturer's recommendations. PCR products and restriction fragments were separated using electrophoresis on 3% agarose gels stained with GelRed™ (Olerup SSP®, Saltsjöbaden, Sweden).

Statistical analysis

Normality of distribution was tested with the Kolmogorov-Smirnov test. The Pearson chi square test was used to examine differences in genotype and allele frequencies between various groups of participants. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to determine the association between *DNMT* gene polymorphisms and SPTB. The *t* test was used for comparison of age and fetal birth weight means between patients and controls, whereas one-way analysis of variance (ANOVA) was used for the comparison of age and fetal birth weight means between the groups with different genotypes of *DNMT* gene polymorphisms. The level of statistical significance was set at

TABLE 1. Characteristics of women with spontaneous preterm birth (SPTB) and controls

	No (%) of		<i>P</i>
	cases*	controls†	
Maternal characteristics			
Mean age at delivery (years)	30 (17-44)	30 (20-42)	0.755 [‡]
Gestational age at delivery		37-41	
extremely preterm <28 week	10 (6.4)		
very preterm 32-28 weeks	20 (12.7)		
moderate to late preterm 32-37 weeks	127 (80.9)		
Smoking before pregnancy			
yes	45 (71.3)	23 (20.7)	0.184 [§]
no	112 (28.7)	88 (79.3)	
Smoking during pregnancy			
yes	19 (12.1)	13 (11.7)	0.925 [§]
no	138 (87.9)	98 (88.3)	
Previous PTB			
yes	13 (8.3)	0	
no	144 (91.7)		
Familial PTB			
yes	48 (30.6)	0	
no	109 (69.4)		
Newborn characteristics			
birth weight (grams)	2403 (620-3915)	3456 (1570-4560)	<0.001 [‡]
congenital anomalies	0	0	
evidence of infection	0	0	

*epidemiological data were available for 157/162 (97%) women with SPTB.

†epidemiological data were available for 111/162 (69%) controls.

‡*t*-test.

§ χ^2 test.

||median and range.

P less than 0.05. Statistical analyses were performed with Statistica for Windows, version 13.3 (StatSoft, Inc., Tulsa, OK, USA) and MedCalc for Windows, version 14.12.0. (Med-

TABLE 2. Genotype and allele frequencies of DNA methyltransferase (DNMT) gene polymorphisms in women with spontaneous preterm birth (SPTB) and controls

	No (%) of		X ²	P
	cases	controls		
DNMT1				
rs2228611				
genotype				
AA	62 (38.3)	54 (33.3)	1.09	0.581
AG	74 (45.7)	83 (51.2)		
GG	26 (16.0)	25 (15.5)		
allele				
A	198 (61.1)	191 (58.9)	0.32	0.575
G	126 (38.9)	133 (41.1)		
DNMT3A				
rs1550117				
genotype				
GG	135 (83.3)	128 (79.0)	1.02	0.601
AG	26 (16.1)	33 (20.4)		
AA	1 (0.6)	1 (0.6)		
allele				
G	296 (91.4)	289 (89.2)	0.86	0.353
A	28 (8.6)	35 (10.8)		
DNMT3B				
rs1569686				
genotype				
GG	67 (41.4)	57 (35.2)	2.54	0.281
TG	76 (46.9)	77 (47.5)		
TT	19 (11.7)	28 (17.3)		
allele				
G	210 (64.8)	191 (58.9)	2.36	0.124
T	114 (35.2)	133 (41.1)		
rs2424913				
genotype				
CC	60 (37.0)	48 (29.6)	2.62	0.270
TC	79 (48.8)	83 (51.2)		
TT	23 (14.2)	31 (19.2)		
allele				
C	199 (61.4)	179 (55.2)	2.54	0.111
T	125 (38.6)	145 (44.8)		
DNMT3L				
rs2070565				
genotype				
CC	56 (34.5)	50 (30.9)	1.89	0.389
TC	89 (55.0)	87 (53.7)		
TT	17 (10.5)	25 (15.4)		
allele				
C	201 (62.0)	187 (57.7)	1.26	0.262
T	123 (38.0)	137 (42.3)		

Calc Software, Mariakerke, Belgium). Statistical power was calculated with ClinCalc LLC (<https://clincalc.com/stats/samplesize.aspx>) and Hardy-Weinberg equilibrium was calculated using Simple Hardy-Weinberg Calculator – Court Laboratory (Washington State University College of Veterinary Medicine, Pullman, WA, USA).

RESULTS

Genetic association between DNMT gene polymorphisms and SPTB

Cases and controls did not significantly differ in the distribution of genotype or allele frequencies of *DNMT1* rs2228611, *DNMT3A* rs1550117, *DNMT3B* rs1569686, *DNMT3B* rs2424913, and *DNMT3L* rs2070565 SNPs (Table 2). Neither of the polymorphisms was associated with SPTB (data not shown). All genotype frequencies in cases and controls were in Hardy-Weinberg equilibrium (data not shown). The study had 80% power to detect a 2-fold increase in the minor alleles of all SNPs.

Association of DNMT gene polymorphisms with clinical characteristics of women with SPTB and their newborns

Individually, both *DNMT3B* rs1569686 and rs2424913 minor alleles (T) were more frequent in women with famil-

TABLE 3. Genotype and allele frequencies of *DNMT3B* gene polymorphisms in women with SPTB according to family history of PTB*

	No. (%) of women with		X ²	P
	non-familial PTB	familial PTB		
DNMT3B				
rs1569686				
Genotype				
GG	54 (49.6)	11 (22.9)	10.31	0.006
TG	45 (41.3)	28 (58.3)		
TT	10 (9.1)	9 (18.8)		
Allele				
G	153 (70.2)	50 (52.1)	9.55	0.002
T	65 (29.8)	46 (47.9)		
rs2424913				
Genotype				
CC	49 (45.0)	9 (18.8)	13.96	<0.001
TC	50 (45.9)	26 (54.2)		
TT	10 (9.1)	13 (27.0)		
Allele				
C	148 (67.9)	44 (45.8)	13.65	<0.001
T	70 (32.1)	52 (54.2)		

*DNMT – DNA methyltransferase; SPTB – spontaneous preterm birth.

ial PTB than in women with non-familial PTB ($X^2=10.31$, $P=0.006$ and $X^2=13.96$, $P<0.001$, respectively) (Table 3) and increased the odds for familial PTB 3.30 and 3.54 times under the dominant genetic models (TT + TG vs GG and TT + TC vs CC) (95% CI 1.53-7.14, $P=0.003$ and 95% CI= 1.56-8.01, $P=0.002$, respectively) (Table 4).

The individual analysis of *DNMT3B* SNPs showed that rs1569686 and rs2424913 T alleles were also significant-

ly more frequent in patients with SPTB who had smoked than patients who had not smoked before pregnancy ($X^2=10.12$, $P=0.001$ and $X^2=5.35$, $P=0.021$, respectively) (Table 5), reaching the most significant association under the additive genetic models (TT vs GG and TT vs CC) (OR 6.86, 95% CI 2.25-20.86, $P<0.001$ and OR 3.77, 95% CI 1.36-10.52, $P=0.011$, respectively, Table 6). None of the other polymorphisms contributed to the clinical charac-

TABLE 4. Association of *DNMT3B* gene polymorphisms with familial PTB*

Genetic models	Familial vs non-familial PTB	
	OR (95% CI)	P
rs1569686		
TT vs TG+GG	2.28 (0.86-6.05)	0.096
TT+TG vs GG	3.30 (1.53-7.14)	0.003
TT vs TG	1.45 (0.52-3.99)	0.477
TT vs GG	4.42 (1.46-13.40)	0.009
GG vs TG	0.33 (0.15-0.73)	0.006
T vs G	2.17 (1.32-3.55)	0.002
rs2424913		
TT vs TC+CC	3.68 (1.48-9.14)	0.005
TT+TC vs CC	3.54 (1.56-8.01)	0.002
TT vs TC	2.50 (0.96-6.47)	0.059
TT vs CC	7.07 (2.38-21.02)	<0.001
CC vs TC	0.35 (0.15-0.83)	0.017
T vs C	2.49 (1.53-4.09)	<0.001

*OR – odds ratio; CI – confidence interval; DNMT – DNA methyltransferase; PTB – preterm birth.

TABLE 6. Association of *DNMT3B* gene polymorphisms with smoking before pregnancy*

Genetic models	SPTB		Controls	
	OR (95% CI)	P	OR (95% CI)	P
rs1569686				
TT vs TG+GG	5.45 (1.98-14.99)	0.001	1.33 (0.39-4.59)	0.649
TT+TG vs GG	2.13 (1.01-4.49)	0.045	1.58 (0.59-4.24)	0.361
TT vs TG	4.54 (1.57-13.17)	0.005	1.11 (0.30-4.09)	0.874
TT vs GG	6.86 (2.25-20.86)	<0.001	1.71 (0.43-6.89)	0.447
GG vs TG	0.66 (0.29-1.47)	0.311	0.65 (0.23-1.83)	0.412
T vs G	2.24 (1.36-3.71)	0.002	1.35 (0.69-2.60)	0.376
rs2424913				
TT vs TC+CC	3.34 (1.35-8.28)	0.009	1.11 (0.33-3.77)	0.864
TT+TC vs CC	1.65 (0.78-3.49)	0.187	1.25 (0.45-3.53)	0.668
TT vs TC	3.05 (1.16-8.01)	0.023	1.03 (0.29-3.68)	0.960
TT vs CC	3.77 (1.36-10.52)	0.011	1.29 (0.31-5.32)	0.729
CC vs TC	0.81 (0.36-1.80)	0.604	0.80 (0.27-2.36)	0.690
T vs C	1.79 (1.08-2.94)	0.022	1.13 (0.59-2.17)	0.711

*OR – odds ratio; CI – confidence interval; DNMT – DNA methyltransferase.

TABLE 5. Genotype and allele frequencies of *DNMT3B* gene polymorphisms according to smoking before pregnancy*

<i>DNMT3B</i>	No. (%) of			X^2	P	No. (%) of			X^2	P
	SPTB non-smokers	SPTB smokers				controls non-smokers	controls smokers			
rs1569686										
genotype										
GG	52 (46.4)	13 (28.9)	13.49	0.001	36 (40.9)	7 (30.4)	0.87	0.647		
TG	53 (47.3)	20 (44.4)			40 (45.5)	12 (52.2)				
TT	7 (6.3)	12 (26.7)			12 (13.6)	4 (17.4)				
allele										
G	157 (70.1)	46 (51.1)	10.12	0.001	112 (63.6)	26 (56.5)	0.79	0.376		
T	67 (29.9)	44 (48.9)			64 (36.4)	20 (43.5)				
rs2424913										
genotype										
CC	45 (40.2)	13 (28.9)			27 (30.7)	6 (26.1)	0.19	0.911		
TC	56 (50.0)	20 (44.4)	7.53	0.023	47 (53.4)	13 (56.5)				
TT	11 (9.8)	12 (26.7)			14 (15.9)	4 (17.4)				
allele										
C	146 (65.2)	46 (51.1)	5.35	0.021	101 (57.4)	25 (54.4)	0.14	0.711		
T	78 (34.8)	44 (48.9)			75 (42.6)	21 (45.6)				

*DNMT – DNA methyltransferase; SPTB – spontaneous preterm birth.

teristics of women with SPTB and their newborns (data not shown).

DISCUSSION

This study indicates that maternal DNMT3B rs1569686 and rs2424913 SNPs might be susceptibility factors for SPTB in women who had a positive family history of PTB and had smoked before pregnancy. Although genotype and allele frequencies of DNMT3B rs1569686 and rs2424913 SNPs were similar in cases and controls, a subgroup analysis of women with SPTB yielded two significant associations for both polymorphisms. First, the minor (T) allele of rs1569686 or rs2424913 DNMT3B polymorphism, in both homozygous and heterozygous form, increased the odds for familial PTB 3.30 and 3.54-fold, respectively, compared with the homozygous form of the major alleles (GG and CC). Positive family history is an independent risk factor and one of the main risk factors for PTB (37-39). Intergenerational influences include both genetic and epigenetic factors, meaning that both the inherited genetic predisposition to PTB and the mother's lifestyle affect her own and the next generation's health status (37). DNMT3B rs2424913 and rs1569686 are located in the 3'-untranslated and promoter regions of DNMT3B gene, 149 and 579 base pairs, respectively, upstream from the transcription start site. The role of rs2424913 SNP is to regulate the expression of DNMT3B gene, while the T allele increases promoter activity (38,39) and affects miRNA binding site (40). The functional role of rs1569686 SNP is still controversial, although *in silico* analysis showed that the T allele might affect the binding activity for several transcription factors (40). A previous study reported that both maternal and infant DNMT3B rs1569686 and rs2424913 gene polymorphisms influenced inter-individual variation in global DNA methylation (41). In addition, the T alleles of both variants, both in homozygous and heterozygous forms, were associated with the risk of several diseases, mostly different cancer types (42-44). Interestingly, rs1569686 TT genotype and T allele were overrepresented in patients with schizophrenia and positive family history of psychiatric illness (40).

The second important finding in our study was the association of the minor (T) alleles of DNMT3B rs1569686 and rs2424913 with maternal smoking, one of the previously confirmed environmental risk factors for SPTB (11,45). Maternal smoking in the pre- and peri-conception period (46,47), as well as throughout pregnancy (45), significantly increased the risk for PTB. For example, Haas et al (47) showed that pre-conception smoking increased the odds

for PTB 2-fold (95% CI 1.29-3.75). In our study, women who smoked and were homozygous for DNMT3B rs1569686 TT genotype and rs2424913 TT genotype had respectively 6.86-fold and 3.77-fold higher odds for SPTB compared with GG and CC carriers. Interestingly, the lack of significant difference in genotype and allele frequencies between control non-smokers and smokers confirms that smoking before pregnancy combined with TT genotype is an additional risk factor for SPTB. This finding shows that smoking can negatively affect epigenetic modifications in the pre-conception period, especially during ovarian follicular development (48). Previously, maternal smoking has been shown to adversely affect ovarian reserve and oocyte quality (49) and clinical outcomes of assisted reproductive technologies (50), which most likely have epigenetic etiology. As shown by a large epigenome wide association study, smoking changed DNA methylation pattern at multiple genomic loci, which was only partially reversible upon smoking cessation (51). Also, maternal smoking was independently associated with reduced site-specific DNA methylation among preterm infants at birth, both in mothers who quit smoking before pregnancy and those who continued to smoke (52). The spatially and temporally indispensable roles of *de novo* methyltransferase DNMT3B during oogenesis and early embryonic development might be affected by the exposure to harmful environmental factors. In humans, DNMT3B transcript is present from the primordial follicle stage onwards, but at the germinal vesicle stage its protein is no longer detected in the nucleus, indicating that *de novo* DNA methylation in oogenesis occurs during the earliest stages of follicular development (53,54). Moreover, DNMT3B seems to be the major DNMT that ensures global DNA remethylation during blastocyst formation before implantation (54). Although the effect of maternal smoking during pregnancy on global and site-specific DNA methylation in the placenta and neonates has been well documented (55-58), its precise impact on DNA methylation and expression on DNMT3B in growing oocytes, as well as the long-term consequences on fetal growth and the timing of birth, is yet to be determined. Moreover, the implied associations between genetic polymorphisms and the tendency to smoke could be confounded by patient selection. However, studies on the association between smoking-related cancers and epigenomic alterations showed that cigarette smoke influenced DNMT3B gene expression, thus changing DNA methylation patterns (59-61).

Although our study was the first study conducted in women with SPTB, the association between DNMT1

rs2162560, *DNMT3A* rs734693, *DNMT3B* rs2424913, and *DNMT3L* rs7354779 and birth outcome was evaluated in one previous study (27). In that study, only maternal *DNMT3B* rs2424913 minor allele was associated with an increased risk for PTB, confirming *DNMT3B* as a potential candidate gene for PTB. Furthermore, three independent studies found *DNMT3B* rs1569686 and rs2424913 to be maternal risk factors for Down syndrome (30,31,62), again confirming the importance of *DNMT3B* gene polymorphisms in human reproduction.

Although our study did not find an association between SPTB and the other tested polymorphisms in *DNMT1*, *DNMT3A*, and *DNMT3L* genes, they still represent good candidate genes for SPTB considering their functionality and the role DNMTs play in modifications during gametogenesis and pregnancy. Although *DNMT1* rs2228611 is located within exon 17 and is considered to be a synonymous mutation, according to *in silico* analysis it might affect splicing regulation (40). This polymorphism was also reported to affect LINE-1 methylation in women exposed to cadmium (63). *DNMT3A* rs1550117 is located 448 base pairs upstream of the transcription start site, and the A allele decreases its expression (64). Intronic *DNMT3L* rs2070565 is also a splice site variant (40). Additionally, there are other polymorphisms within these genes that should be considered for future analysis.

The potential limitations of our study include the analysis of only the maternal genotypes and the low number of patients in the subgroup analysis, which reduces the study power. Moreover, we did not adjust P value for multiple comparisons and multiple presented analyses. On the other hand, the strengths of our study include patient selection according to the standard clinical definition of SPTB, sufficient statistical power, and the use of peripheral blood samples for DNA analysis. Further genetic association and expression studies in different populations should evaluate the role of *DNMT* gene polymorphisms in SPTB.

Funding This study was supported by research grants (16.06.2.1.02, 17.07.2.1.04) from the University of Rijeka, Croatia) and program P3-0326 (Slovenian Research Agency, Slovenia).

Ethical approval given by the Slovenian National Medical Ethics Committee (98/12/10, 2010) and the Ethics Committee for Biomedical Research of the Faculty of Medicine, University of Rijeka (2170-29-02/15-17-2, 2017).

Declaration of authorship AB, NT, SO, BP and NP conceived and designed the study; AB, MK, AP, NT, MGK, and NP acquired, analysed, and interpreted the data; all authors drafted the manuscript; AB, NT, SO, BP, and NP critically revised the manuscript for important intellectual content; all authors gave approval of the version to be submitted; all authors agree to be accountable for all aspects of the work.

Competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

References

- Lockwood CJ, Kuczynski E. Risk stratification and pathological mechanisms in preterm delivery. *Paediatr Perinat Epidemiol*. 2001;15 Suppl 2:78-89. [Medline:11520402](#) [doi:10.1046/j.1365-3016.2001.00010.x](#)
- Pennell CE, Jacobsson B, Williams SM, Buus RM, Muglia LJ, Dolan SM, et al. Genetic epidemiologic studies of preterm birth: guidelines for research. *Am J Obstet Gynecol*. 2007;196:107-18. [Medline:17306646](#) [doi:10.1016/j.jajog.2006.03.109](#)
- Moutquin JM. Classification and heterogeneity of preterm birth. *BJOG*. 2003;110 Suppl 20:30-3. [Medline:12763108](#) [doi:10.1046/j.1471-0528.2003.00021.x](#)
- Menon R. Spontaneous preterm birth, a clinical dilemma: etiologic, pathophysiologic and genetic heterogeneities and racial disparity. *Acta Obstet Gynecol Scand*. 2008;87:590-600. [Medline:18568457](#) [doi:10.1080/00016340802005126](#)
- Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. *Science*. 2014;345:760-5. [Medline:25124429](#) [doi:10.1126/science.1251816](#)
- Anum EA, Springel EH, Shriver MD, Strauss JF III. Genetic contributions to disparities in preterm birth. *Pediatr Res*. 2009;65:1-9. [Medline:18787421](#) [doi:10.1203/PDR.0b013e31818912e7](#)
- Esplin MS. Preterm birth: a review of genetic factors and future directions for genetic study. *Obstet Gynecol Surv*. 2006;61:800-6. [Medline:17107629](#) [doi:10.1097/01.ogx.0000248747.52343.5f](#)
- Fulka H, Mrazek M, Tepla O, Fulka J Jr. DNA methylation pattern in human zygotes and developing embryos. *Reproduction*. 2004;128:703-8. [Medline:15579587](#) [doi:10.1530/rep.1.00217](#)
- Schroeder DI, Blair JD, Lott P, Yu HO, Hong D, Crary F, et al. The human placenta methylome. *Proc Natl Acad Sci U S A*. 2013;110:6037-42. [Medline:23530188](#) [doi:10.1073/pnas.1215145110](#)
- Mansell T, Saffery R. The end of the beginning: epigenetic variation in utero as a mediator of later human health and disease. *Epigenomics*. 2017;9:217-21. [Medline:28234019](#) [doi:10.2217/epi-2017-0007](#)
- Knight AK, Craig JM, Theda C, Bckvad-Hansen M, Bybjerg-Grauholm J, Hansen CS, et al. An epigenetic clock for gestational age at birth based on blood methylation data. *Genome Biol*. 2016;17:206. [Medline:27717399](#) [doi:10.1186/s13059-016-1068-z](#)
- Toure DM, El Rayes W, Barnes-Josiah D, Hartman T, Klinkebiel D, Baccaglioni L. Epigenetic modifications of human placenta associated with preterm birth: a systematic review. *J Matern Fetal*

- Neonatal Med. 2018;31:530-41. [Medline:28282769](#) [doi:10.1080/14767058.2017.1291620](#)
- 13 Barcelona de Mendoza V, Wright ML, Agaba C, Prescott L, Desir A, Crusto CA, et al. A systematic review of DNA methylation and preterm birth in African American women. *Biol Res Nurs*. 2017;19:308-17. [Medline:27646016](#) [doi:10.1177/1099800416669049](#)
 - 14 Sparrow S, Manning JR, Cartier J, Anblagan D, Bastin ME, Piyasena C, et al. Epigenomic profiling of preterm infants reveals DNA methylation differences at sites associated with neural function. *Transl Psychiatry*. 2016;6:e716. [Medline:26784970](#) [doi:10.1038/tp.2015.210](#)
 - 15 Fernando F, Keijser R, Henneman P, van der Kevie-Kersemaekers AM, Mannens MM, van der Post JA, et al. The idiopathic preterm delivery methylation profile in umbilical cord blood DNA. *BMC Genomics*. 2015;16:736. [Medline:26419829](#) [doi:10.1186/s12864-015-1915-4](#)
 - 16 Behnia F, Parets SE, Kechichian T, Yin H, Dutta EH, Saade GR, et al. Fetal DNA methylation of autism spectrum disorders candidate genes: association with spontaneous preterm birth. *Am J Obstet Gynecol*. 2015;212:533.e1-9. [Medline:25687563](#) [doi:10.1016/j.ajog.2015.02.011](#)
 - 17 Parets SE, Conneely KN, Kilaru V, Fortunato SJ, Syed TA, Saade G, et al. Fetal DNA methylation associates with early spontaneous preterm birth and gestational age. *PLoS One*. 2013;8:e67489. [Medline:23826308](#) [doi:10.1371/journal.pone.0067489](#)
 - 18 Kantake M, Yoshitake H, Ishikawa H, Araki Y, Shimizu T. Postnatal epigenetic modification of glucocorticoid receptor gene in preterm infants: a prospective cohort study. *BMJ Open*. 2014;4:e005318. [Medline:25023132](#) [doi:10.1136/bmjopen-2014-005318](#)
 - 19 Vidal AC, Benjamin Neelon SE, Liu Y, Tuli AM, Fuemmeler BF, Hoyt C, et al. Maternal stress, preterm birth, and DNA methylation at imprint regulatory sequences in humans. *Genet Epigenet*. 2014;6:37-44. [Medline:25512713](#) [doi:10.4137/GEG.S18067](#)
 - 20 Maccani JZ, Koestler DC, Houseman EA, Marsit CJ, Kelsey KT. Placental DNA methylation alterations associated with maternal tobacco smoking at the RUNX3 gene are also associated with gestational age. *Epigenomics*. 2013;5:619-30. [Medline:24283877](#) [doi:10.2217/epi.13.63](#)
 - 21 Piyasena C, Cartier J, Provençal N, Wiechmann T, Khulan B, Sunderasan R, et al. Dynamic changes in DNA methylation occur during the first year of life in preterm infants. *Front Endocrinol (Lausanne)*. 2016;7:158. [Medline:28018293](#) [doi:10.3389/fendo.2016.00158](#)
 - 22 Montirosso R, Provenzi L, Giorda R, Fumagalli M, Morandi F, Sirgiovanni I, et al. SLC6A4 promoter region methylation and socio-emotional stress response in very preterm and full-term infants. *Epigenomics*. 2016;8:895-907. [Medline:27381173](#) [doi:10.2217/epi-2016-0010](#)
 - 23 Subramaniam D, Thombre R, Dhar A, Anant S. DNA methyltransferases: a novel target for prevention and therapy. *Front Oncol*. 2014;4:80. [Medline:24822169](#) [doi:10.3389/fonc.2014.00080](#)
 - 24 Lan J, Hua S, He X, Zhang Y. DNA methyltransferases and methyl-binding proteins of mammals. *Acta Biochim Biophys Sin (Shanghai)*. 2010;42:243-52. [Medline:20383462](#) [doi:10.1093/abbs/gmq015](#)
 - 25 Cheng P, Chen H, Zhang RP, Liu SR, Zhou-Cun A. Polymorphism in DNMT1 may modify the susceptibility to oligospermia. *Reprod Biomed Online*. 2014;28:644-9. [Medline:24631383](#) [doi:10.1016/j.rbmo.2014.01.003](#)
 - 26 Liu Y, Zheng H, Guo P, Feng S, Zhou X, Ye D, et al. DNA methyltransferase 3A promoter polymorphism is associated with the risk of human spontaneous abortion after assisted reproduction techniques and natural conception. *J Assist Reprod Genet*. 2017;34:245-52. [Medline:27817038](#) [doi:10.1007/s10815-016-0837-7](#)
 - 27 Haggarty P, Hoad G, Horgan GW, Campbell DM. DNA methyltransferase candidate polymorphisms, imprinting methylation, and birth outcome. *PLoS One*. 2013;8:e68896. [Medline:23922667](#) [doi:10.1371/journal.pone.0068896](#)
 - 28 Dong Y, Pan Y, Wang R, Zhang Z, Xi Q, Liu RZ. Copy number variations in spermatogenic failure patients with chromosomal abnormalities and unexplained azoospermia. *Genet Mol Res*. 2015;14:16041-9. [Medline:26662397](#) [doi:10.4238/2015.December.7.17](#)
 - 29 Borghese B, Santulli P, Héquet D, Pierre G, de Ziegler D, Vaiman D, et al. Genetic polymorphisms of DNMT3L involved in hypermethylation of chromosomal ends are associated with greater risk of developing ovarian endometriosis. *Am J Pathol*. 2012;180:1781-6. [Medline:22401780](#) [doi:10.1016/j.ajpath.2012.01.009](#)
 - 30 Jaiswal SK, Sukla KK, Kumari N, Lakhota AR, Kumar A, Rai AK. Maternal risk for down syndrome and polymorphisms in the promoter region of the DNMT3B gene: a case-control study. *Birth Defects Res A Clin Mol Teratol*. 2015;103:299-305. [Medline:25656965](#) [doi:10.1002/bdra.23348](#)
 - 31 Coppede F, Bosco P, Tannorella P, Romano C, Antonucci I, Stuppia L, et al. DNMT3B promoter polymorphisms and maternal risk of birth of a child with Down syndrome. *Hum Reprod*. 2013;28:545-50. [Medline:23081874](#) [doi:10.1093/humrep/des376](#)
 - 32 Perez N, Pleša I, Peterlin A, Jan Ž, Tul N, Kapović M, et al. Functional polymorphisms of matrix metalloproteinases 1 and 9 genes in women with spontaneous preterm birth. *Dis Markers*. 2014;2014:171036. [Medline:25530657](#) [doi:10.1155/2014/171036](#)
 - 33 Khatami F, Noorinayer B, Ghiasi S, Mohebi R, Hashemi M, Zali MR. Lack of effects of single nucleotide polymorphisms of the DNA methyltransferase 1 gene on gastric cancer in Iranian patients: a case control study. *Asian Pac J Cancer Prev*. 2009;10:1177-82.

Medline:20192608

- 34 Fan H, Liu D, Qiu X, Qiao F, Wu Q, Su X, et al. A functional polymorphism in the DNA methyltransferase-3A promoter modifies the susceptibility in gastric cancer but not in esophageal carcinoma. *BMC Med.* 2010;8:12. [Medline:20128888](#) [doi:10.1186/1741-7015-8-12](#)
- 35 Fan H, Zhang F, Hu J, Liu D, Zhao Z. Promoter polymorphisms of DNMT3B and the risk of colorectal cancer in Chinese: a case-control study. *J Exp Clin Cancer Res.* 2008;27:24. [Medline:18662374](#) [doi:10.1186/1756-9966-27-24](#)
- 36 Huang JX, Scott MB, Pu XY, Zhou-Cun A. Association between single-nucleotide polymorphisms of DNMT3L and infertility with azoospermia in Chinese men. *Reprod Biomed Online.* 2012;24:66-71. [Medline:22116073](#) [doi:10.1016/j.rbmo.2011.09.004](#)
- 37 Sherf Y, Sheiner E, Vardi IS, Sergienko R, Klein J, Bilenko N. Recurrence of preterm delivery in women with a family history of preterm delivery. *Am J Perinatol.* 2017;34:397-402. [Medline:27606779](#) [doi:10.1055/s-0036-1592131](#)
- 38 Shen H, Wang L, Spitz MR, Hong WK, Mao L, Wei Q. A novel polymorphism in human cytosine DNA-methyltransferase-3B promoter is associated with an increased risk of lung cancer. *Cancer Res.* 2002;62:4992-5. [Medline:12208751](#)
- 39 Xiao Y, Word B, Hammons G, Lyn-Cook B. Transcriptional activity of DNMT3B in pancreatic cancer cells: effects of -149 (C→T) promoter polymorphism. *Biochem Biophys Res Commun.* 2011;415:220-3. [Medline:21854760](#) [doi:10.1016/j.bbrc.2011.07.115](#)
- 40 Saradalekshmi KR, Neetha NV, Sathyan S, Nair IV, Nair CM, Banerjee M. DNA methyl transferase (DNMT) gene polymorphisms could be a primary event in epigenetic susceptibility to schizophrenia. *PLoS One.* 2014;9:e98182. [Medline:24859147](#) [doi:10.1371/journal.pone.0098182](#)
- 41 Potter C, McKay J, Groom A, Ford D, Coneyworth L, Mathers JC, et al. Influence of DNMT genotype on global and site specific DNA methylation patterns in neonates and pregnant women. *PLoS One.* 2013;8:e76506. [Medline:24098518](#) [doi:10.1371/journal.pone.0076506](#)
- 42 Li H, Li W, Liu S, Zong S, Wang W, Ren J, et al. DNMT1, DNMT3A and DNMT3B polymorphisms associated with gastric cancer risk: a systematic review and meta-analysis. *EBioMedicine.* 2016;13:125-31. [Medline:27789275](#) [doi:10.1016/j.ebiom.2016.10.028](#)
- 43 Duan F, Cui S, Song C, Dai L, Zhao X, Zhang X. Systematic evaluation of cancer risk associated with DNMT3B polymorphisms. *J Cancer Res Clin Oncol.* 2015;141:1205-20. [Medline:25515408](#) [doi:10.1007/s00432-014-1894-x](#)
- 44 Pesmatzoglou M, Lourou M, Goulielmos GN, Stiakaki E. DNA methyltransferase 3B gene promoter and interleukin-1 receptor antagonist polymorphisms in childhood immune thrombocytopenia. *Clin Dev Immunol.* 2012;2012:352059. [Medline:23049596](#) [doi:10.1155/2012/352059](#)
- 45 Ion R, Bernal AL. Smoking and preterm birth. *Reprod Sci.* 2015;22:918-26. [Medline:25394641](#) [doi:10.1177/1933719114556486](#)
- 46 Lassi ZS, Imam AM, Dean SV, Bhutta ZA. Preconception care: caffeine, smoking, alcohol, drugs and other environmental chemical/radiation exposure. *Reprod Health.* 2014;11 Suppl 3:56. [Medline:25415846](#) [doi:10.1186/1742-4755-11-S3-S6](#)
- 47 Haas JS, Fuentes-Afflick E, Stewart AL, Jackson RA, Dean ML, Brawarsky P, et al. Prepregnancy health status and the risk of preterm delivery. *Arch Pediatr Adolesc Med.* 2005;159:58-63. [Medline:15630059](#) [doi:10.1001/archpedi.159.1.58](#)
- 48 Steegers-Theunissen RP, Twigt J, Pestinger V, Sinclair KD. The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. *Hum Reprod Update.* 2013;19:640-55. [Medline:23959022](#) [doi:10.1093/humupd/dmt041](#)
- 49 Firms S, Cruzat VF, Keane KN, Joesbury KA, Lee AH, Newsholme P, et al. The effect of cigarette smoking, alcohol consumption and fruit and vegetable consumption on IVF outcomes: a review and presentation of original data. *Reprod Biol Endocrinol.* 2015;13:134. [Medline:26669322](#) [doi:10.1186/s12958-015-0133-x](#)
- 50 Waylen AL, Metwally M, Jones GL, Wilkinson AJ, Ledger WL. Effects of cigarette smoking upon clinical outcomes of assisted reproduction: a meta-analysis. *Hum Reprod Update.* 2009;15:31-44. [Medline:18927070](#) [doi:10.1093/humupd/dmn046](#)
- 51 Tsaprouni LG, Yang TP, Bell J, Dick KJ, Kanoni S, Nisbet J, et al. Cigarette smoking reduces DNA methylation levels at multiple genomic loci but the effect is partially reversible upon cessation. *Epigenetics.* 2014;9:1382-96. [Medline:25424692](#) [doi:10.4161/15592294.2014.969637](#)
- 52 Piyasena C, Cartier J, Provençal N, Wiechmann T, Khulan B, Sunderasan R, et al. Dynamic changes in DNA methylation occur during the first year of life in preterm infants. *Front Endocrinol (Lausanne).* 2016;7:158. [Medline:28018293](#) [doi:10.3389/fendo.2016.00158](#)
- 53 Huntriss J, Hinkins M, Oliver B, Harris SE, Beazley JC, Rutherford AJ, et al. Expression of mRNAs for DNA methyltransferases and methyl-CpG-binding proteins in the human female germ line, preimplantation embryos, and embryonic stem cells. *Mol Reprod Dev.* 2004;67:323-36. [Medline:14735494](#) [doi:10.1002/mrd.20030](#)
- 54 Petrusa L, Van de Velde H, De Rycke M. Dynamic regulation of DNA methyltransferases in human oocytes and preimplantation embryos after assisted reproductive technologies. *Mol Hum Reprod.* 2014;20:861-74. [Medline:24994815](#) [doi:10.1093/molehr/gau049](#)
- 55 Rotroff DM, Joubert BR, Marvel SW, Hlberg SE, Wu MC, Nilsen RM, et al. Maternal smoking impacts key biological pathways in newborns through epigenetic modification in utero. *BMC Genomics.* 2016;17:976. [Medline:27887572](#) [doi:10.1186/s12864-016-3310-1](#)
- 56 Morales E, Vilahur N, Salas LA, Motta V, Fernandez MF, Murcia M,

- et al. Genome-wide DNA methylation study in human placenta identifies novel loci associated with maternal smoking during pregnancy. *Int J Epidemiol*. 2016;45:1644-55. [Medline:27591263](#) [doi:10.1093/ije/dyw196](#)
- 57 Flom JD, Ferris JS, Liao Y, Tehranifar P, Richards CB, Cho YH, et al. Prenatal smoke exposure and genomic DNA methylation in a multiethnic birth cohort. *Cancer Epidemiol Biomarkers Prev*. 2011;20:2518-23. [Medline:21994404](#) [doi:10.1158/1055-9965.EPI-11-0553](#)
- 58 Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, et al. DNA methylation in newborns and maternal smoking in pregnancy: genome-wide consortium meta-analysis. *Am J Hum Genet*. 2016;98:680-96. [Medline:27040690](#) [doi:10.1016/j.ajhg.2016.02.019](#)
- 59 Liu F, Killian JK, Yang M, Walker RL, Hong JA, Zhang M, et al. Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. *Oncogene*. 2010;29:3650-64. [Medline:20440268](#) [doi:10.1038/onc.2010.129](#)
- 60 Liu H, Zhou Y, Boggs SE, Belinsky SA, Liu J. Cigarette smoke induces demethylation of prometastatic oncogene synuclein-gamma in lung cancer cells by downregulation of DNMT3B. *Oncogene*. 2007;26:5900-10. [Medline:17369845](#) [doi:10.1038/sj.onc.1210400](#)
- 61 Tang M, Xu W, Wang Q, Xiao W, Xu R. Potential of DNMT and its epigenetic regulation for lung cancer therapy. *Curr Genomics*. 2009;10:336-52. [Medline:20119531](#) [doi:10.2174/138920209788920994](#)
- 62 Moura CM, Bastos PR, Ribeiro JSV, Ribeiro MG, Amorim MR, Costa-Lima MA. DNA (cytosine-5)-methyltransferase 3B (DNMT 3B) polymorphism and risk of Down syndrome offspring. *Saudi J Biol Sci*. 2018;25:101-4. [Medline:29379364](#) [doi:10.1016/j.sjbs.2017.09.008](#)
- 63 Hossain MB, Vahter M, Concha G, Broberg K. Low-level environmental cadmium exposure is associated with DNA hypomethylation in Argentinean women. *Environ Health Perspect*. 2012;120:879-84. [Medline:22382075](#) [doi:10.1289/ehp.1104600](#)
- 64 Wang J, Li C, Wan F, Li Z, Zhang J, Zhang J, et al. The rs1550117 A>G variant in DNMT3A gene promoter significantly increases non-small cell lung cancer susceptibility in a Han Chinese population. *Oncotarget*. 2017;8:23470-8. [Medline:28423585](#)