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Induction of the apoptotic pathway by oxidative stress in spontaneous preterm birth: Single nucleotide polymorphisms, maternal lifestyle factors and health status

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Abstract. The purpose of the present study was to search for associations between spontaneous preterm birth (sPTB), single nucleotide polymorphisms (SNPs) associated with the apoptotic pathway as triggered by oxidative stress, maternal lifestyle and health status. SNP genotyping [rs7560 for c-Jun N-terminal kinase (JNK), rs9517320 for mammalian STE20-like protein kinase 3 (MST3), rs1049216 for caspase 3 (CASP3)] in the placenta and maternal blood of 300 controls with at-term birth and 43 cases of sPTB was performed. No association was identified in genotype frequencies or combinations of foetal/maternal genotypes between single SNPs and sPTB. The risk of sPTB was significantly reduced by physical activity and significantly increased by current hypertensive diseases, premature rupture of membranes (PROM) or preterm PROM (P-PROM) and previous sPTB. The TT/GA genotype of JNK/CASP3 in maternal blood and maternal health status (current hypertensive diseases, current PROM/P-PROM, previous sPTB) were independently associated with sPTB. The present findings suggested that, independently of other maternal factors, pregnant women carrying the TT/GA genotype of JNK/CASP3 were more susceptible to sPTB than women bearing the GT/GA (our reference) genotype; that the apoptotic pathway triggered by oxidative stress was involved; and that genetic and non-genetic factors contributed to sPTB. Knowledge of these aspects may aid to improve the management of pregnancies by indicating the lifestyle to be adopted on the basis of sPTB susceptibility.

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Key words: spontaneous preterm birth, single nucleotide polymorphism, maternal lifestyle factors, maternal health status

Introduction

Although the normal duration of pregnancy is 40 weeks, 5-10% (mean average 8%) of births take place preterm in Europe, with prematurity constituting a main cause of perinatal morbidity and mortality (1). Pregnancy duration is primarily determined by a 'placental clock', which is activated in early stage pregnancy (2) and involves the triggering of several mechanisms including apoptotic events. Apoptosis occurs physiologically in all regions of the human placenta throughout pregnancy and, in its early stages, ensures successful placentation and embryo development; at term increased apoptosis of all villous components indicates initiation of delivery (3).

In response to hypoxia and oxidative stress, the signalling pathways of hypoxia-induced apoptosis in human trophoblasts are considered to involve activation and/or upregulation of certain genes, including c-Jun N-terminal kinase (JNK), a key apoptosis regulatory gene (4,5). JNK serves as a mediator, bridging upstream hypoxia and oxidative stress events and the downstream mammalian STE20-like protein kinase 3 (MST3) gene (the human counterpart of the protein serine/threonine kinase Ste20 in yeast), with subsequent caspase 3 (CASP3) activation (6). In human placentas following at-term births and caesarean deliveries without labour, Wu *et al* (4) identified that MST3 served a crucial role in apoptosis and detected significantly increased MST3 expression, trophoblast apoptosis, oxidative stress and hypoxia, and reported that these events were significantly correlated. In their study, trophoblast apoptosis was induced by hypoxia via nitric oxide synthase activation, which generated diverse components including O_2^- that upregulated MST3 via JNK activation (5). Other previous study has investigated apoptotic genes in the placenta as contributors to spontaneous preterm birth (sPTB). Dutta *et al* (7) screened a panel of phosphorylated proteins to identify potential markers of activation and identified higher JNK phosphorylation in sPTB patients than in patients with premature rupture of membranes, suggesting specific pathways are involved in sPTB.

As apoptosis triggered by oxidative stress may serve a role in the pathophysiology of sPTB, we hypothesized that susceptibility may be linked to genes encoding proteins that are involved in apoptosis regulation. Consequently, single nucleotide polymorphisms (SNPs) affecting expression levels of apoptotic pathway genes (JNK, MST3 and CASP3) could plausibly contribute to the risk of sPTB.

Evidence exists that inflammation serves a role in the pathophysiology of sPTB (8-10). Given that inflammation contributes to the initiation of sPTB, genes encoding proteins involved in the regulation of inflammatory mediators are plausible candidate genes, and genetic polymorphisms affecting inflammatory-associated gene transcription and subsequent expression levels may contribute to the development and progression of inflammatory disorders (11). In this regard, as apoptosis occurs in the inflammatory process, it was hypothesized that mechanisms associated with apoptosis may be involved.

Although previous data demonstrated that genetic factors contributed to gestational length and risk of preterm birth (12), non-genetic factors may also influence oxidative stress and redox system imbalances in the maternal-foetal intrauterine compartment (13). Maternal lifestyle (14) and health status, including diseases with onset prior to or during pregnancy (15), may influence oxidative stress and affect the apoptotic pathway leading to at-term birth or sPTB, both of which also appear consequent to a mechanism involving genetic and non-genetic factors, which may modulate the oxidative stress-induced apoptosis.

For these reasons, the present study first evaluated the role of certain SNPs (JNK: rs7560; MST3: rs9517320; CASP3: rs1049216) in influencing expression of apoptotic pathway genes in the placenta and maternal blood, and subsequently investigated whether maternal lifestyle factors and health status may potentially affect oxidative stress.

Materials and methods

Subjects. The present single-centre study enrolled 300 pregnant women at term (gestational age >37 weeks) as controls and 43 preterm pregnant women (gestational age 22 weeks + 0 days to 36 weeks + 6 days) as sPTB cases on admission to the Department of Obstetrics and Gynaecology of Hospital 'S. Maria della Misericordia' (Perugia, Italy), for spontaneous delivery from November 2013 to June 2015. Gestational age was determined by the last menstrual period and confirmed by ultrasound dating before 20 weeks. Exclusion criteria were twin pregnancies, known genetic foetal anomalies and indicated preterm delivery.

Each case and control completed a self-reported questionnaire to record medical and obstetrical history, maternal lifestyle factors and health status including the onset of diseases prior to or during pregnancy, and smoking and eating habits.

The pregnant women were divided into 5 classes according to the number of Mediterranean diet (MD) criteria fulfilled as described by Meltzer *et al* (16). The present study used five MD criteria as follows: Intake of ≥5 vegetables and fruit per day; ≥2 servings of fish per week; use of olive oil for cooking and salad dressing; ≤2 servings of red meat per week; and ≤2 cups of coffee per day.

The following pathologies with onset during pregnancy were analysed: Genital and urinary tract infections, current hypertensive diseases (high blood pressure, preeclampsia, haemolysis/elevated liver enzymes/low platelet count syndrome) and PROM or preterm PROM (P-PROM). sPTBs in previous pregnancies were also considered.

Written informed consent was obtained from all participants. The study protocol was approved by the Medical Ethics Committee of the Region of Umbria (CEAS Umbria; approval no. 2157113). The experimental procedures adhered to the ethical standards for human experimentation of the 1975 Declaration of Helsinki (revised in 1983).

Selection of SNPs. To select SNPs a computational analysis was performed using the polymiRTS database 3.0 (http://compbio.uthsc.edu/miRSNP/) to identify genetic variants responsible for transcriptional variation (17) and the Preterm Birth database (dbPTB) (http://ptbdb.cs.brown.edu/dbPTBv1.php) to identify links with sPTB. Candidate SNPs were selected for analysis based on biological plausibility [their position in micro (mi)RNA target sites] and their potential role in sPTB.

DNA sampling and genotyping. Placenta tissue samples at time of delivery were frozen in liquid nitrogen within 10 min of delivery and stored at -80˚C until further analysis. Following delivery, 10 ml venous blood was drawn from each participant, collected in tubes containing EDTA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), immediately centrifuged at 1,600 x g per 10 min at room temperature to separate the buffy coat from plasma and frozen at -80˚C for subsequent DNA extraction.

DNA was extracted from placenta tissue with a QiAmp DNA Mini kit (Qiagen GmbH, Hilden, Germany) and from the buffy coat with a NucleoSpin Blood kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany) according to the manufacturer's instructions.

SNP genotyping was performed by real-time polymerase chain reaction (7300 Real Time PCR System; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using TaqMan SNP Genotyping Assays (Thermo Fisher Scientific, Inc.). The following polymorphisms were analysed: JNK -rs7560 (T>G), MST3 -rs9517320 (A>C) and CASP3 -rs1049216 (A>G) with minor allele frequencies (MAFs) of 0.31, 0.47 and 0.40, respectively. These polymorphisms were selected based on MAF >0.05 to avoid rare polymorphisms and enable identification in our population, and with localization in the sites of interaction of miRNAs, suggestive of an influence on expression by interfering with miRNA function.

The amplification reaction for all SNPs was performed by Real-Time PCR 7300 detection system (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) in a total volume of 20 μl containing 10 μl TaqMan Universal PCR Master Mix [containing dNTPs, uracil-DNA glycosylase (UNG) and appropriate buffer; Thermo Fisher Scientific, Inc.] 5 μl extracted DNA and 1 μl TaqMan SNP Genotyping Assay (Thermo Fisher Scientific, Inc.) specific for each SNP. The thermal profile consisted of a 2-min cycle at 50˚C for UNG treatment, a 10-min cycle at 95˚C for DNA denaturation

Table I. Demographic characteristics of enrolled subjects.

Characteristic	Controls	Cases	P-value
n	300	43	
Age, years	34 (19-46)	32 (20-45)	0.181
Pregravid body mass index, kg/m^2	21.5 (15.8-42.2)	22.4 (16.9-38.1)	0.326
Ethnicity (%)			
Caucasian	285 (96.0)	37 (86.0)	
African	3 (1.0)	3 (7.0)	0.001
Oriental	1 (0.3)	2 (4.7)	
Hispanic	7 (2.4)	0 (0.0)	
Indo-Asian	1 (0.3)	1 (2.3)	
Gestational age at delivery, weeks	39 (37-42)	35 (23-36)	<0.001
Placenta weight, g	575 (358-938)	472 (200-830)	<0.001
Neonatal weight, g	3.340 (1,128-4,570)	2,163 (350-3,420)	<0.001

Data are expressed as the median (min-max) unless otherwise stated.

and 50 cycles of 15 sec at 92˚C and 1 min at 60˚C for DNA denaturation, primer hybridization and probe-DNA targeting and amplification, respectively. In each experiment, unknown samples in a single well and two wells for no template control (NTC) reactions (reactants + sterile distilled water) were amplified for each SNP that was analysed. Following PCR, fluorescence signals were analysed by the Applied Biosystems 7300 Real Time-PCR System (software 7300 System v1.4.0) using TaqMan SNP Genotyping assay to determine the distribution of gene-specific polymorphisms in the study population. For each sample, a unique pair of fluorescent dye detectors is used: One fluorescent dye detector is a perfect match to the wild-type (allele 1, VIC 551 nm) and the other fluorescent dye detector is a perfect match to the mutation (allele 2, FAM 517 nm). Each pregnant woman was classified as homozygous when the polymorphism was present in both alleles (homozygous for the mutant allele) or in neither of the two alleles (homozygous for the wild-type allele). Subjects were defined as heterozygous when the polymorphism was present in only one allele.

Statistical analysis. Differences in allele/genotype frequencies and categorical variables between cases and controls were analysed using the χ^2 test. The Mann-Whitney U test was used to analyse differences in continuous variables. As variables were distributed asymmetrically, the results were expressed as median, minimum and maximum.

Multiple logistic regression analysis was used to assess the association between the incidence of sPTB and dichotomous variables (presence/absence of alleles), in which odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. The logistic regression model predicts the probability of disease according to the explanatory variables. Our multivariate analysis selected variables (genetic factors, maternal lifestyle and health status) with a statistical difference between sPTB cases and controls reaching P<0.25 in a univariate analysis. Data analysis was performed using IBM SPSS version 20.0 (IBM Corp., Armonk, NY, USA).

Results

Demographic characteristics. The current study recruited a total of 343 pregnant women: 300 who delivered at term (controls) and 43 who delivered spontaneously preterm (sPTB). Table I lists the demographic characteristics of these subjects. Age and pre-pregnancy body mass index were similar between the groups. Placenta and neonatal weights were significantly lower (P<0.001) in the sPTB group, confirming premature births. The majority of women in each group was Caucasian, with smaller populations of subjects of African, Oriental, Hispanic and Indo-Asian origin. An increased proportion of African women were included in the sPTB group compared with in the control group (P=0.001).

SNPs: Foetal and maternal genetic aspects. Table II presents the genotype frequencies of all SNPs analysed in placenta and maternal blood samples from cases and controls. No significant differences were identified in genotype distribution between the two groups. The genotype results of paired combinations of the SNPs were combined (JNK/CASP3; JNK/MST3; MST3/CASP3) yielding nine classes for each combination in placenta and maternal blood, respectively. No significant inter-group differences were identified in any of these combinations (Tables III and IV).

To determine whether foetal/maternal genotype interactions impacted on the risk of sPTB, placenta (foetal) and maternal blood (maternal) genotypes were combined for each SNP, obtaining nine classes of foetal/maternal genotypes. The foetal/maternal genotype frequencies for the SNPs of the JNK, MST3 and CASP3 genes did not differ significantly between the controls and sPTB cases (Table V).

Maternal lifestyle factors. With regard to maternal lifestyle factors, the majority of controls and sPTB cases declared that they had never smoked (cases, 79.1%; controls, 70.3%); a percentage of each group stated they gave up smoking immediately following conception (cases, 11.6%; controls,

Table II. Genotype frequencies of analysed single nucleotide polymorphisms in placenta and maternal blood samples of cases and controls.

		Genotype frequencies										
		JNK				MST3				CASP3			
Allele 1/allele 2		G/T	G/G	T/T	P-value	A/C	A/A	C/C	P-value	G/A	G/G	A/A	P-value
Placenta													
Controls	n	116	29	155	0.277	143	56	100	0.764	118	15	167	0.358
	%	38.7	9.7	51.7		47.8	18.7	33.4		39.3	5.0	55.7	
Cases	n	22	4	17		18	9	16		19	4	20	
	%	51.2	9.3	39.5		41.9	20.9	37.2		44.2	9.3	46.5	
Maternal blood													
Controls	n	108	39	153	0.965	139	55	106	0.414	108	18	174	0.409
	%	36.0	13.0	51.0		46.3	18.3	35.3		36.0	6.0	58.0	
Cases	n	16	5	22		24	8	11		20	2	21	
	%	37.2	11.6	51.2		55.8	18.6	25.6		46.5	4.7	48.8	

JNK, c-Jun N-terminal kinase; MST3, mammalian STE20-like protein kinase 3; CASP3, caspase 3.

Table III. Genotype frequencies of paired single nucleotide polymorphisms in placenta samples of cases and controls.

		JNK/CASP3									
Placenta		GT/GA	GG/GA	TT/GA	GT/AA	GG/AA	TT/AA	GT/GG	GG/GG	TT/GG	P-value
Controls	n	49	9	52	9	3	3	69	17	89	0.145
	%	16.3	3.0	17.3	3.0	1.0	1.0	23.0	5.7	29.7	
Cases	n	10	2	7	1	0	3	11	2	7	
	%	23.3	4.7	16.3	2.3	0.0	7.0	25.6	4.7	16.3	

		JNK/MST3									
Placenta		GT/AC	GT/AA	GT/CC	GG/AC	GG/AA	GG/CC	TT/AC	TT/AA	TT/CC	P-value
Controls	n	53	12	72	14	9	32	54	8	45	0.459
	%	17.7	4.0	24.1	4.7	3.0	10.7	18.1	2.7	15.1	
Cases	n	12	1	5	4	1	4	6	2	8	
	%	27.9	2.3	11.6	9.3	2.3	9.3	14.0	4.7	18.6	

		MST3/CASP3									
Placenta		AC/GA	AC/GG	AC/AA	AA/GA	AA/GG	AA/AA	CC/GA	CC/GG	CC/AA	P-value
Controls	n	63	18	36	7	5	3	72	33	62	0.258
	%	21.1	6.0	12.0	2.3	1.7	1.0	24.1	11.0	20.7	
Cases	n	5	4	10	3	1	0	10	4	6	
	%	11.6	9.3	23.3	7.0	2.3	0.0	23.3	9.3	14.0	

JNK, c-Jun N-terminal kinase; MST3, mammalian STE20-like protein kinase 3; CASP3, caspase 3.

20.0%) and smaller percentages of each group had continued smoking during pregnancy (cases, 9.3%; controls, 9.7%). No statistical significance was identified regarding smoking status in the sPTB group versus the control group (P=0.407). No

Table IV. Genotype frequencies of paired single nucleotide polymorphisms in maternal blood samples of cases and controls.

		JNK/CASP3									
Maternal blood		GT/GA	GG/GA	TT/GA	GT/AA	GG/AA	TT/AA	GT/GG	GG/GG	TT/GG	P-value
Controls	n	38	17	53	4	3	11	66	19	89	0.194
	%	12.7	5.7	17.7	1.3	1.0	3.7	22.0	6.3	29.7	
Cases	n	3	3	14	0	0	2	13	2	6	
	%	7.0	7.0	32.6	0.0	0.0	4.7	30.2	4.7	14.0	

		JNK/MST3									
Maternal blood		GT/AC	GT/AA	GT/CC	GG/AC	GG/AA	GG/CC	TT/AC	TT/AA	TT/CC	P-value
Controls	n	43	23	72	18	7	30	47	9	51	0.379
	%	14.3	7.7	24.0	6.0	2.3	10.0	15.7	3.0	17.0	
Cases	n	11	1	12	1	2	5	4	2	5	
	%	25.6	2.3	27.9	2.3	4.7	11.6	9.3	4.7	11.6	

		MST3/CASP3									
Maternal blood		AC/GA	AC/GG	AC/AA	AA/GA	AA/GG	AA/AA	CC/GA	CC/GG	CC/AA	P-value
Controls	n	50	23	35	8	5	5	81	27	66	0.827
	%	16.7	7.7	11.7	2.7	1.7	1.7	2.7	9.0	22.0	
Cases	n	10	4	6	1	1	0	13	3	5	
	%	23.3	9.3	14.0	2.3	2.3	0.0	30.2	7.0	11.6	

JNK, c-Jun N-terminal kinase; MST3, mammalian STE20-like protein kinase 3; CASP3, caspase 3.

pregnant woman fulfilled all of the MD criteria. The majority of controls (58.0%) had a good MD (3 or 4 criteria fulfilled) while the majority of sPTB cases (60.5%) had a mediocre MD (1 or 2 criteria fulfilled). This difference tended towards significance (P=0.069). A significant difference was identified in the practice of physical activities. Unlike the majority of controls (51.5% practising physical activity), the majority of sPTB women (69.8%) did not practice any physical activities P=0.014). Urinary and genital tract infections were similarly distributed between the groups (cases, 7.0%; controls, 11.0%; P=0.596) while hypertensive diseases and PROM/P-PROM were significantly more frequent among sPTB women (9.3 and 32.6%, respectively; P=0.001; P<0.001) than controls (0.3 and 2.0%, respectively). The frequency of sPTB in previous pregnancies was similar in both groups (cases, 4.7%; controls, 1.0%; Table VI).

Combinations of genetic and lifestyle factors. The JNK/CASP3 genotype combination in maternal blood, maternal age, physical activity, diet, current hypertensive diseases, current P-PROM/PROM and previous sPTB were the selected variables to be analysed. The GT/GA genotype was selected as the reference genotype. The TT/GA genotype of JNK/CASP3 was significantly associated with sPTB; pregnant women carrying the TT/GA genotype had higher risk of sPTB than those with the reference genotype GT/GA (OR, 5.7; P=0.037) independently of all other maternal factors. Physical activity reduced the risk of sPTB (OR, 0.309; P=0.009). Current hypertensive diseases, PROM/P-PROM in the current pregnancy and previous sPTB significantly increased the risk of sPTB (ORs, 47.2, 65.6 and 11.7; P=0.004, <0.001 and 0.03, respectively; Fig. 1).

Discussion

In the current preliminary study investigating SNPs in genes involved in placental apoptosis in at-term births and sPTB, SNPs were selected by computational analysis using the polymiRTS database 3.0 (17) and dbPTB, and PubMed to search for literature supportive of the database findings. To the best of our knowledge, the current study has evaluated SNPs, namely rs7560, rs9517320 and rs1049216 SNPs of the JNK, MST3 and CASP3 genes, respectively, that have not been studied previously in the context of sPTB.

The JNK variant rs7560 is a G/T single-nucleotide variation on human chromosome 14. The long arm of chromosome 14 contains protein-coding genes and two loci of key importance to the immune system; more than 60 disease-associated genes have been localized on several loci of chromosome 14 (18). The MST3 variant rs9517320 is an A/C transversion substitution on human chromosome 13. It participates in the mitogen-activated protein kinase cascade, and this variant

Table V. Genotype frequencies of placenta/maternal blood combinations of analysed SNPs in controls and cases.

Placenta/ maternal blood		JNK									P-value
		GT/GT	GT/GG	GT/TT	GG/GT	GG/GG	GG/TT	TT/GT	TT/GG	TT/TT	
Controls	n	49	30	39	18	7	3	40	1	113	0.329
	%	16.3	10.0	13.0	6.0	2.3	1.0	13.3	0.3	37.7	
Cases	n	10	2	9	1	3	0	6	0	12	
	%	23.2	4.7	20.9	2.3	7.0	0.0	14.0	0.0	27.9	

Placenta/ maternal blood		MST3								P-value
		AC/AC	AC/AA	AC/CC	AA/AC	AA/AA	AA/CC	CC/AC	CC/CC	
Controls	n	66	34	42	35	20	1	36	65	0.312
	%	22.1	11.3	14.0	11.7	6.7	0.3	12.0	21.7	
Cases	n	11	4	3	5	4	0	6	9	
	%	25.6	9.3	7.0	11.6	9.3	0.0	14.0	20.9	

Placenta/ maternal blood		CASP3							P-value
		GA/GA	GA/GG	GA/AA	GG/GA	GG/GG	AA/GA	AA/AA	
Controls	n	61	10	47	7	8	40	127	0.505
	%	20.3	3.3	15.7	2.3	2.7	2.3	42.3	
Cases	n	10	2	6	3	1	8	13	
	%	23.3	4.7	14.0	7.0	2.3	18.6	30.2	

JNK, c-Jun N-terminal kinase; MST3, mammalian STE20-like protein kinase 3; CASP3, caspase 3.

is considered as a longevity variant, based on its potential association with the life-spans of individuals sharing similar environmental and living conditions (19). The CASP3 variant rs1049216 is a G/A transition substitution on human chromosome 4. It is located in the 3'UTR and, through ribosome binding, initiation or elongation, may alter mRNA transcript stability (20). In putative microRNA target sites, these SNPs may serve a biological role in regulating post-transcriptional gene expression through mRNA destabilization or translation repression, thus impacting on gene functions and phenotypes that are involved in trophoblast apoptosis. Consequently, they may underlie sPTB risk in pregnant women.

The present findings demonstrated that, in the placenta and maternal blood, any of the three individual SNPs or their paired combinations (JNK/CASP3; JNK/MST3; MST3/CASP3) in placenta or maternal blood samples were not associated with sPTB susceptibility. Furthermore, no significant associated was identified when the placental genotype was combined with the maternal for each SNP in order to assess the foetal-maternal interaction.

Since sPTB involves complex interactions between SNPs, maternal lifestyle and health status (21), the present study also investigated non-genetic factors. Although sPTB did not associate with smoking or diet, a significant association was identified with physical activity, in concurrence with a previous report that physical exercise could decrease the risk of sPTB (22). Multivariate analysis indicated that the risk of sPTB was higher in pregnant women carrying the TT genotype for JNK and the GA genotype for CASP3, and in pregnant

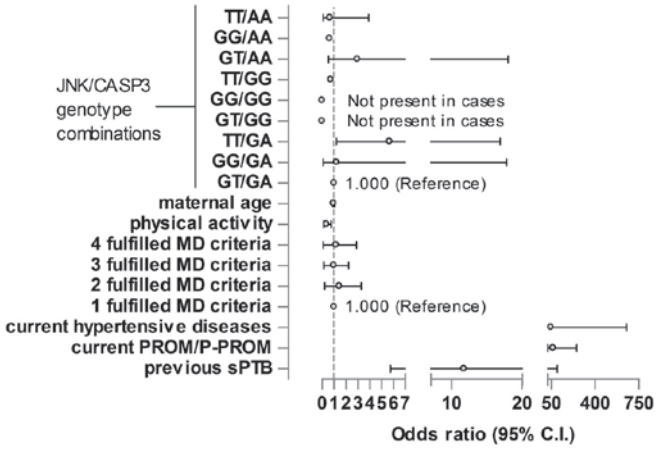

Figure 1. Multivariate analysis of JNK/CASP3 genotypes in maternal blood and maternal characteristics (age, physical activity, eating habits, current health status and previous sPTB). JNK, c-Jun N-terminal kinase; CASP3, caspase 3; sPTB, spontaneous preterm birth; MD, Mediterranean diet; (P-) PROM, (preterm) premature rupture of membranes; C.I., confidence interval.

Table VI. Maternal lifestyle factors and maternal health status during and prior to pregnancy in the studied groups.

Maternal lifestyle factor	Controls, n (%)	Cases, n (%)	P-value
Smoking			
Never smoked	211 (70.3)	34 (79.1)	0.407
Active smoker	29 (9.7)	4 (9.3)	
Ex-smoker	60 (20.0)	5 (11.6)	
Eating habits according to Mediterranean diet criteria			
1 criterion	18 (6.0)	6 (14.0)	0.069
2 criteria	108 (36.0)	20 (46.5)	
3 criteria	121 (40.3)	13 (30.2)	
4 criteria	53 (17.7)	4 (9.3)	
Physical activity			
Absent	145 (48.5)	30 (69.8)	
Present	154 (51.5)	13 (30.2)	0.014
Maternal health status	**Controls, n (%)**	**Cases, n (%)**	**P-value**
Current urinary and genital tract infections			
Absent	267 (89.0)	40 (93.0)	0.596
Present	33 (11.0)	3 (7.0)	
Current hypertensive diseases			
Absent	299 (99.7)	39 (90.7)	0.001
Present	1 (0.3)	4 (9.3)	
Current PROM/preterm PROM			
Absent	294 (98.0)	29 (67.4)	<0.001
Present	6 (2.0)	14 (32.6)	
Previous spontaneous preterm birth			
Absent	297 (99.0)	41 (95.3)	0.120
Present	3 (1.0)	2 (4.7)	

PROM, premature rupture of membranes.

women with current hypertensive diseases, PROM/P-PROM or previous sPTB. Current urinary and genital tract infections did not appear to impact on sPTB risk.

In investigating the etiopathogenesis of sPTB, studies have focused on genetically determined molecular mechanisms in the inflammatory process (23,24), identifying that cytokine gene polymorphisms were related to sPTB (24). Placental apoptosis may also be involved: An imbalance between reactive oxygen species and antioxidants may result in excessive oxidative stress that triggers trophoblast apoptosis and initiates labour by upregulating expression of the JNK, MST3 and CASP3 genes, as depicted in Fig. 2.

Consequently, as in the inflammatory process, SNPs were hypothesized to influence genes involved in placental apoptosis in the current study. It was also speculated that sPTB susceptibility may be increased by the combination of foetal and maternal genotypes. Romero *et al* (25) observed that different combinations of maternal and foetal genetic variants [including those of the genes interleukin-6 receptor (IL6R1), tissue inhibitor of metalloproteinases 2 (TIMP2), insulin like growth factor 2 (IGF2) and collagen type iv alpha 3 chain (COL4A3)] involved in inflammation and extracellular matrix metabolism, influenced sPTB susceptibility

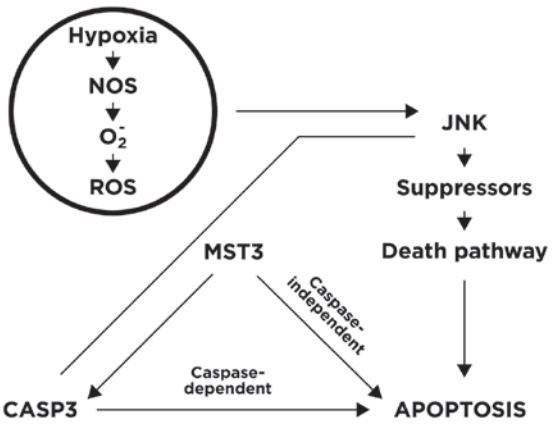

Figure 2. Schematic diagram detailing the hypoxia-induced signaling pathway in placental apoptosis. NOS, nitric oxide synthase; ROS, reactive oxygen species; JNK, c-Jun N-terminal kinase; MST3, mammalian STE20-like protein kinase 3, CASP3, caspase 3.

differently. Notably, rs8192282 in foetal IL6R1 significantly doubled the risk of sPTB (OR=2.07, 95% CI=1.42-3-02; P=0.000148). Furthermore, haplotypes for COL4A3 in the mother and for IGF2 and IL2 in the foetus were associated with preterm labour/delivery (25). Similar findings were reported by Ota *et al* (26) and Roberts and Cooper (27), with these studies demonstrating a foetal-maternal interaction in the aetiology of preeclampsia. In pregnancy the interaction between maternal and foetal genetic backgrounds may also influence a polymorphism-pathology association and increase sPTB susceptibility independently or synergistically. The significant association with combined JNK and CASP3 genotypes suggested the apoptotic pathway was involved in the pathogenesis of sPTB. Additionally, the identification that maternal lifestyle and health status factors were involved in sPTB confirmed a multifactorial mechanism involving genetic and non-genetic factors.

The present study considered SNPs that, to the best of our knowledge, have not been studied previously; all of which have associations with the apoptotic pathway that triggered by oxidative stress, with maternal lifestyle factors and with health status. However, there were limitations to the current study. Firstly, the cohort of women with sPTB was relatively small, considering the frequency of preterm births and the MAF of each SNP. The lack of significant association between any single SNP and sPTB risk may have thus been due to the low number of women in the sPTB group. However, even with the present sample size, subtle difference in ethnicity was highlighted, which was analysed only in terms of inter-group frequencies, but emerged as statistically significant. Indeed, these preliminary results concur with those of Dunlop *et al* (28), who reported that the incidence of PTB was significantly higher among African-American women. Since the present study detected some significant associations and trends towards significance in other parameters, confirmation of the findings and expanding the scope of results by recruiting more pregnant women at risk of or having experienced sPTB is warranted in the future. Secondly, as phenotype is a function of a combination of alleles, some of which may increase/decrease the risk of disease, multiple genetic interactions in the process of apoptosis may serve a greater role in disease susceptibility than any single variant. This hypothesis is the basis of future planned investigation into the combination of these SNPs in a consistent number of individuals to identify predictive markers of sPTB.

In conclusion, considering that sPTB is multifactorial, further studies are warranted to confirm the present results in a larger cohort of women at risk. The ultimate aim is to improve pregnancy care by introducing screening tests for sPTB and drawing up guidelines on maternal lifestyle to reduce the risk of sPTB.

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FT and EP contributed to conception and design of the study, processed all placenta and blood samples, performed the SNP genotyping and were primarily responsible for the writing of the manuscript. VB performed the statistical analysis and interpreted the results. GC enrolled the pregnant women and aided in writing the manuscript. SM, CA and IG were responsible for placenta and blood sampling and contributed to the interpretation of data. MC performed SNP selection via the database searches and contributed to the acquisition of patient demographic data. GCDR contributed to conception and design of the study and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All participants provided written informed consent for participation in the study. The study protocol was approved by the Ethics Committee of Region Umbria (approval no. 2157113). The experimental procedures adhered to the ethical standards for human experimentation of the 1975 Declaration of Helsinki (revised in 1983).

Consent for publication

Patients provided written informed consent for the publication of associated data.

Competing interests

The authors declare that they have no competing interests.

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