Matrix metalloproteinases-2, -3 and tissue inhibitors of metalloproteinases-1, -2 in placentas from preterm pregnancies and their association with one-carbon metabolites

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Abstract

Maternal nutrition is an important determinant of one-carbon metabolism and defects in the one-carbon metabolism may lead to poor obstetric outcomes. This study was designed to test the hypothesis that altered intake/metabolism of micronutrients (folic acid and vitamin B₁₂) and docosahexaenoic acid (DHA) contributes to increased homocysteine and oxidative stress leading to altered levels of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in women delivering preterm. We have earlier reported increased vitamin B₁₂, homocysteine, and oxidative stress along with reduced placental DHA in women delivering preterm. In this study, we further examine the placental levels of MMP2, MMP3, TIMP1, and TIMP2 in 75 women delivering at term and 73 women delivering preterm. Placental levels of MMPs and TIMPs were determined by ELISA. Placental MMP2 and MMP3 levels were higher ($P < 0.01$) in women delivering preterm as compared with term. There was no difference in the placental TIMP1 and TIMP2 levels in women delivering preterm and at term. Further placental MMP2 and MMP3 levels were higher ($P < 0.01$) in women with preterm labor as compared with those in labor at term, suggesting that MMPs may favor degradation of extracellular matrix in the placenta during preterm labor. Our study for the first time suggests a crucial role of micronutrients and MMPs in preterm birth. Future studies need to examine if epigenetic modifications through the one-carbon cycle contribute to increased levels of MMPs leading to preterm deliveries.

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Introduction

Maternal nutritional status before and during pregnancy plays a key role in the development of the embryo and fetus and is important for a healthy pregnancy outcome (Simpson *et al.* 2010, Imdad & Bhutta 2012). Both excess and deficiency of micronutrients are suggested to have detrimental effects on fetal tissues and organs (Ashworth & Antipatis 2001). Suboptimal levels of micronutrients like folate or vitamin B₁₂ lead to elevated plasma homocysteine concentrations and are observed to be associated with a greater risk of adverse pregnancy outcomes (Allen 2005). Marginal folate and elevated maternal homocysteine levels impair cellular growth and replication in the fetus and placenta, and could increase the risk of preterm birth (Scholl *et al.* 1996, Ronnenberg *et al.* 2002).

Preterm birth (<37 weeks of gestation) contributes to 9.6% of all births worldwide (Beck *et al.* 2010). A number of factors are known to increase the risk of preterm birth, but the mechanisms underlying the

initiation of preterm labor are not clear (Goldenberg *et al.* 2008). Matrix metalloproteinases (MMPs) are reported to contribute during pregnancy in extracellular matrix (ECM) remodeling/degradation leading to cervical ripening, fetal membrane rupture, and finally placental separation from maternal uterus (Stygar *et al.* 2002, Xu *et al.* 2002, Goldman *et al.* 2003). Under physiological conditions, the activities of MMPs are regulated at the levels of transcription, by zymogen activation and inhibition by tissue inhibitors of metalloproteinases (TIMPs; Brew & Nagase 2010).

An imbalance in the MMPs/TIMPs activity ratio may lead to preterm labor, preeclampsia, premature rupture of membranes (PROM), and intrauterine growth restriction (IUGR) (Cockle *et al.* 2007). We have recently reported increased placental levels of MMP1 (collagenase) and MMP9 (gelatinase) in women delivering preterm (Sundrani *et al.* 2012). The activity of MMPs is reported to be regulated by homocysteine, oxidative stress, and long-chain polyunsaturated fatty acids (LCPUFA; Guo *et al.* 2006, Kundu *et al.* 2009, Solakivi

et al. 2009, Tyagi *et al.* 2009, Zainal *et al.* 2009, Zeydanli & Turan 2009, Ke *et al.* 2010).

In view of this, we have recently hypothesized that altered intake or metabolism of maternal vital micro-nutrients (folic acid, vitamin B₁₂) and omega 3 fatty acids especially docosahexaenoic acid (DHA) influences the one-carbon cycle thereby contributing to increased homocysteine and oxidative stress leading to altered epigenetic regulation of *MMP* and *TIMP* genes expression in women delivering preterm (Sundrani *et al.* 2011). In order to test our hypothesis, we now report the placental levels of MMPs (MMP2 and MMP3) and TIMPs (TIMP1 and TIMP2) and their association with maternal micronutrients and LCPUFA in women delivering preterm.

Results

Maternal and neonatal characteristics

The maternal characteristics and birth outcome are given in Table 1. All the women recruited in the study had similar age and education. The BMI and gestation age for women delivering preterm were significantly lower ($P<0.01$) than that of women delivering at term. The baby weight, length, and head and chest circumference were lower ($P<0.01$ for all) in the preterm group compared with term.

Placental MMP2 levels

Placental MMP2 levels were higher in the preterm group (4.4 ± 1.2 ng/ml) ($P<0.01$) compared with control (3.6 ± 1.1 ng/ml) (Fig. 1A). Placental MMP2 levels were higher ($P<0.01$) in women with preterm labor (4.4 ± 1.3 ng/ml) compared with those in labor at term (3.6 ± 1.2 ng/ml) (Fig. 1B). When the levels were examined based on the mode of delivery, it was observed that in women undergoing spontaneous vaginal delivery, placental MMP2 levels were higher in preterm group (4.6 ± 1.3 ng/ml) ($P<0.01$) compared with control (3.6 ± 1.2 ng/ml) (Fig. 1C). In contrast, in women

undergoing cesarean sectioning, there was no difference in placental MMP2 levels in preterm group (4.1 ± 1.3 ng/ml) compared with control (3.4 ± 1.0 ng/ml) (Fig. 1D). In the control cesarean sectioning subgroup, there was no difference in placental MMP2 levels between women in labor and those not in labor. Similarly in the preterm cesarean sectioning subgroup, there was no difference in placental MMP2 levels between women in labor and those not in labor. In the control group, there was no difference in placental MMP2 levels between women with PROM and those with intact membranes. Similarly in the preterm group, there was no difference in placental MMP2 levels between women with preterm PROM (PPROM) and those with intact membranes.

Placental MMP3 levels

Placental MMP3 levels were higher in preterm group (1.5 ± 0.7 ng/ml) ($P<0.01$) compared with control (1.2 ± 0.5 ng/ml) (Fig. 2A). Placental MMP3 levels were significantly higher ($P<0.01$) in women with preterm labor (1.5 ± 0.7 ng/ml) compared with those in labor at term (1.3 ± 0.6 ng/ml) (Fig. 2B). When the levels were examined based on the mode of delivery, it was observed that in women undergoing spontaneous vaginal delivery, placental MMP3 levels were higher in preterm group (1.6 ± 0.7 ng/ml) ($P<0.01$) compared with control (1.2 ± 0.5 ng/ml) (Fig. 2C). In contrast, in women undergoing cesarean sectioning there was no significant difference in placental MMP3 levels in preterm group (1.4 ± 0.6 ng/ml) compared with control (1.4 ± 0.5 ng/ml) (Fig. 2D). In the control cesarean sectioning subgroup, there was no difference in placental MMP3 levels between women in labor and those not in labor. Similarly in the preterm cesarean sectioning subgroup, there was no difference in placental MMP3 levels between women in labor and those not in labor. In the control group, there was no difference in placental MMP3 levels between women with PROM and those with intact membranes. Similarly in the preterm group, there was no difference in placental MMP3 levels between women with PPRM and those with intact membranes.

Placental TIMP1 and TIMP2 levels

There was no significant difference in the placental TIMP1 and TIMP2 levels in preterm group (56.9 ± 36.7 and 110.3 ± 39.6 ng/ml respectively) compared with control (51.4 ± 26.9 and 107.7 ± 47.8 ng/ml respectively) (Figs 3A and 4A). Placental TIMP1 and TIMP2 levels showed no difference between women with preterm labor (58.0 ± 36.7 and 110.7 ± 41.3 ng/ml respectively) and those with at term labor (53.1 ± 27.0 and 105.0 ± 47.6 ng/ml respectively) (Figs 3B and 4B).

Table 1 Maternal and neonatal characteristics.

Maternal and neonatal characteristics	Term (<i>n</i> =75)	Preterm (<i>n</i> =73)
Age (years)	23.1 ± 3.7	22.6 ± 4.0
BMI (kg/m ²)	21.9 ± 4.4	19.0 ± 6.5*
Gestation age at delivery (weeks)	39.3 ± 1.1	34.5 ± 1.9*
Education (grade)	9.6 ± 4.0	8.7 ± 3.8
Baby weight (kg)	2.9 ± 0.2	2.0 ± 0.4*
Baby length (cm)	47.5 ± 6.3	44.9 ± 3.4*
Baby head circumference (cm)	33.5 ± 4.2	31.5 ± 2.2*
Baby chest circumference (cm)	32.2 ± 4.0	27.6 ± 6.5*

Type of analysis: independent Student's *t*-test. Values given are mean ± s.d. * $P<0.01$ when compared with term.

Figure 1 Placental MMP2 levels. (A) Comparison of placental MMP2 levels in preterm and term groups, ** $P < 0.01$. (B) Comparison of placental MMP2 levels in preterm and term labors, ** $P < 0.01$. (C) Comparison of placental MMP2 levels in preterm and term groups undergoing spontaneous vaginal delivery, ** $P < 0.01$. (D) Comparison of placental MMP2 levels in preterm and term groups undergoing cesarean sectioning. Type of analysis: independent Student's *t*-test. Values given are mean $\pm$ s.d. ** $P < 0.01$ when compared with term.

When the levels were examined based on the mode of delivery, it was observed that in women undergoing spontaneous vaginal delivery there was no significant difference in the placental TIMP1 and TIMP2 levels in preterm group (60.6 ± 33.1 and 109.7 ± 40.9 ng/ml respectively) compared with control (54.7 ± 27.1 and 103.1 ± 49.2 ng/ml respectively). Similarly, in women undergoing cesarean sectioning there was no significant difference in placental TIMP1 and TIMP2 levels in preterm group (54.8 ± 26.4 and 111.8 ± 36.9 ng/ml respectively) compared with control (39.1 ± 23.1 and 124.6 ± 39.3 ng/ml respectively) (Figs 3C and 4C; 3D and 4D). In the control cesarean sectioning subgroup, there was no difference in placental TIMP1 and TIMP2 levels between women in labor and those not in labor. Similarly in the preterm cesarean sectioning subgroup, there was no difference in placental TIMP1 and TIMP2 levels between women in labor and those not in labor. In the control group, there was no difference in placental TIMP1 and TIMP2 levels between women with PROM and those with intact membranes. Similarly in the preterm group, there was no difference in placental TIMP1 and TIMP2 levels between women with PPRM and those with intact membranes.

Associations of placental MMPs and TIMPs with placental DHA levels

The associations between MMP/TIMP and DHA levels were studied on a subset of term ($n=38$) and preterm ($n=56$) women from this study for which placental DHA levels were estimated in our previous study (Dhobale *et al.* 2011). In this study, a negative association between placental MMP2 levels and placental DHA levels was seen in the whole cohort ($r = -0.353$, $P = 0.003$, $n = 94$) and in the control group ($r = -0.346$, $P = 0.033$, $n = 38$) but not in the preterm group. A negative association between placental MMP3 levels and placental DHA levels was also seen in the whole cohort ($r = -0.232$, $P = 0.024$, $n = 94$) but not in the individual groups. There was no association of TIMP1 and TIMP2 with placental DHA in the whole cohort or individual groups (Table 2).

Associations of placental MMPs and TIMPs with maternal plasma vitamin B₁₂ levels

The associations between MMP/TIMP and vitamin B₁₂ levels were studied on a subset of term ($n=26$) and

preterm ($n=51$) women from this study for which maternal plasma vitamin B₁₂ levels were estimated in our previous study (Dhobale *et al.* 2012a). In this study, a positive association between placental MMP3 levels and maternal plasma vitamin B₁₂ levels was seen in the whole cohort ($r=0.265$, $P=0.020$, $n=77$) and only in the preterm group ($r=0.277$, $P=0.049$, $n=51$). There was no association of MMP2, TIMP1, and TIMP2 with maternal vitamin B₁₂ levels in the whole cohort or individual groups (Table 2).

Associations of placental MMPs and TIMPs with maternal plasma folate levels

The associations between MMP/TIMP and folate levels were studied on a subset of term ($n=26$) and preterm ($n=52$) women from this study for which maternal plasma folate levels were estimated in our previous study (Dhobale *et al.* 2012a). In this study, a positive association between placental MMP2 levels and maternal plasma folate levels ($r=0.374$, $P=0.007$, $n=52$) was seen in the preterm group. There was no

association of MMP3 with folate levels in the whole cohort or individual groups. There was no association of TIMP1 and TIMP2 with maternal folate levels in the whole cohort or individual groups (Table 2).

Associations of placental MMPs and TIMPs levels with maternal plasma malondialdehyde levels

The associations between MMP/TIMP and malondialdehyde (MDA) levels were studied on a subset of term ($n=58$) and preterm ($n=58$) women from this study for which maternal plasma MDA levels were estimated in our previous study (Dhobale *et al.* 2012b). In this study, a positive association between placental MMP2 levels and maternal plasma MDA levels was seen in the whole cohort ($r=0.236$, $P=0.011$, $n=116$). A positive association between placental MMP3 levels and maternal plasma MDA levels was seen in the whole cohort ($r=0.283$, $P=0.002$, $n=116$) and control group ($r=0.354$, $P=0.006$, $n=58$). There was no association of TIMP1 and TIMP2 with maternal MDA levels in the whole cohort or individual groups (Table 2).

Figure 2 Placental MMP3 levels. (A) Comparison of placental MMP3 levels in preterm and term groups, $**P<0.01$. (B) Comparison of placental MMP3 levels in preterm and term labors, $**P<0.01$. (C) Comparison of placental MMP3 levels in preterm and term groups undergoing spontaneous vaginal delivery, $**P<0.01$. (D) Comparison of placental MMP3 levels in preterm and term groups undergoing cesarean sectioning. Type of analysis: independent Student's *t*-test. Values given are mean $\pm$ s.d. $**P<0.01$ when compared with term.

Discussion

This study for the first time reveals several novel and interesting key findings in pregnant women delivering at term and preterm. Our results show i) increased placental MMP2 and MMP3 levels in women delivering preterm compared with those delivering at term; ii) mode of delivery affects placental MMPs in the preterm group; iii) negative association of placental MMP2 and MMP3 levels with placental DHA levels in the whole cohort; iv) positive association between placental MMP3 levels and maternal plasma vitamin B₁₂ levels in the whole cohort and v) positive association of placental MMP2 and MMP3 levels with maternal plasma MDA levels in the whole cohort.

MMPs are important enzymes that, along with ECM degradation, also contribute in cell proliferation, migration, differentiation, apoptosis, and angiogenesis. They are almost undetectable in normal adult tissue, but when affected by injury, disease, or pregnancy, they have elevated expression (Bellayr *et al.* 2009). MMP2 is an important member of the gelatinase subgroup which mainly digests peptide bonds in denatured collagens (gelatins) to yield small peptides, while MMP3 is a member of the stromelysin subgroup

that has a similar domain structure to those of collagenases, but they cannot cleave native fibrillar collagens (Visse & Nagase 2003, Swarnakar *et al.* 2011). MMP3 cleaves a number of substrates including proteoglycans, fibronectins, gelatins, collagen IV, and V (Hulboy *et al.* 1997). A number of studies have suggested that these enzymes have been implicated in the process of parturition, rupture of membranes, and intra amniotic infection (Athayde *et al.* 1999, Maymon *et al.* 2000a, 2000b, 2000c). However, there is no information on the placental levels of these MMPs in preterm deliveries.

In our study, placental MMP2 levels were higher in women delivering preterm compared with those delivering at term. Fortunato *et al.* (1999a) have reported increased MMP2 levels in the amniotic fluid of women with PPROM, suggesting that MMP2 is implicated in the mechanism responsible for rupture of membranes. In contrast, other reports suggest no changes in amniotic fluid MMP2 levels in women with spontaneous labor (term and preterm) and rupture of membranes (term and preterm) (Maymon *et al.* 2000c, 2001). However in our study, the placental MMP2 levels were higher in women with preterm labor compared with those in labor at term.

Figure 3 Placental TIMP1 levels. (A) Comparison of placental TIMP1 levels in preterm and term groups. (B) Comparison of placental TIMP1 levels in preterm and term labors. (C) Comparison of placental TIMP1 levels in preterm and term groups undergoing spontaneous vaginal delivery. (D) Comparison of placental TIMP1 levels in preterm and term groups undergoing cesarean sectioning. Type of analysis: independent Student's *t*-test. Values given are mean $\pm$ s.d.

MMP3 is a novel member of the stromelysin family, which is produced in the context of infection and is able to activate the latent forms of other MMPs (Park *et al.* 2003). In our study, placental MMP3 levels were significantly increased in women delivering preterm compared with those delivering at term. Further, women in preterm labor also showed increased placental MMP3 levels compared with those in term labor, suggesting that MMP2 and MMP3 play a crucial role in human preterm labor probably by degradation of ECM in the placenta. This is consistent with other studies which have examined MMP3 levels in the amniotic fluid and fetal membranes in women with preterm labor and PROM (Fortunato *et al.* 1999b, Park *et al.* 2003). Further, Reister *et al.* (2006) have reported reduced expression of MMP3 in extravillous trophoblast cells obtained from placental bed biopsies from patients with early-onset preeclampsia combined with IUGR. However, to our knowledge our study for the first time examines the placental levels of these MMPs in preterm deliveries.

TIMPs comprise a four-member family of homologous MMP inhibitors which includes TIMP1, 2, 3, and 4

(Nagase & Woessner 1999). TIMPs are specific inhibitors that bind MMPs in a 1:1 stoichiometry. All the four TIMPs inhibit MMPs but differ in their affinities for different inhibitor–protease pairs (Brew & Nagase 2010). A number of studies have examined the levels of TIMP1 and TIMP2 in the amniotic fluid and fetal membranes both in term and preterm pregnancy (Athayde *et al.* 1999, Fortunato *et al.* 1999a, Maymon *et al.* 2001, Goldman *et al.* 2003). However, the results are inconsistent. Fortunato *et al.* (1999a) have shown elevated amniotic fluid TIMP1 levels in women with PROM, while studies have reported that TIMP1 levels decrease before labor (Makrakis *et al.* 2003) and following PPROM (Nishihara *et al.* 2008). Further, it is suggested that spontaneous labor (term and preterm) and infection are associated with significant decreased concentrations of TIMP2 (Maymon *et al.* 2001). In our study, placental TIMP1 and TIMP2 levels showed no difference in the preterm and term group. Further, the mode of delivery also did not influence the levels of TIMPs in the term and preterm groups. The current study suggests that there was a change in the MMP levels

Figure 4 Placental TIMP2 levels. (A) Comparison of placental TIMP2 levels in preterm and term groups. (B) Comparison of placental TIMP1 levels in preterm and term labors. (C) Comparison of placental TIMP2 levels in preterm and term groups undergoing spontaneous vaginal delivery. (D) Comparison of placental TIMP2 levels in preterm and term groups undergoing cesarean sectioning. Type of analysis: independent Student's *t*-test. Values given are mean $\pm$ s.d.

Table 2 Association of MMPs with micronutrients, DHA, and oxidative stress marker.

	Whole cohort			Term			Preterm		
	<i>n</i>	<i>r</i>	<i>P</i>	<i>n</i>	<i>r</i>	<i>P</i>	<i>n</i>	<i>r</i>	<i>P</i>
Placental MMP2 levels (ng/ml)									
Placental DHA levels (g/100 g fatty acids)	94	−0.353	0.001 [†]	38	−0.346	0.033*	56	−0.135	0.324
Maternal plasma vitamin B ₁₂ (pg/ml)	77	−0.039	0.735	26	−0.091	0.658	51	−0.089	0.537
Maternal plasma folate levels (ng/ml)	78	−0.051	0.660	26	−0.041	0.844	52	0.374	0.007 [†]
Maternal plasma MDA levels (nM/ml)	116	0.236	0.011*	58	0.199	0.134	58	−0.060	0.655
Placental MMP3 levels (ng/ml)									
Placental DHA levels (g/100 g fatty acids)	94	−0.232	0.024*	38	−0.034	0.837	56	0.058	0.669
Maternal plasma vitamin B ₁₂ (pg/ml)	77	0.265	0.020*	26	−0.204	0.318	51	0.277	0.049*
Maternal plasma folate levels (ng/ml)	78	−0.090	0.433	26	−0.158	0.440	52	0.188	0.182
Maternal plasma MDA levels (nM/ml)	116	0.283	0.002 [†]	58	0.354	0.006 [†]	58	0.076	0.570

MMP, matrix metalloproteinase; DHA, docosahexaenoic acid; MDA, malondialdehyde. * $P < 0.05$; [†] $P < 0.01$.

without any underlying change in the TIMP levels possibly due to altered expression of MMPs. It is known that TIMP1 is a multifunctional protein and MMP inhibition is just one of its many properties (Hayakawa 1994). TIMPs also have biological activities that are independent of metalloproteinases; these include effects on cell growth and differentiation, cell migration, anti-angiogenesis, anti- and pro-apoptosis, and synaptic plasticity (Goldman *et al.* 2003).

In this study among women undergoing cesarean sectioning, there was no difference in the placental MMP and TIMP levels between women in labor vs those not in labor in the preterm group and the similar results were seen in the term group. Further, there was no difference in the placental MMP and TIMP levels between women with PROM vs those with intact membranes in the term group and between women with PPRM vs those with intact membranes in the preterm group. This could be possibly attributed to the small sample size in women with PROM/PPROM and those not in labor.

In this study, a negative association of placental MMP2 and MMP3 levels with placental DHA levels was seen in the whole cohort. This is consistent with our earlier study where we have reported a negative association between placental MMP9 levels and DHA levels in the whole cohort (Sundrani *et al.* 2012). It is suggested that reduced omega 3 fatty acids may up regulate two-series prostaglandin release thereby increasing the levels of MMPs. Further studies are needed to confirm this mechanism.

Our findings for the first time show a positive association between placental MMP3 and maternal plasma vitamin B₁₂ levels in the whole cohort and preterm group. In contrast, a negative trend was seen in the control group. We have recently reported increased maternal plasma vitamin B₁₂ levels in women delivering preterm (Dhobale *et al.* 2012a). Thus, a positive association between MMP3 and vitamin B₁₂ in the preterm group may be attributed to altered pathology in preterm pregnancy with the following possible mechanisms.

In the one-carbon cycle, methionine synthase (MS) requires vitamin B₁₂ as a cofactor and catalyzes the remethylation of homocysteine to methionine. In this reaction, the methyl group of 5-methyltetrahydrofolate (5-MTHF) is transferred to the enzyme-bound cob(I)-alamin to generate methylcobalamin followed by the transfer of the methyl group to homocysteine to reform methionine (Al Farra 2010). The elevation of vitamin B₁₂ could be attributed to impaired one-carbon metabolism due to defects in MS and/or enzymes involved in methyl transfer from folate. This altered one-carbon metabolism could be responsible for the increased homocysteine levels in preterm deliveries. It is known that hyperhomocysteinemia may be involved in matrix degradation and inflammation (Tobin *et al.* 2009). Further, elevated circulating vitamin B₁₂ levels have been observed in inflammatory diseases, particularly with underlying liver disease (Geissbühler *et al.* 2000). Thus, it could be possible that the increased vitamin B₁₂ may be related to inflammation which is also one of the underlying causes of preterm birth. Increased inflammatory cytokine levels stimulate prostaglandin production, which in turn are observed to upregulate the expression/activity of MMPs (Locksmith *et al.* 1999, Ulug *et al.* 2001). So, it is likely that increased placental MMP3 levels in women delivering preterm may be due to increased vitamin B₁₂ levels through the inflammatory pathway. However, further studies are needed to confirm this mechanism.

The human placenta has a specific receptor for binding transcobalamin II–vitamin B₁₂ (TC II–B₁₂) complex called transcobalamin II–vitamin B₁₂ receptor (TC II–B₁₂ R; Schneider & Miller 2010) which is a heavily glycosylated protein (Seetharam & Li 2000). It is known that MMP3 degrades a wide range of substrates including proteoglycans (Hulboy *et al.* 1997). In future, it needs to be explored whether the increased vitamin B₁₂ may be a consequence of degradation of TC II–B₁₂ receptors by MMP3.

Increased oxidative stress may be involved in the upregulation of proinflammatory cytokines and increased activity of MMPs (Kundu *et al.* 2009, Sundrani

et al. 2011). This supports the positive association of placental MMP2 and MMP3 levels with maternal plasma MDA levels observed in the whole cohort. Future studies are needed to examine the role of MMPs in various regions of preterm placenta. This would help in understanding the spatial regulation of these enzymes in the preterm placenta.

In conclusion, this study for the first time indicates increased placental levels of MMP2 and MMP3 in women delivering preterm. Further, among women with spontaneous vaginal delivery, placental MMP2 and MMP3 were increased in women delivering preterm compared with term, indicating their crucial role in the mechanism of preterm labor. Our study for the first time indicates positive associations of MMPs with vitamin B₁₂ and MDA levels. In addition, lower placental DHA levels were associated with increased placental MMP2 and MMP3 levels. There is growing evidence that maternal nutritional status can alter the epigenetic patterns by stable alterations in gene expression through DNA methylation and histone modifications (Wu *et al.* 2004) and may be a possible mechanism for the impact of maternal nutrition on MMPs and TIMPs in the preterm placenta. Further studies are needed to determine whether gene–nutrient interactions and epigenetic modifications of genes affect the risk of preterm birth.

Materials and Methods

Subjects

This study was conducted at the Department of Obstetrics and Gynecology, Bharati Hospital, Pune. The study was approved by the Bharati Vidyapeeth Medical College Institutional Ethical Committee and a written consent was taken from each subject. A total number of 148 pregnant women with singleton pregnancy were recruited for this study: 75 women delivered at term (control) and 73 women delivered preterm. In the control group, 59 (78.66%) women had spontaneous vaginal delivery (in labor) and 16 (21.33%) had cesarean sectioning (nine in labor, five with no labor, and no data for two). The control group included five women with early rupture of membrane (EROM), three women with premature rupture of the membranes (PROM), and 67 with intact membranes. In the preterm group, 51 (69.86%) had spontaneous vaginal delivery (in labor) and 22 (30.14%) had cesarean sectioning (15 in labor, six with no labor, and no data for one). The preterm group included one woman with EROM, 15 women with PPROM, 56 with intact membranes, and no data for one woman. Considering both mode of deliveries, there were 68 women in term labor and 66 women in preterm labor. The inclusion and exclusion criteria were the same as reported in our earlier study (Sundrani *et al.* 2012).

Tissue collection and processing

Fresh placental tissues were obtained from normal and preterm pregnancies, immediately after delivery, processed,

and stored until assayed as described by us earlier (Sundrani *et al.* 2012).

MMP and TIMP levels from placental tissue lysates

Placental tissue lysates were prepared as described by us earlier (Sundrani *et al.* 2012). MMP2, MMP3, TIMP1, and TIMP2 levels were measured from placental tissue lysates using commercial ELISA kits (Abnova, Taipei, Taiwan for MMP2, MMP3, and TIMP2; Abcam, Inc., Cambridge, MA, USA for TIMP1). The detection limit (sensitivity) of the assay was <10 pg/ml for MMP2, <10 pg/ml for MMP3, <30 pg/ml for TIMP1, and <2 pg/ml for TIMP2. The concentrations of MMPs and TIMPs were normalized for 1 mg of total protein content.

Placental MMP and TIMP levels were compared between term and preterm groups. We also compared the placental MMP and TIMP levels between women in preterm labor and those in term labor. Further based on mode of delivery (spontaneous vaginal delivery and cesarean sectioning), the placental MMP and TIMP levels were compared between term and preterm groups.

Statistical analysis

Values are mean $\pm$ s.d. The data were analyzed using SPSS/PC + package (Version 20.0). Mean values of the various parameters were compared using independent sample *t*-tests to identify statistically significant differences ($P < 0.05$). Skewed variables were transformed to normality using the following transformations: log to the base 10 (MMP2, MMP3, TIMP1, and TIMP2). The extent of linear relationship between several variables was studied in the whole cohort and individual groups using bivariate correlation analysis. Statistical power was calculated using placental MMP values from our earlier studies in term and preterm women, where we have reported significant group differences in MMP1 and MMP9 from 74 preterm and 75 women delivering at term ($P < 0.05$) (Sundrani *et al.* 2012). Thus, with such an effect size we proposed the sample size of 75 women in each group, which would give >90% probability of detecting a difference at an alpha of 0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S Joshi and S Mehendale conceived and designed the experiments. D Sundrani and P Chavan-Gautam performed

the experiments. D Sundrani, P Chavan-Gautam, and S Joshi analyzed the data. D Sundrani, H Pisal, and S Mehendale contributed reagents, materials, and analysis tools. D Sundrani, P Chavan-Gautam, and S Joshi wrote the paper.

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