Ind. J. Phys. Anthrop & Hum. Genet. Vol. 42, No. 2, (2023) : 101-109

GLOBAL AND *MTHFR* GENE SPECIFIC METHYLATION PATTERNS IN PRETERM PREMATURE RUPTURE OF MEMBRANES: A HOSPITAL-BASED CASE CONTROL STUDY (INDIA)

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ABSTRACT

The subjects comprised of preterm premature rupture of membranes (PPROM) cases and gestation matched controls. All the participants provided a fasting blood sample (5ml), which was then processed for DNA extraction, MTHFR gene- specific methylation, and global DNA methylation. PCR and RFLP were used to examine the MTHFR C677T polymorphism. Levels of folate and vitamin B12 were estimated.

Preterm premature rupture of membranes cases were characterised with hypo global DNA methylation levels (p <0.0001) and hyper MTHFR *gene specific methylation (p= 0.005) at the fetal front as compared to controls. Similar patterns were observed among PPROM cases with folate repletion. It was discovered that PPROM cases with the MTHFR CC genome had considerably hypo global DNA methylation (p=0.02) and hyper MTHFR gene-specific methylation (p=0.002) at the foetal front. Intergenerationally, reverse patterns of MTHFR gene-specific methylation and global DNA methylation among PPROM cases and controls were observed at maternal and fetal fronts. It can be concluded that preterm birth seems to be favouring mother as against fetus from the evolutionary perspective.*

Key Words: *Methylation; PPROM; Preterm births; Pregnancy complications;* MTHFR *gene specific methylation; global DNA methylation.*

INTRODUCTION

Preterm birth (PTB) is remarkably a leading cause of perinatal mortality and long term morbidity. Preterm premature rupture of membranes (PPROM) is

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one of the spontaneous types and leading cause of PTBs and is defined as the rupture of the fetal membranes before 37 weeks of gestation. PPROM is considered to be a multifactorial condition. It remains a critical public health problem and is one of the pivotal factors associated with adverse pregnancy outcomes. Despite of extensive advancements in perinatal care, PPROM is still prevalent as an important obstetrical complication. Moreover, the challenges posed by PPROM to obstetricians and paediatricians, demand more studies to find out the risk factors adding to the etiology of PPROM. Studies have suggested a link between babies born preterm and future risk of development of cardiovascular diseases (Bavineni *et al*., 2019: 1107-1112). In addition, studies have also reported an association of preterm, premature infants with type 2 diabetes, high blood pressure, and stroke in adulthood (Barker *et al*., 1993: 62-67; Lawlor *et al*., 2005: 1414-1418). It has also been reported that mothers with high intakes of folic acid and vitamin B12 deficiency are having increased risk of type 2 diabetes in the offspring suggesting that defects in one- carbon metabolism might involve intrauterine programming of adult disease (Yajnik and Deshmukh 2008: 203-211). Altered DNA methylation in PTB is suggested to be a causal factor for adverse long-term health consequences (Maddalena 2013: 137-139). One carbon metabolic pathway (OCMP) is the major source of methyl groups whereas folate acts as one of the critical cofactors playing a major role in the fetal epigenetic programming (Crider *et al*., 2011: 370-384). Global DNA methylation patterns established during embryogenesis remain largely unchanged in adult cells (Finnell *et al*., 2002: 181-208). Further, methylenetetrahydrofolate reductase (MTHFR) is responsible for catalyzing the reduction of 5,10- methylenetetrahydrofolate to 5- methyltetrahydrofolate involving S- adenosyl- L- methionine (SAM) as a methyl donor. Moreover, MTHFR *C677T* polymorphism results in a thermolabile enzyme with reduced activity that is predicted to influence DNA methylation status. Hence, the present study aims to evaluate and understand the association and intergenerational variation of global DNA methylation and MTHFR gene specific methylation in PPROM in light of MTHFR *C677T* polymorphism, folate and vitamin B12 levels.

MATERIALS AND METHODS

PPROM cases and controls were recruited from the Department of Obstetrics and Gynaecology, Lady Hardinge Medical College, and Smt. S.K Hospital, New Delhi, India (after obtaining the ethical clearance). Blood Samples were collected from cases that include women with PPROM (n= 16) <37 weeks of gestational age and their age and gestation matched controls (n=20) after obtaining informed written consent. From the above-recruited cases and controls, placentae of 11 PPROM cases and 11 controls were also collected. The selection criteria are mentioned in Table-1.

Table-1: Details and selection criteria of the recruited participants

	Gestation	Selection criteria	Tissue type	No. of samples
PPROM Cases	20-34 weeks	**Inclusion**: Women presented with a diagnosis of spontaneous rupture of the fetal (chorio-amniotic) membranes with gestation ranging from 20 to 34 weeks. Non reactive for HIV, HBsAg and VDRL	Blood	16
			Placenta	11
		Exclusion: Women with polyhydramnios, multiple gestation, positive for high vaginal swab (HVS), any uterine tract infection (UTI) and bacterial infection. Clinical evidence of chorioamnionitis and elevated leucocyte counts. The women with Pregnancy induced hypertension (PIH) and diabetes.		
Controls	20-34 weeks	**Inclusion:** Women with one or more consecutive normal deliveries (gestation ranging from 20 to 34 weeks)	Blood	20
			Placenta	11
		Non reactive for HIV, HBsAg and VDRL		
		The controls were followed up till successful pregnancy outcome.		
		Exclusion: Women with the history of any pregnancy complications. The women with Pregnancy induced hypertension (PIH) and diabetes.		

DNA was extracted from blood samples using the protocol given by *Miller et al., 1988*, whereas placental genomic DNA extraction was done using Qiagen Blood and Tissue kit followed by MTHFR *C677T* polymorphism genotyping (Frosst *et al*., 1995: 111-113). MTHFR gene-specific promoter region methylation was done by mass array technique, MALDI TOF Sequenom (ACE-PROBE) which captures 17 CpG sites in the 1st island of the MTHFR gene promoter region (Mishra *et al*., 2019: 68-73) and global DNA methylation by Epigentek Methylflash kit (Epigentek Group Inc., New York, U.S.A.). Serum folate and vitamin B12 levels were estimated using the chemiluminiscence technique (Immulite 1000, Seimens). As the data in the present study data were found to be skewed; median levels have been calculated for the data analysis.

RESULTS

In the placental tissues (fetal front), PPROM cases were having a significantly higher MTHFR gene specific methylation ($p= 0.005$) and lower global DNA methylation ($p<0.0001$) as compared to controls while at the maternal front; no such differences were observed between PPROM cases and controls (Table-2).

Table-2: Global and MTHFR gene-specific methylation at the maternal and foetal fronts in PPROM cases and gestation-matched controls.

	PPROM cases Median (IQR)	Controls Median (IQR)	Mann Whitney TestP value (cases vs controls)
Blood (global)	0.49 (0.22- 1.08)	0.59 (0.26- 0.72)	1
Blood (MTHFR gene specific)	5 (5- 6)	5 (4.71- 5.94)	0.7
Placenta (global)	0.18 (0.14- 0.27)	0.98 (0.42- 1.22)	<0.0001
Placenta (MTHFR gene specific)	7.75 (6.96- 9.01)	6 (5.77- 6.71)	0.005
Mann Whitney TestP value	**P_1** 0.008**P_2**<0.0001	**P_3** 0.04**P_4** 0.06	
Folate Normal			
Blood (global)	0.39 (0.19- 1.16)	0.53 (0.22- 0.72)	0.83
Blood (MTHFR gene specific)	5 (5-6)	4.92 (4.6- 5.79)	0.52
Placenta (global)	0.2 (0.15- 0.28)	0.97 (0.42- 1.06)	<0.0001
Placenta (MTHFR gene specific)	7.71 (6.6- 8.31)	6.17 (5.4- 6.83)	0.02
Mann Whitney TestP value	**P_1** 0.02**P_2** 0.001	**P_3** 0.09**P_4** 0.15	
Vitamin B12 Normal			
Blood (global)	0.32 (0.19- 1.16)	0.35 (0.16- 0.56)	0.65
Blood (MTHFR gene specific)	5 (4- 6)	4.54 (4.38- 6.39)	0.79
Placenta (global)	0.23 (0.18- 0.26)	0.96 (0.71- 1.13)	0.02
Placenta (MTHFR gene specific)	7.71 (7.31- 8.5)	6.67 (4.25- 8)	0.17
Mann Whitney TestP value	**P_1** 0.23**P_2** 0.005	**P_3** 0.02**P_4** 0.41	
Vitamin B12 Deficiency			
Blood (global)	0.59 (0.27- 0.99)	0.64 (0.36- 0.95)	0.89
Blood (MTHFR gene specific)	5 (5- 6)	5.04 (4.85- 5.94)	0.49
Placenta (global)	0.17 (0.13- 0.31)	0.98 (0.36- 1.23)	0.006
Placenta (MTHFR gene specific)	8.42 (5- 9.6)	5.96 (5.75- 6.58)	0.29
Mann Whitney TestP value	**P_1** 0.02**P_2** 0.28	**P_3** 0.58**P_4** 0.13	

p value significant at d" 0.05; P_1:within PPROM cases maternal vs fetal global DNA methylation, P_2: within PPROM cases maternal vs fetal MTHFR gene specific methylation, P_3: within controls maternal vs fetal global DNA methylation, P_4: within PPROM controls maternal vs fetal MTHFR gene specific methylation

To understand the intergenerational effect, cases and controls at the maternal front are compared with their respective fetal fronts w. r. t. global and MTHFR gene-specific methylation levels (Table-3). Among the PPROM cases, global DNA methylation at the fetal front is found to be significantly lower as compared to that of the maternal front (p= 0.008). However, MTHFR gene-specific methylation is found to be significantly high (p< 0.0001) in the placental tissue as compared to that of the maternal front. A reverse trend was observed among the controls, wherein global DNA methylation (p= 0.04) and MTHFR gene-specific methylation (p= 0.06) at the fetal front is found to be significantly higher as compared to that of maternal front.

Similar trends were seen when PPROM cases and controls with folate

repletion were analysed. In the placental tissues, PPROM cases with normal vitamin B12 levels and deficiency were having a significantly lower global DNA methylation (p= 0.02; p= 0.006), while no significant differences were observed in the MTHFR gene specific methylation both among PPROM cases and controls matched for age and gestation. Intergenerationally, among the PPROM cases with vitamin B12 deficiency, global DNA methylation at fetal fronts is found to be significantly lower as compared to that of maternal front (p= 0.02).

On analysing the effect of MTHFR *C677T* gene polymorphism on global and MTHFR gene specific methylation, at the fetal front, PPROM cases carrying CC genotype were found to have significantly lower levels of global DNA methylation (p= 0.002) and higher MTHFR gene specific methylation (p= 0.02) as compared to controls (p=0.002). No such differences were observed at the maternal front. *MTHFR* gene specific methylation does not seem to vary with *MTHFR C677T* genotypic status of the women, be it PPROM cases or controls. Intergenerationally, within the PPROM cases, MTHFR gene specific methylation is found to be significantly (p= 0.001) high in the placental tissue as compared to that of maternal front and no such differences were observed in global DNA methylation patterns.

Table-3: Global and *MTHFR* gene specific methylation of Maternal and Placental DNA among PPROM cases and gestation matched controls with respect to *MTHFR* gene *C677T* polymorphism

MTHFR C677T genotypes (maternal DNA)	PPROM CASES		CONTROLS		P value (Mann Whitney test)
	MTHFR GENE SPECIFIC	GLOBAL	MTHFR GENE SPECIFIC	GLOBAL	
CC	5 (4.25- 6)	0.48 (0.22- 1.08)	4.92 (4.67- 5.42)	0.59 (0.35- 1.02)	p_1 – 0.76 p_2 – 0.68
CT	6 (5.25- 6)	0.55 (0.22- 1.07)	5.75 (4.88- 6.83)	0.48 (0.14- 0.66)	p_3 - 1 p_4 – 0.41
P value (Mann Whitney test)	CC-CT= 0.26	CC-CT= 0.86	CC-CT= 0.19	CC-CT= 0.39	
MTHFR C677T genotypes (placental DNA)					
CC	7.88 (6.6- 9.3)	0.18 (0.14- 0.26)	5.92 (5.04- 6.67)	0.98 (0.39- 1.18)	P_5 -0.02 P_6 -0.002
CT	7.67	0.22 (0.11- 0.31)	6.63 (6.04- 7.27)	0.74 (0.42- 1.34)	P_7 – 0.4 P_8 – 0.1
P values	CC-CT= 0.67	CC-CT= 1	CC-CT= 0.13	CC-CT= 0.87	

p values for PPROM cases vs controls (**In maternal DNA:** P_1 –gene specific methylation w.r.t. CC genotype, P_2 –global DNA methylation w.r.t. CC genotype, P_3 –gene specific methylation w.r.t. CT genotype, P_4 global DNA methylation w.r.t. CT genotype **In placental DNA:** P_5 - gene specific methylation w.r.t. CC genotype, P_6- global DNA methylation w.r.t. CC genotype, P_7 gene specific methylation w.r.t. CT genotype, P_8- global DNA methylation w.r.t. CT genotype) (p value significant at d" 0.05).

DISCUSSION

PTB has become a global health challenge including enormous burden on society. The neonatal mortality and morbidity associated with PTB has been linked to long term adverse health consequences (Bavineni *et al*., 2019: 1107-1112). Placenta serves as a blue print for intrauterine life (Marsit *et al*., 2012: 854-

860) and reflects the highest variability in global DNA methylation as compared to other tissues (Christensen *et al*., 2009: e1000602). The hypo global DNA methylation observed in the placenta of PPROM cases is in concordance with a study (Schuster *et al*., 2019: 1-12) revealing hypo methylation in placental tissue. Similar findings with respect to MTHFR gene specific methylation were observed in the case of preeclampsia (Mishra *et al*., 2019: 68-73). But, unlike preeclampsia where the women have double the risk for developing stroke and heart diseases and are 3 to 4 times more at risk for high blood pressure later in life, no such future complications have been reported in the case of women with PPROM (Turbeville and Sasser 2020: 1315-1326).

The differences in the methylation patterns of both global and *MTHFR* gene specific methylation in the fetal tissue between PPROM cases and controls could be a triggering factor for spontaneous rupture of membranes and preterm birth. Thus, PTB could be an adaptive response to the aberrant methylation patterns at the fetal end and a strategy to prioritize mother's survival for increased chances of having successful subsequent pregnancies (Pike 2005: 55-65).

Aberrant methylation patterns in the placental tissues of PPROM cases would deprive the fetus from obtaining enough nutrients through placenta. This results in altered metabolic programming, impaired nutritional environment and decreased energy metabolism (Sookoian *et al*., 2013: 531-542). Alterations in DNA methylation have been linked to early life events and late diseases risk (Godfrey *et al*., 2007: 5-10).

The opposing trends of intergenerational variations between PPROM cases and controls are in agreement with maternal- fetal conflict hypothesis (Haig 1993: 495-532); that highlights the maternal interest which lies in producing as many healthy progenies by limiting her expenditure on single progeny hence marks the evolutionary perspectives deciding the timing of the birth. These observations suggest the association of babies born preterm for the development of adverse consequences and long term sequalae of non-communicable diseases in future. The results of the present study hints towards the evolutionary perspective of PTB which seems to be favouring maternal front as against fetal front (Figure-1). This evolutionary perspective against fetus is justified by the fact that PTB is a significant contributor to less than five year children mortality and is a leading cause of neonatal mortality (Cupen *et al*., 2017: 108). A few studies have shown no association between MTHFR *C677T* polymorphism and decreased global DNA methylation (Narayanan *et al*., 2004: 1436-1443; Ono *et al*., 2012: 2159-2164). Further, in the present study, fetal hypo global DNA methylation does not seem to get altered with folate repletion questioning the indiscriminate supplementation of folate (Mishra *et al*., 2020: e23388).

Revealing the pathophysiology, in terms of unfolding the epigenetic mechanisms (global and MTHFR gene specific methylation) of PPROM would aid in designing and implementing preventive and therapeutic strategies to control this condition. The study strengthens the role of altered global DNA

methylation patterns in the placenta far beyond intrauterine environment; in programming the future health of the baby delivered preterm.

The limitation of the present study is the small sample size. Moreover, the cause and effect relationship between PPROM and methylation can be evaluated through pregnancy follow up studies with larger sample size.

CONCLUSIONS

The opposing trends of intergenerational variations among PPROM cases and controls could possibly be leading to preterm premature rupture of membranes which in turn may be favouring mother as against fetus from the evolutionary perspective. PPROM babies seem to carry an epigenetic burden/load which might make them susceptible to future onset of non- communicable diseases as opposed to mothers who get a chance for subsequent pregnancy. The results of the present study are in line with Developmental Origins of Health and Disease (DOHaD) Hypothesis.

ACKNOWLEDGEMENTS

The authors are extremely thankful to the Department of Biotechnology, Government of India for the financial support. (LETTER NO/Award No. BT/PR14504/MED/12/481/2010, Department of Anthropology, University of Delhi).

CONFLICT OF INTEREST: All the authors have no conflict of interest to declare.

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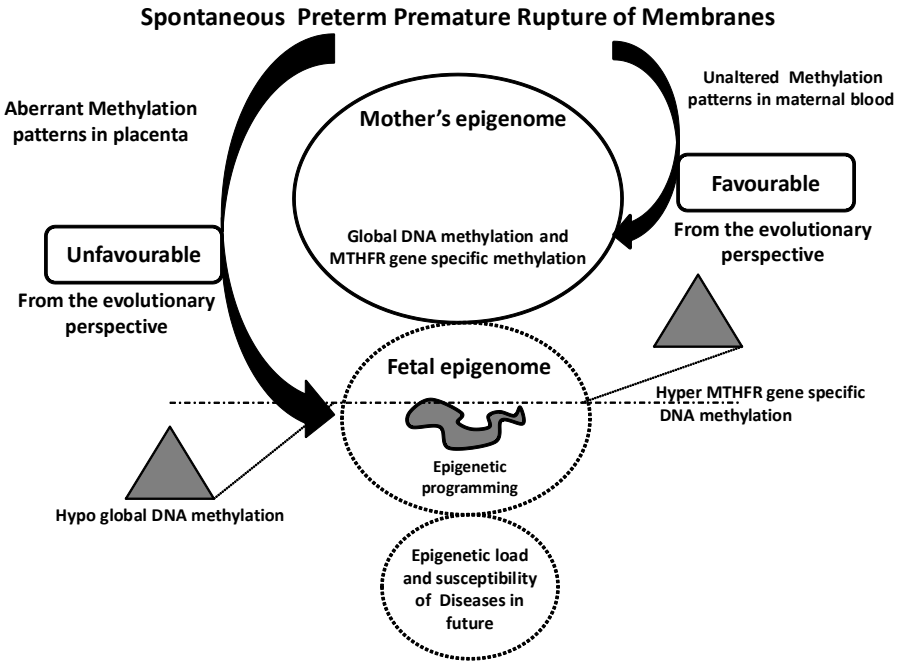
Spontaneous Preterm Premature Rupture of Membranes
Aberrant Methylation patterns in placenta
Mother's epigenome
Unaltered Methylation patterns in maternal blood
Favourable
From the evolutionary perspective
Global DNA methylation and MTHFR gene specific methylation
Unfavourable
From the evolutionary perspective
Fetal epigenome
Hyper MTHFR gene specific DNA methylation
Epigenetic programming
Hypo global DNA methylation
Epigenetic load and susceptibility of Diseases in future