# Inflammatory response in maternal serum during preterm labour

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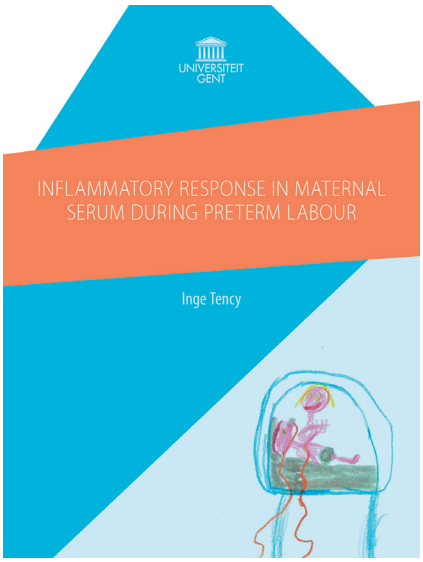


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## *Abstract*

**Preterm birth (PTB), defined as a delivery before 37 weeks of gestation, is the leading cause of perinatal morbidity and mortality worldwide. Diagnosis of preterm labour as well as accurate prediction of PTB is notoriously difficult. Preterm birth is initiated by multiple mechanisms including infection or inflammation which is the only pathological process for which a firm causal link with PTB has been established. Intrauterine infection evokes an immune response that involves the release of cytokines and chemokines, prostaglandins and matrix-degrading enzymes. These substances trigger uterine contractions, membrane rupture and cervical ripening. Most intra-uterine infections are chronic and subclinical in nature and consequently hard to diagnose before labour or rupture of the membranes. The best studied site of infection is amniotic fluid, but this requires an invasive procedure. A non-invasive approach seems to be more relevant to clinical practice. However, few studies have investigated the maternal inflammatory response during preterm labour. Therefore, the overall objective of this study was to determine several inflammatory markers in maternal serum from pregnant women in labour (either term or preterm) vs. non-labouring controls.**

**We completed a nested case control study in which singleton pregnancies were recruited at Ghent University Hospital and divided into groups according to gestational age and labour status. Multiple proteins were evaluated in maternal serum using enzyme-linked or multiplex bead immunoassays including soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), matrix metalloproteinases (MMP)-9 and MMP-3, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, TIMP-3 and TIMP-4 and a panel of 30 cytokines, chemokines and growth factors.**

*Key words:* **preterm labor, maternal serum, human parturition, sTREM-1, MMP, TIMP, MMP:TIMP imbalance, biomarkers, cytokines.**

## Introduction

Preterm birth (PTB), referring to all births before 37 completed weeks of gestation, is a major perinatal health problem because it is the leading cause of perinatal morbidity and mortality worldwide (Beck et al., 2010; Goldenberg et al., 2008). Although survival rates have markedly improved over the last 15 years, these infants have an increased risk of neurodevelopmental impairments and respiratory and gastrointestinal complications (Goldenberg et al., 2008). The serious effects of PTB on parents, infant and society make PTB an important issue to public health worldwide (Khan and Honest, 2007).

In 2010, the global incidence of PTB has been estimated at 11.1% of all births, with 14.9 million PTBs occurring annually (Blencowe et al., 2012). Europe has the lowest rate of PTB worldwide with prevalence's varying from 5.5% to 11.4, with an average of 7.1% (or 500 000 births annually) (Keller et al., 2011). In Flanders, 7.6% of the babies were born preterm in 2012. In singleton pregnancies about 6.7% of the deliveries (n = 4395) occur preterm, while in multiple pregnancies, accounting for 1.8% of the deliveries in Flanders, 59.4% (n = 717) ended preterm (Cammu et al., 2012).

Preterm birth rates have increased in most industrialized and many developing countries (Beck et al., 2010; Blencowe et al., 2012; Goldenberg et al., 2008). Also in Flanders, the PTB rate has increased from 5.3% in 1991 to 7.6% in 2012, but the number remained relatively stable during the last 10 years (2001-2011) (Cammu et al., 2012). Keirse et al. (2009) examined the pattern of PTB over a 12-year period (1991-2002) and showed that there was an almost linear increase in PTB rate of 0.23% a year (Keirse et al., 2009).

Despite advances in perinatal medicine, improved perinatal health indicators and advancing knowledge of risk factors and mechanisms associated with PTB, there has been little progress in reducing PTB rate (Goldenberg et al., 2008; Khan and Honest, 2007; Kramer et al., 2012). Prediction and prevention remains difficult and poses a continuing and significant challenge to modern obstetrics. An increasing number of maternal and foetal risk factors have been associated with PTB (Table I), but the precise mechanisms by which risk factors are related to PTB are often unknown (Goldenberg et al., 2008).

Infection and inflammation are important mechanisms leading to PTB. Intrauterine infection is the

Table I. — Risk factorsfor preterm birth*

| Primary risk factors | Secondary risk factors |
|---|---|
| *Sociodemographic factors* | *Biophysical predictors* |
| Black race<br>Low socioeconomic and educational status<br>Low (<18 years) and high (> 40 years) maternal age<br>Single marital status | Digital examination<br>Short cervix<br>Absence of foetal breathing movements |
| *Lifestyle, psychosocial and environmental factors* | Biochemical predictors (non-exhaustive) |
| Smoking, cocaine and heroin use<br>Heavy alcohol consumption<br>Hard working conditions<br>Anxiety, stress and depression<br>Low and high prepregnancy body-mass index (BMI) | Cervicovaginal foetal fibronectin (fFN)<br>Cervicovaginal phosphorylated form of insulin-like growth factor binding protein 1 (phIGFBP-1)<br>Cervicovaginal β-human chorionic gonadotropin (β-hCG)<br>Cervicovaginal interleukin-8 (IL-8)<br>Plasma matrix metalloproteinase-9 (MMP-9)<br>Maternal serum and amniotic fluid C-reactive protein (CRP)<br>Amniotic fluid interleukin-6 (IL-6)<br><br>Insulin-like growth factor binding protein 1 (IGFBP-1)<br>Placental alpha macroglobulin-1 (PAMG-1) |
| *Obstetrical history and pregnancy characteristics* | |
| Previous preterm birth<br>Second trimester pregnancy loss<br>Multiple pregnancy and higher order pregnancy<br>Short interpregnancy interval (< 6 months)<br>Singleton pregnancy after in-vitro fertilisation<br>Inadequate prenatal care<br>Low gestational weight gain<br>Vaginal bleeding<br>**Infection/inflammation**<br>Extreme amniotic fluid volume (oligo- or polyhydramnios)<br>Abdominal surgery in the 2nd or 3rd trimester<br>Cervical procedures<br>Uterus anomalies | |

* Modified after Goffinet, F. BJOG, 2005; Goldenberg, RL et al. Lancet, 2008; Honest, H. et al. Health Technol Assess,2009.

only pathological process for which both a firm causal link with PTB has been established and a direct molecular pathophysiology defined (Goldenberg et al., 2008; Romero et al., 2006). Intrauterine infection evokes an immune response that involves the release of cytokines and chemokines, prostaglandins and matrix-degrading enzymes. These substances trigger uterine contractions, membrane rupture and cervical ripening. However, most intrauterine infections are chronic and subclinical in nature and consequently hard to diagnose before labour or rupture of the membranes (Goldenberg et al., 2000). Therefore, tremendous research efforts have been performed to identify inflammatory markers to predict PTB in both asymptomatic and symptomatic women and to improve our understanding of the mechanisms and pathways leading to PTB. Despite these efforts, the puzzle of PTB seems far from being resolved, at least because the nature of PTB is heterogeneous and complex, but also because the process of term labour is poorly understood. The best studied site of infection is amniotic fluid. Although these studies increase our understanding in the foetal inflammatory response, it is unlikely that one will perform invasive procedures to test foetal biomarkers for the prediction of PTB. A non-invasive approach (e.g. maternal blood, vaginal or cervical secretions) seems to be more relevant to clinical practice because of the feasibility and accessibility. Therefore, the overall objective of this thesis was to determine several inflammatory markers in maternal serum from pregnant women in labour (either term or preterm) vs. non labouring controls.

## Objectives

*Objective 1: To assess sTREM-1 concentrations in maternal serum during term and preterm labour*

Recently, a family of cell surface receptors, the triggering receptor expressed on myeloid cells (TREM) proteins, has been discovered that seems to play an important role in fine-tuning the immune response during infectious diseases. TREM-1 is a transmembrane glycoprotein, mainly expressed in monocytes and neutrophils (Bouchon et al., 2001). It has been demonstrated that sTREM-1 is involved in bacterial infection (Jiyong et al., 2009) as well as in non-infectious inflammatory conditions (Collins et al., 2009; Park et al., 2009). Research in septic patients demonstrated that sTREM is of substantial value for early diagnosis of bacterial infection (Jiyong et al., 2009), and represents an excellent biomarker for the prognosis of sepsis (Gibot et al., 2005), bacteraemia (Su et al., 2012) and sepsis severity (Zhang et al., 2011). Moreover, it has been shown that sTREM-1 levels reflect the infection status more accurately than C-reactive protein (CRP) and procalcitonin (PCT) levels do (Su et al., 2012).

Few studies have investigated the role of sTREM-1 in PTB. Menon and Fortunato (2008) demonstrated that both lipopolysaccharide (LPS) and preterm labour induced foetal membrane TREM-1 expression. In the presence of IUI, amniotic fluid sTREM-1 concentrations were significantly higher in patients with preterm labour or PPROM (Kusanovic et al., 2010; Menon and Fortunato, 2008). To our knowledge, only two studies (Cobo et al., 2013; Tsiartas et al., 2012) evaluated serum sTREM-1 concentrations during preterm labour. However, these studies did not include women with term labour. There is accumulating evidence that inflammation has also been implicated in the mechanism of spontaneous term parturition (Romero et al., 2006) and upregulation of sTREM-1 in myometrium and cervix has been demonstrated with the onset of term labour (Youssef et al., 2009). Therefore, the purpose of this study was to assess sTREM-1 concentrations in maternal serum during term and preterm labour.

*Objective 2: To determine the maternal serum concentrations of MMP-3, MMP-9 and all four TIMPs as well as the MMP:TIMP ratios during term and preterm labour*

Matrix metalloproteinases (MMPs) are proteolytic, zinc-dependent enzymes, capable of degrading extracellular matrix (ECM) components, including collagen. Their activity is regulated by tissue inhibitors of metalloproteinases (TIMPs) of which four have been identified, TIMP-1 through TIMP-4. Inhibition of MMP activity occurs in a 1:1 stoichiometric relationship. The balance between collagenolysis and its inhibition is critical during ECM remodelling. An imbalanced MMP:TIMP ratio has been involved in various medical conditions in humans including cancer, rheumatoid arthritis, osteoarthritis, endometriosis and vascular diseases (Amalinei et al., 2007; Brew and Nagase, 2010).

Human pregnancy is characterized by a steady remodelling of the collagenous ECM in order to adapt foetal membranes and cervix to uterine and foetal growth as gestation progresses. Matrix metalloproteinases are undoubtedly involved in ECM remodelling during pregnancy as well as in the generation of labour through membrane weakening and rupture and cervical ripening and dilatation (Cockle et al., 2007; Weiss et al., 2007). Some MMPs (e.g. MMP-1, MMP-2 and MMP-3) are constitutively expressed during gestation, while the

production of others (e.g. MMP-9) are induced by active labour (Cockle et al., 2007). Aberrant ECM degradation by MMPs or imbalanced MMP:TIMPs ratios have been implicated in the pathogenesis of preterm labour. Intrauterine infection triggers MMP production via inflammatory mediators (Goldenberg et al., 2000). Activation of the MMP cascade causes ECM degradation, predisposing membrane rupture and cervix ripening (Cockle et al., 2007). The involvement of MMP-9 in IUI and parturition has been clearly demonstrated in amniotic fluid, but few studies have investigated MMP-3. The majority of studies focused only on TIMP-1 and TIMP-2 concentrations in amniotic fluid. Few studies have investigated MMPs or TIMPsin maternal serum. We hypothesized that aberrant MMP expressions at local level implicated in ECM degradation of the chorio-amnion and cervix, are associated with aberrant changes in circulating MMPs, resulting in imbalanced MMP:TIMP ratios and leading to preterm labour. We therefore sought to determine the maternal serum concentrations of MMP-3, MMP-9 and all four TIMPs as well as the MMP:TIMP ratios during term and preterm labour.

*Objective 3: To evaluate the maternal inflammatory response during term and preterm labour by simultaneous measurement of 30 biomarkers in maternal serum*

Human term and preterm parturition are inflammatory processes in which cytokines play a pivotal role. Deregulated cytokine networks may lead to adverse pregnancy outcomes such as preterm labour. A number of studies have explored amniotic fluid cytokine profiles in women with PTB, but few studies have evaluated circulating cytokines during term and preterm labour. We hypothesized that inflammatory serum markers are differently expressed in labour, especially in women with preterm labour. Therefore, we sought to evaluate a panel of relevant maternal cytokines in labouring vs. non labouring women (either term or preterm) by using a multiplex immunoassay. This novel technological approach makes it possible to measure multiple proteins simultaneously, thereby requiring only small sample volumes.

## Materials and Methods

*Study design and study population*

An observational study was conducted and included 768 pregnant women between 24 and 42 weeks' gestation, presenting to the labour and delivery ward of Ghent University Hospital. We completed a nested case-control study after the major outcomes (such as PTB before 34 weeks' gestation) were determined. All subjects for this study, except patients from group 2 (see below), were selected from the original cohort. A convenience sample of singleton pregnancies was selected and divided into four groups according to gestational age (GA) and labour status. Table II provides an overview of the study population and immunoassays for the different inflammatory markers.

*Group 1* consisted of women with preterm labour, allocated to the PTB group when delivered before 34 weeks gestation ( = *PTB*), including patients with preterm prelabour rupture of the membranes (PPROM) or with preterm labour (PTL) and intact membranes. *Group 2* consisted of women not in labour, attending the prenatal clinic and matched for week of gestation with the PTB group. All these women had an uncomplicated pregnancy that proceeded to term delivery ( = *GA matched controls*). *Group 3* consisted of healthy pregnant women at term in labour ( = *AT in labour*), including patients in labour with intact membranes and women with prelabour rupture of the membranes (PROM). *Group 4* consisted of healthy pregnant women at term not in labour, undergoing a primary Caesarean section *( = AT not in labour*).

Eligibility criteria included age ≥ 18 years, gestational age ≥ 24 weeks, absence of foetal (congenital) malformations, absence of infectious disease or acute infection and Dutch speaking. Data on maternal demographics, medical and obstetrical history and pregnancy outcome were recorded. Written informed consent was obtained from every patient. The study was approved by the Ethical Committee of Ghent University Hospital (EC/2009/010).

*Sample collection, processing and analysis*

Blood samples of labouring women (either term or preterm) were collected by the attending midwife upon admission to the labour and delivery ward using serum separating tubes. Women at term not in labour were sampled prior to their Caesarean section. *Gestational age matched controls* were enrolled from the prenatal clinic. These pregnant women were screened at 20-22 weeks (structural ultrasound) to verify whether they fulfilled the inclusion criteria. These women were matched for week of gestation with a PTB case. Sampling was performed during a subsequent prenatal consultation at the appropriate gestational age.

Samples were stored at 4°C until centrifugation. Blood samples were centrifuged at 1000 g for 10 minutes at room temperature to harvest serum. All samples were stored at -80 °C until analysis.

Table II — Overview of the study population and immunoassays for the different inflammatory markers.

| Inflammatory marker | Study population | | | | | Immunoassay | |
|---|---|---|---|---|---|---|---|
| | Total patients | Group 1 PTB | Group 2 GA matched controls | Group 3 AT in labour | Group 4 AT not in labour | | |
| sTREM-1 | 176 | 52 | 52 | 40 | 32 | ELISA | R&D systems, Minneapolis, MN |
| MMP-9 | 166 | 47 | 47 | 40 | 32 | Luminex | Human Matrix Metalloproteinases 3-Plex Panel* Invitrogen, Inc. Carlsbad, CA |
| TIMP-1 to -4 | 166 | 47 | 47 | 40 | 32 | Luminex | Human TIMP Multiplex Kit R&D systems, Minneapolis, MN |
| MMP-3 | 116 | 34 | 34 | 27 | 21 | Luminex | Human Matrix Metalloproteinases 3-Plex Panel Invitrogen, Inc. Carlsbad, CA |
| Multiplex | 144 | 39 | 39 | 34 | 32 | Luminex | Human Cytokine 30-plex Panel** Invitrogen, Inc. Carlsbad, CA |

*The 3-plex was validated for serum by performing spike and recovery and linearity-of-dilution experiments. MMP-13 concentrations in maternal serum were undetectable in all samples using this method. At a twofold dilution of serum, concentrations were below the detection limit and recovery fell outside the range 70-130%.

** The panel consisted of epidermal growth factor (EGF), eotaxin, basic fibroblast growth factor (FGF-basic), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF), interferon alpha (IFN-α), interferon gamma (IFN-γ), interleukin (IL) receptor antagonist (IL-1RA), IL-1β, IL-2, soluble IL-2 receptor (sIL2R), IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, inducible protein-10 (IP-10), monocyte chemoattractant protein (MCP)-1, monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP)-1α, MIP-1β, Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES), tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF).

sTREM-1 concentrations were determined using an enzyme-linked immunoassay. Concentrations of MMP-3, -9 and -13 and TIMP-1, -2, -3 and -4 were assessed using a Human Matrix Metalloproteinases 3-Plex Panel and Human TIMP Multiplex Kit. Multiple proteins were analysed using a multiplex bead immunoassay (Human Cytokine 30-plex Panel). All multiplexes were run on a Luminex 200 platform equipped with Bio-Plex software.

*Statistical analysis*

Univariate group differences were tested with $\chi^2$ or Fisher's Exact test for categorical and Mann-Whitney *U*-test or Student's t-test for continuous variables. As multiple markers were considered as outcome variables (e.g. multiplexes), we accounted for multiple testing by applying the Bonferroni correction: where appropriate, adjusted p-values were obtained by multiplying with the total number of markers and ratios and used to evaluate significance. The normality of the continuous data was tested using the Kolmogorov-Smirnov test and visual inspection of QQ-plots. Since the distribution of inflammatory markers were positively skewed, their natural log transformed values were used so as to have a normally distributed outcome variables for the multiple regression analysis, which was performed on the full dataset.

The subgroups were translated into three variables: preterm (vs. at term), labour (vs. not in labour) and rupture of membranes (ROM) (vs. intact membranes). Because these key covariates were the focus of our investigation, they remained in the models regardless of their significance. To adjust for possible confounding effects, the following covariates were considered in the model selection procedure: maternal age, education level, marital status, smoking, BMI, history of PTB, storage time and time delay between sampling and processing (referred to as sample age). This set of covariates was included in the initial model of the selection procedure for each outcome. Model selection was carried out for each outcome independently and occurred in two steps. First, a backward selection of main terms was applied in which covariates were sequentially removed in order of increasing significance until only terms with p-value below 0.10 remained. In the second step, first order interactions were considered between the covariates remaining in the model. The forward selection of interaction terms was performed with an inclusion criterion of $p = 0.05$. When no further interactions met this criterion, the final model was obtained for that outcome.

All statistical analyses and tests were performed two-sided at the 5% significance level using SPSS statistics 19 software (IBM, Chicago, Illinois).

Table III. — Demographic and clinical characteristics of the study population (sTREM-1).

| Variables | PTB (n = 52) | GA matched controls (n = 52) | AT in labor (n = 40) | AT not in labor (n = 32) | P[a] | P[b] | P[c] |
|---|---|---|---|---|---|---|---|
| Maternal age (mean ± SD, y) | 28.7 ± 5.6 | 29.8±4.1 | 29.1 ± 4.6 | 31.4 ± 4.4 | P = 0.26 | P = 0.03 | P = 0.69 |
| Pre-pregnancy BMI (Me, IQR, kg/m$^2$) | 21.5 [19.7-24.8] | 21.8 [20.1-23.1] | 21.9 [19.9-24.0] | 21.6 [19.9-25.0] | P = 0.98 | P = 0.43 | P = 0.77 |
| Educational level (n, %) | | | | | P = 0.002 | P = 0.58 | P = 0.09 |
| Secondary education or less | 24 (46.2) | 9 (17.3) | 11 (27.5) | 7 (21.9) | | | |
| Higher education | 28 (53.8) | 43 (82.7) | 29 (72.5) | 25 (78.1) | | | |
| Marital status (n, %) | | | | | P = 0.70 | P = 1.00 | P = 0.38 |
| Married or cohabiting | 47 (92.2) | 48 (94.1) | 39 (97.5) | 31 (96.9) | | | |
| Living alone | 4 (7.8) | 3 (5.9) | 1 (2.5) | 1 (3.1) | | | |
| Smoking at recruitment | 9 (17.3) | 8 (15.4) | 0 (0.0) | 4 (12.5) | P = 0.79 | P = 0.04 | P = 0.005 |
| Ethnicity (n, %) | | | | | P = 1.00 | P = 0.12 | P = 0.16 |
| White/Caucasian | 51 (98.1) | 50 (96.2) | 36 (90.0) | 32 (100.0) | | | |
| Other | 1 (1.9) | 2 (3.8) | 4 (10.0) | 0 (0.0) | | | |
| GA at recruitment (Me, IQR, wk) | 29.0 [26.0-31.0] | 29.0 [26.0-31.0] | 40.0 [39.0-40.0] | 38.0 [38.0-39.0] | P = 1.0 | P < 0.001 | P < 0.001 |
| Conception (n, %) | | | | | P = 0.78 | P = 0.09 | P = 0.96 |
| Spontaneous | 44 (84.6) | 45 (86.5) | 34 (85.0) | 31 (96.9) | | | |
| Assisted reproductive technology | 8 (15.4) | 7 (13.5) | 6 (15.0) | 1 (3.1) | | | |
| Nullipara (n, %) | 32 (61.5) | 26 (50.0) | 20 (50.0) | 12 (37.5) | P = 0.24 | P = 0.29 | P = 0.27 |
| History of PTB | 4 (7.7) | 2 (3.8) | 2 (5.0) | 1 (3.1) | P = 0.68 | P = 1.00 | P = 0.69 |
| GA at delivery (Me, IQR, wk) | 30.0 [28.0-32.0] | 40.0 [39.0-40.0] | 40.0 [39.0-40.0] | 38.0 [38.0-39.0] | P < 0.001 | P < 0.001 | P < 0.001 |
| Delivery mode (n, %) | | | | | P = 0.21 | P < 0.001 | P = 0.38 |
| Vaginal birth | 48 (92.3) | 43 (84.3) | 39 (97.5) | 0 (0.0) | | | |
| Caesarean section | 3 (5.9) | 8 (15.7) | 1 (2.5) | 32 (100.0) | | | |
| Birth weight (mean, ±SD, g) | 1517.3 ± 514.4 | 3484.9 ± 498.0 | 3461.9 ± 396.2 | 3236.9 ± 360.0 | P < 0.001 | P = 0.01 | P < 0.001 |
| Gender (n, %) | | | | | P = 0.04 | P = 0.75 | P = 0.04 |
| ♀ | 16 (30.8) | 26 (51.0) | 21 (52.5) | 18 (56.3) | | | |
| ♂ | 36 (69.2) | 25 (49.0) | 19 (47.5) | 14 (43.8) | | | |

[a] PTB vs. GA matched controls; [b] AT in labor vs. AT not in labor; [c] PTB vs. AT in labor.
SD, standard deviation; BMI, body mass index; Me, Median; IQR, interquartile range; GA, gestational age; PTB, preterm birth; AT, at term.
Group differences were evaluated with Fisher's Exact test for categorical variables and Mann-Whitney *U*-test for continuous variables.

## Results

### *Demographic and clinical characteristics of the study population*

As shown in Table II, the number of included women varied among the different immunological analyses, but in general the population characteristics were similar. No significant differences were found regarding pre-pregnancy BMI, marital status, ethnicity, conception, parity and history of PTB. Women with PTB had significantly lower education levels than *GA matched controls*. There were significantly more smokers among women with PTB compared to women *AT in labour*. Significant differences were also found in maternal age and the proportion of smokers between women *AT in labour* vs. *AT not in labour*. Table III summarizes maternal and obstetrical characteristics of the study population for sTREM-1 determination.

### *Evaluation of sTREM-1 levels in maternal serum*

sTREM-1 was detected in all serum samples collected in the study. Figure 1 shows sTREM-1 concentrations among the different groups. Significantly higher median concentrations of sTREM-1 were seen in women with *PTB* compared to *GA matched controls* ($p < 0.001$). Median sTREM-1 concentrations were significantly increased in women *AT in labour* compared with *AT not in labour* ($p < 0.001$).

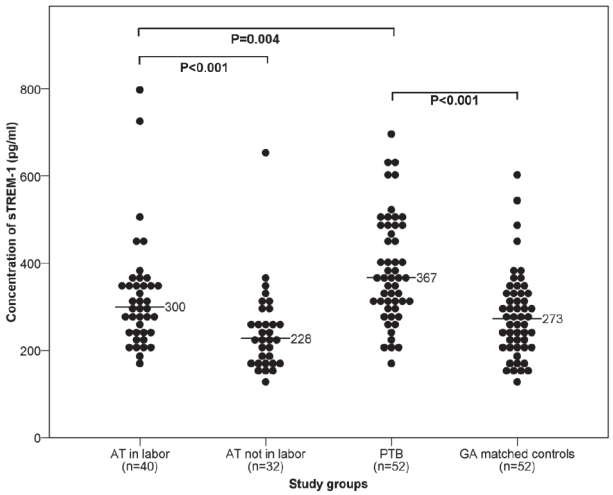


***Fig. 1.*** — Serum sTREM-1 concentrations among groups. Horizontal bars denote the median value for each study group.

Table IV. — Multiple regression model for ln(sTREM-1 concentration).

| Parameter | Model coefficient (95%CI) | Exponentiated coefficient (95%CI) | *P*-value |
|---|---|---|---|
| Intercept | 5.416 [5.323, 5.508] | 224.9 [205.1, 246.7] | < 0.001 |
| Preterm [vs. at term] | 0.142 [0.043, 0.241] | 1.152 [1.044, 1.272] | 0.005 |
| Labor [vs. not in labor] | 0.258 [0.126, 0.391] | 1.295 [1.134, 1.479] | < 0.001 |
| ROM [vs. intact membranes] | -0.021 [-0.156, 0.113] | 0.979 [0.856, 1.120] | 0.76 |
| Secondary education (or less) [vs. higher education] | 0.128 [0.020, 0.236] | 1.136 [1.020, 1.266] | 0.02 |
| History of PTB [vs. no history] | -0.324 [-0.542, -0.105] | 0.724 [0.582, 0.900] | 0.004 |
| Sample age (in hours) | 0.0039 [0.0003, 0.0076] | 1.004 [1.000, 1.008] | 0.04 |

Results of the model fitted on the full dataset (n = 176), obtained from the backward selection procedure outlined in the text. Covariates considered but not retained were: maternal age, marital status, smoking, body mass index and storage time. Coefficients of the model (additive on the log scale) were exponentiated to multiplicative factors, allowing interpretation on the concentration scale.
Sample age = time delay between blood sampling and processing
ROM, rupture of the membranes; PTB, preterm birth; CI, confidence interval
$R^2$=0.28

Women with *PTB* had significantly higher sTREM-1 levels than women *AT in labour* (p = 0.004).

The regression model (Table IV) showed that labour (vs. not in labour) and preterm (vs. not preterm) remained significantly associated with sTREM-1 concentration after adjusting for educational level, history of PTB and sample age. On average, the sTREM-1 concentration was 30% higher in labour (vs. not in labour) and 15% higher preterm (vs. at term).

*Evaluation of serum MMP-9, MMP-3 and TIMP-1 to -4 levels as well as MMP:TIMP balances*

MMP-3, TIMP-1, TIMP-2 and TIMP-4 were detectable in all serum samples, MMP-9 in 97.2% and TIMP-3 in only 26.7% of samples. Serum MMP-9 concentrations of 4 samples (all patients from the PTB group) were outside the linear range and TIMP-3 levels were below detection limits of the assay in most samples (75.3%). Therefore these values were omitted from further analyses. MMP-13 could not be assessed in serum. Serum MMP-3 and -9, TIMP-1, -2 and -4 concentrations and their ratios are summarized in Table V.

Median levels of MMP-9 and TIMP-4 were significantly higher in women with *PTB* compared to *GA matched controls* (p = 0.001 resp. p < 0.001). The same was true for median MMP-9:TIMP-1 and MMP-9:TIMP-2 ratios (p < 0.02 resp. p < 0.001).

No significant differences in MMP-9, MMP-3, all TIMP concentrations or any of the MMP:TIMP ratios were observed between women *AT in labour* vs. *AT not in labour*.

Median TIMP-2 levels were significantly lower in women with *PTB* compared to those *AT in labour* (p < 0.001). A significant higher MMP-9:TIMP-1 and MMP-9:TIMP-2 ratio was observed in women with *PTB* (p = 0.006 resp. p < 0.001). Higher MMP-9 and lower TIMP-1 concentrations were observed in women with *PTB*, but these differences were only weakly significant (p = 0.07 resp. p = 0.09).

Multiple regression analysis was performed on the full dataset. The $R^2$ of our regression models varied from 7 to 33%, indicating a large amount of variation in log marker concentration not explained by the covariates included. Our regression results showed that, after adjusting for other covariates, MMP-9 and all MMP-9:TIMP ratios were significantly higher for *preterm* (vs. at term, p < 0.001), whereas TIMP-1 and TIMP-2 were significantly lower for *preterm* (p = 0.002 resp. p < 0.001). The regression model showed that MMP-9 concentrations and MMP-9:TIMP-2 ratios were significantly higher for *labour* (vs. no labour, p = 0.03 resp. p = 0.04), the same was true for TIMP-4 concentrations (p < 0.001). Regression results show no significant association between *preterm*or *labour*status and MMP-3 or any of the MMP-3:TIMP ratios.

*Evaluation of multiple biomarkers in maternal serum*

Of the 30 inflammatory markers analysed, only 8 were detectable in more than 50% of the maternal serum samples: EGF, HGF, IL-12, eotaxin, MIP-1β, MCP-1, IP-10 and sIL-2R. Concentrations of RANTES were all outside the linear range. Levels of the other 21 inflammatory markers were below the detection limits of the assay in more than 50% of samples in all groups. To avoid biased results in subsequent statistical analyses, these censored values were imputed at half the detection limit.

Table V. — Comparison of MMP-3, MMP-9, TIMPs levels and MMP:TIMPs ratios in maternal serum among groups.

| | **Group 1 PTB (n = 47)** | **Group 2 GA matched controls (n = 47)** | **Group 3 AT in labor (n = 40)** | **Group 4 AT not in labor (n =32)** | **Group 1 vs. 2 *P*-value$** | **Group 3 vs. 4 *P*-value$** | **Group 1 vs. 3 *P*-value$** |
|---|---|---|---|---|---|---|---|
| MMP-9 | 1125.1 [694.1-1977.4] | 639.5 [513.8-924.5] | 828.7 [420.7-1259.8] | 554.6 [377.5-711.3] | < 0.001 (**0.001**) | 0.06 (NS) | 0.006 (0.07) |
| TIMP-1 | 132.04 [118.25-152.27] | 125.65 [111.07-139.21] | 149.49 [133.01-185.46] | 137.98 [125.18-149.44] | 0.08 (NS) | 0.02 (NS) | 0.008 (0.09) |
| TIMP-2 | 121.76 [107.62-132.19] | 121.26 [109.35-135.82] | 156.12 [139.67-190.19] | 137.71 [124.57-164.27] | 0.68 (NS) | 0.03 (NS) | < 0.001 (**< 0.001**) |
| TIMP-4 | 1.46 [1.09-1.96] | 1.04 [0.83-1.26] | 1.24 [1.12-1.46] | 1.08 [0.81-1.35] | 0.001 (**< 0.001**) | 0.01 (NS) | 0.08 (NS) |
| MMP-9:TIMP-1 ratio | 8.21 [4.82-13.88] | 5.26 [3.89-7.09] | 4.85 [2.86-8.98] | 3.94 [3.07-5.33] | 0.002 (**0.02**) | 0.35 (NS) | 0.001 (**0.006**) |
| MMP-9:TIMP-2 ratio | 9.68 [6.06-14.34] | 5.23 [3.95-7.35] | 4.61 [2.80-6.75] | 3.69 [2.92-5.45] | 0.001 (**< 0.001**) | 0.26 (NS) | < 0.001 (**< 0.001**) |
| MMP-9:TIMP-4 ratio | 847.3 [386.8-1463.3] | 657.4 [434.0-995.9] | 578.0 [342.7-980.3] | 567.7 [337.6-765.7] | 0.29 (NS) | 0.57 (NS) | 0.13 (NS) |
| | **PTB (n = 34)** | **GA matched controls (n = 34)** | **AT in labor (n = 27)** | **AT not in labor (n = 21)** | ***P*-value$** | ***P*-value$** | ***P*-value$** |
| MMP-3 | 9.10 [5.83-15.53] | 8.78 [4.68-14.97] | 9.55 [3.96-15.83] | 7.14 [4.76-13.46] | 0.55 (NS) | 0.76 (NS) | 0.52 (NS) |
| MMP-3:TIMP-1 ratio | 0.065 [0.046-0.111] | 0.065 [0.032-0.136] | 0.062 [0.033-0.108] | 0.057 [0.035-0.093] | 0.60 (NS) | 0.88 (NS) | 0.25 (NS) |
| MMP-3:TIMP-2 ratio | 0.073 [0.058-0.118] | 0.074 [0.041-0.111] | 0.048 [0.031-0.103] | 0.045 [0.030-0.102] | 0.47 (NS) | 0.99 (NS) | 0.03 (NS) |
| MMP-3:TIMP-4 ratio | 6.13 [3.86-12.40] | 7.95 [4.84-13.37] | 8.45 [3.94-11.37] | 7.36 [4.06-11.37] | 0.44 (NS) | 0.96 (NS) | 0.74 (NS) |

Results are expressed as median (interquartile range) (ng/ml), group differences were evaluated with the Mann-Whitney *U*-test
PTB, preterm birth; PT, preterm; AT, at term; NS, not significant
$ Unadjusted P values and Bonferroni-adjusted P values (adjusted for 11 tests) between brackets

Median levels of EGF and HGF were significantly higher in women with *PTB* compared to *GA matched controls* (respectively $p < 0.01$ and $p < 0.05$). A significantly lower median IL-12 concentration was observed in women *AT in labour* compared to *AT not in labour* ($p < 0.05$).

Median IL-12, eotaxin and IP-10 were significantly lower in women with *PTB* compared to *AT in labour* (respectively $p < 0.01$, $p < 0.05$ and $p < 0.01$). Lower median MCP-1 levels were observed in women with *PTB*, although this difference was marginally not significant ($p = 0.05$).

Multiple regression analysis was performed on the full dataset. The $R^2$ of our regression models varied from 10 to 39%, indicating a large amount of variation in log marker concentration not explained by the covariates included. Our regression results showed that, after adjusting for other covariates, average IP-10 concentrations were 47% lower *preterm* (vs. at term, $p < 0.001$) and average IL-12 concentrations were 23% *lower* in labour. We observed no similar significant association of *preterm* or *labour* status with any of the other 7 markers.

## Discussion

### *Elevated sTREM-1 levels in maternal serum during term and preterm labour*

In line with previous observations in amniotic fluid (Kusanovic et al., 2010), serum sTREM-1 levels were significantly increased in women with preterm labour compared to GA matched controls. We also observed elevated sTREM-1 concentrations during spontaneous term parturition vs. non labouring controls. This observation is consistent with Youssef et al. who demonstrated increased TREM-1 RNA expression in myometrium and cervix after labour at term (Youssef et al., 2009). In contrast, no differences were observed in amniotic fluid concentrations of sTREM-1 between labouring and non-labouring women at term (Kusanovic et

al., 2010). These data suggest that the maternal inflammatory response during labour may be different form the foetal response. We also found higher sTREM-1 levels in women with preterm labour compared to term labour. The increased circulating sTREM-1 levels during preterm labour may indicate a contributory role of sTREM-1 in infectious-associated PTB and is in agreement with previous studies who demonstrated that sTREM-1 may play a role during preterm parturition and IUI (Cobo et al., 2012; Holst et al., 2011; Kusanovic et al., 2010; Menon and Fortunato, 2008).

Our observations support the involvement of an inflammatory process in human parturition (either term or preterm). However, additional research is recommended to explore the role of sTREM-1 during pregnancy and labour and to assess the usefulness of sTREM-1 as diagnostic and prognostic biomarker in maternal blood of both symptomatic and asymptomatic patients compared with standard of care.

*Imbalances between MMPs and TIMPs in maternal serum during preterm labour*

The role of MMPs in human term and preterm parturition, and to a lesser extent that of TIMPs, have been widely investigated over the past decades. The novelty of our study regards the determination of MMP-3, MMP-9 and all four TIMPs in maternal serum as well as the MMP:TIMP ratios. In line with previous observations in maternal plasma and amniotic fluid (Makrakis et al., 2003; Maymon et al., 2000) we found elevated MMP-9 concentrations in maternal serum during preterm labour. Human parturition (either term or preterm) was not associated with changes in serum MMP-3 concentration in our study. In contrast, MMP-3 levels in amniotic fluid were elevated during spontaneous labour at term and preterm (Park et al., 2003).

A fully functional TIMP network has been demonstrated in human foetal membranes, placenta and decidua and in second trimester amniotic fluid (Fortunato et al., 1998; Riley et al., 1999). To the best of our knowledge, we demonstrated for the first time that all four TIMPs were expressed in maternal serum. Levels TIMP-1 and TIMP-2 were lower during gestation compared to term gestation, irrespective of labour status. This is in agreement with Clark et al., who demonstrated that TIMP-1 is suppressed during pregnancy with increasing serum levels from 37 weeks onwards, back to pre-pregnant levels (Clark et al., 1994). The similar observation was made in amniotic fluid for TIMP-2 showing an increase in concentration with advancing gestational age (Athayde et al., 1998).

Little is known about the role of TIMP-4 during pregnancy and parturition. It has been shown that TIMP-4 is involved in reproductive processes and cancers (Chegini et al., 2003; Melendez-Zajgla et al., 2008; Pilka et al., 2006). We found that TIMP-4 levels were elevated during labour (either term or preterm), but changes in MMP-9:TIMP-4 ratio were not significant. This may be related to the relatively low sample size of the study, but may also be explained by the different potency of TIMP-4 against different MMPs. It has been shown that, although TIMP-4 effectively inhibits MMP-9, it has the highest affinity for MMP-26 (Zhang et al., 2002). It would be interesting to investigate whether higher MMP-26:TIMP-4 ratios are associated with preterm labour.

Importantly, our study showed that MMP-9:TIMP-1 and MMP-9:TIMP-2 ratios were significantly higher in women with preterm labour, indicating a ratio shift towards matrix degradation.Previous studies in amniotic fluid and *in vitro* studies on foetal membranes demonstrated imbalances between MMPs and TIMPs during PPROM. Fortunato et al. (1999) found that the molar ratio between MMP-2 and TIMP-2 in amniotic fluid was increased during PPROM. Our finding of MMP-9:TIMP imbalances in maternal serum, tilting the balance toward matrix degradation, deserves further consideration. It has been shown that MMP-9 is detectable in serum approximately 24 hours before the initiation of labour or PPROM (Goldenberg et al., 2005), so probably the same is true for the MMP-9:TIMP ratio, thereby limiting the time to initiate an intervention. In conclusion, our results indicate that circulating MMPs and TIMPs may play a role in the pathogenesis of preterm labour at systemic level and suggest that a far less invasive approach could be developed for the measurement of enzymes essential for ECM remodelling during pregnancy and parturition. However, further research is needed to determine the temporal relationship between MMP:TIMP ratio shift and PTB as well as the involvement of other members of the MMP family in pregnancy and (preterm) labour.

*Multiple biomarkers in maternal serum in women with term and preterm labour*

Finally, we evaluated a biomarker panel in maternal serum during term and preterm labour. Among the 30 inflammatory markers we studied, only EGF and HGF levels were associated with preterm labour. However, the multiple regression model for EGF showed that sample age may act as a confounding factor. It has been demonstrated that EGF levels are higher in serum than those measured in

plasma, which may be attributed to platelet derived EGF (Yucel et al., 2007). Therefore, the value of EGF as non-invasive marker for preterm labour seems to be limited. To our knowledge, no other studies investigated HGF serum levels during labour. Although group comparison showed higher HGF levels in preterm labour, none of the key covariates were significant in the multiple regression models. Large studies are needed to confirm the possible role of HGF in the onset of preterm labour.

We observed lower IL-12 levels in labouring women, irrespective of gestational age (term or preterm). Our results are in agreement with those of Dubicke et al. (2010) who demonstrated that mRNA IL-12 was downregulated in cervical tissues of labouring women and expression was lower in preterm than in term labour. These data are contrary to the classical Th1:Th2 paradigm, which assumes that a Th1 response is associated with adverse pregnancy outcomes.However, it has been shown that IL-12 can interact synergistically with IL-18 to provoke a Th1 response. Therefore, the cytokine balance may be of greater importance than the absolute concentrations. High IL-12:IL-18 ratios in women with preterm labour were associated with a twofold increased risk for delivery before 34 weeks (Ekelund et al., 2008). Few studies have reported the relation between IP-10 and preterm labour. We observed higher IP-10 levels in term gestation, irrespective of labour. This is in line with Kraus et al. who observed increasing IP-10 levels in maternal blood with advancing gestational age(Kraus et al., 2010). It has been demonstrated that amniotic fluid levels of IP-10 were elevated in preterm labour cases with chronic chorioamnionitis (Ogge et al., 2011). It would be interesting to investigate whether chronic chorioamnionitis is associated with an altered chemokine profile in maternal blood.

Our study has several strengths. First, we evaluated several biomarkers in maternal serum which can be easily obtained during pregnancy. Secondly, we determined biomarkers in women with either term or preterm labour vs. non-labouring controls. The mechanisms of preterm labour are largely unknown, at least in part because the process of term labour is poorly understood. Further understanding of the inflammatory processes during labour is essential for the development of novel and effective therapies to block preterm labour (Hamilton et al., 2012). Another strength of our study is that we performed multiple regression analysis to determine the effect of three key covariates (preterm vs. at term, labour vs. no labour, ROM vs. intact membranes) and to adjust for possible confounding effects by other covariates including important risk factors of PTB, sample age and storage time. When multiple biomarkers were considered, we also accounted for the number of tests by applying Bonferroni correction. Finally, our analyses were performed by using multiplex technology, when commercially available. This novel technological approach makes it possible to measure multiple proteins simultaneously in a minimal amount of biological sample. This is important, because it is increasingly accepted that single biomarkers lack effectiveness to accurately predict PTB (Menon et al., 2011).

Despite these strengths, some limitations of our study deserve consideration. First, we only evaluated maternal biomarkers in serum, mainly because of the non-invasive approach, but also because amniocentesis and placental and foetal membrane histology are not standard of care in our institution. Hence, our PTB group represents a quite heterogeneous group of patients, because information on foetal or placental conditions was lacking. Secondly, the study was conducted in a single institution which prevented the use of a larger sample size; hence the number of women was inadequate for extensive subgroup analysis. An interesting subgroup analysis would be to evaluate whether biomarker levels differ between women with preterm labour and intact membranes who delivered preterm vs. those with term delivery. Finally, the nested case control design does not allow us to evaluate the accuracy of the analysed inflammatory markers to predict PTB. A large multicentre prospective cohort study is needed to investigate the predictive value of these biomarkers in maternal blood and PTB in asymptomatic women (before preterm labour) and to develop valuable combined multivariable prediction models.

## Conclusion

Until present, few biomarkers have shown clinical usefulness, because they are nonspecific or become positive too late. Among the biomarkers evaluated to date, the most powerful and consistent predictors of PTB are the presence of foetal fibronectin in cervicovaginal fluid and a short cervix on transvaginal ultrasound. The clinical value of both tests primarily lies in their negative predictive value thereby guiding clinicians in decision-making and avoiding unnecessary interventions. During the last decades, it has become clear that single or universal biomarkers will not be capable to predict PTB accurately in all populations. Future research should focus on multiple biomarkers in different PTB subtypes to allow differentiation depending on the underlying causes. The future development of an accurate, minimally invasive multiple marker test is necessary to permit incorporation

into clinical practice. The availability of new technologies capable of probing the genome offers exciting possibilities to gain new insights into the mechanisms leading to PTB and to develop targeted therapies.

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