Serum cytokine patterns in first half of pregnancy

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Abbreviations:

BMI, body mass index; CCL, CC chemokine ligand; CRP, C-reactive protein; CXCL, CXC chemokine ligand; DBP, diastolic blood pressure; FGF, fibroblastic growth factor; G-CSF, granulocyte colony-stimulating factor; GA, gestational age; GM, granulocyte macrophage; IFN, interferon; IL, interleukin; IP, IFN-γ-induced protein; LMM, linear mixed effects models; MBRN, Medical birth registry of Norway; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PCA, principal component analysis; PDGF platelet-derived growth factor; PLS-DA, partial least squares discriminant analysis; Ra, receptor antagonist; SBP, systolic blood pressure; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Abstract

Introduction: Human pregnancy is a state of elevated maternal systemic inflammation, and pregnancy complications are often associated with a dysfunctional immune response. The network of cytokines reflects this complex immune activity, and broad serum cytokine profiling provides a new tool to understand the changes in immune status during pregnancy.

Objective: This study aimed to determine how maternal serum cytokine patterns change during the first half of pregnancy.

Methods: Maternal peripheral serum samples collected at a mean gestation of 10, 13, 18 and 24 weeks were included from a prospective clinical study of healthy women (n=110) in first half of normal pregnancy. The serum samples were analysed for 27 different cytokines using multiplex magnetic bead-based immunoassays, and high sensitivity C-reactive protein (CRP) was analysed by ELISA. Serum cytokine and CRP patterns were explored with linear mixed effects models (LMM) and multilevel partial least squares discriminant analysis (PLS-DA).

Results: Serum cytokine profiling provided partial overview of the maternal immune status and corresponding reference values for serum cytokine levels during the first half of pregnancy. Several cytokines decreased in concentration from first to second trimester. Cytokine pattern analysis revealed that chemokines provided the most sensitive measurement of variation with gestational age in normal pregnancies. The nine inflammatory cytokines showed the highest intra-group correlation during pregnancy, while CRP levels did not correlate with changes in the inflammatory cytokines.

Conclusion: Chemokines showed the greatest gestational variation and inflammatory cytokines showed a strong intra-group correlation during the first half of pregnancy.

Keywords: Pregnancy, inflammation, longitudinal, cytokine, chemokine, C-reactive protein.

1. Introduction

A complex and dynamic immune activity is central to the success of human pregnancy (Aagaard-Tillery, Silver, & Dalton, 2006; Mor & Cardenas, 2010). The maternal immune system must maintain protection against infections, while keeping an immune balance and meeting the demands of the developing fetus. This challenge is reflected by an increased systemic level of inflammation in pregnancy, with elevated serum levels of C-reactive protein (CRP), cytokines like interleukin (IL)-6 and tumor necrosis factor (TNF)-α, and oxidative stress markers such as oxidized low-density lipoprotein, compared to in the non-pregnant state (Belo et al., 2004; Molvarec et al., 2011; Skarzynska, Zborowska, Jakimiuk, Karlinska, & Lisowska-Myjak, 2018; Szarka, Rigo, Lazar, Beko, & Molvarec, 2010). Several pregnancy complications have been associated with further alterations of serum inflammatory markers, reflecting the detrimental role of a dysfunctional maternal immune response. How serum cytokine levels change during normal pregnancy is currently not well described, and characterizing the overall maternal immune status would be clinically useful (Azizieh et al., 2018; Kalagiri et al., 2016). Currently, CRP is the only inflammatory marker used in clinical practice in pregnancy.

Cytokines are cell-signalling proteins important for immune activation, inhibition and regulation. Cytokines are produced by most cell types and have multiple and overlapping functions. This creates a network of cellular communication and provides a basis for understanding the complex immunity of pregnancy. Simultaneous measurement of multiple serum cytokines provides a snapshot of the overall immune status.

Cytokines are abundant at the maternal-fetal interface, and they are involved in regulating the delicate interaction between maternal and fetal cells. Studies in early pregnancy have focused on single cytokine measurements or the shift from inflammatory TH1 cytokines to more anti-inflammatory TH2 cytokines with advancing gestation (Christian & Porter, 2014; Curry et al., 2008; Holtan et al., 2015; Kraus et al., 2010). The literature on cytokine

development during normal pregnancy holds conflicting findings. Some studies report increasing TNF-α and interferon (IFN)-γ levels with gestational age (Christian & Porter, 2014; Kraus et al., 2010; Vassiliadis, Ranella, Papadimitriou, Makrygiannakis, & Athanassakis, 1998), while others find no significant change (Coussons-Read, Okun, & Nettles, 2007; Curry et al., 2008; Vassiliadis et al., 1998) or decreasing levels (Kraus et al., 2010). These diverging results may imply that no single cytokine can explain the complex immunological network needed for maintaining a successful pregnancy, and a broader profiling of multiple cytokines is required. Powerful multivariate discriminating methods such as partial least squares discriminant analysis (PLS-DA) allows for analysis of several cytokines simultaneously while taking interactions between the cytokines into account. In this way, the complex maternal immune responses during pregnancy may be assessed for novel insight. We have recently demonstrated that broad maternal serum cytokine profiling at gestational age (GA) 11-13 weeks provided a sensitive measurement of maternal inflammatory status and could identify gestational hypertension occurring later in pregnancy (Tangeras et al., 2015).

There is a need for establishing the normal range of cytokine pattern variations in pregnancy. This is necessary for revealing disease-specific changes in cytokine patterns in complicated pregnancies. In the present study, we performed a broad characterization of the maternal serum cytokine profile at four different time points in the first half of normal pregnancy.

2. Materials and methods

2.1 Study population and study visits

The present study includes serum samples from the NormalFlow study (Stridsklev et al., 2017). NormalFlow was a prospective clinical study of 124 women included at St. Olavs Hospital, Trondheim University Hospital between June 2008 and May 2010, aiming to construct a reference curve for Doppler measurements of the uterine artery in first and second trimester. The participants were healthy Caucasian women, 18 to 38 years old with an ongoing first trimester, singleton pregnancy. Exclusion criteria were 1) somatic or psychiatric disease, 2) pregnancy complication in previous pregnancies (e.g. preeclampsia, intrauterine fetal death, gestational diabetes or preterm delivery), 3) multiple pregnancy and 4) other reasons (non-Norwegian speaker, long distance to study centre). Missed abortions and severe congenital anomalies were excluded. The study was approved by the Regional Committee for Medical and Health Research Ethics in Mid-Norway, Norway (No. 4.2008.841). All participants gave written informed consent.

Fourteen women were excluded from the original Normalflow cohort before analyses due to: previously undetected polycystic ovary syndrome (n = 1), eating disorder and ADHD (n = 1), hypertensive pregnancy disorders (n = 5), intrauterine fetal death in week 35 (n = 1), delivery before gestational week 37+0 (n = 4), and missing info about pregnancy outcome (n = 2), leaving 110 women for the present study. None of the included women experienced gestational diabetes.

Study characteristics of the participants and the neonatal outcomes were compared to national averages for the same characteristics collected from the Medical Birth Registry of Norway (MBRN) in 2017 (Norwegian Institute of Public Health (NIPH), 2018). Statistical comparisons between the two groups were not done, as only a summary of the MBRN data was available.

Four study visits were performed during the first and second trimester. Mean GA in weeks and days (± standard deviation) at the four study visits were 10+5 (±7 days), 13+0 (±6 days), 18+4 (±5 days) and 24+2 (±7 days), and these time intervals were categorized as GA 10, 13, 18 and 24 weeks. Non-fasting peripheral blood was drawn from the antecubital vein in non-heparinized tubes. A serum sample was separated and stored at -80°C, thawed on ice and aliquots were stored at -80°C until analysis. Serum was sampled and analysed from 104 women (95%) at GA 10 weeks, 96 (87%) at GA 13 weeks, 93 (85%) at GA 18 weeks and 101 (92%) at GA 24 weeks. Seventy-eight women (71%) provided samples at all four time points. This gave a total of 394 serum samples for analysis.

2.2 Serum measurements

Serum levels of 27 cytokines (Bio-Plex Pro Human Cytokine 27-plex Assay) were measured in single replicates using Luminex xMAP Technology on a Bio-Plex 200 System (Bio-Rad Laboratories, CA, USA) according to the manufacturer's protocol. The kit was chosen as it was commercially available, included many pregnancy relevant cytokines and had been shown to provide sensitive measurement of the immunological status in early pregnancy (Tangeras et al., 2015). Cytokine standards and sample diluent provided in the assay were measured in duplicate on each plate. To minimize technical variation when analysing samples run on different plates, duplicates of a pre-made quality control sample were run on each plate for inter-assay comparison. Adjustments were done according to Browne *et al.* resulting in an equal mean value of the quality control sample on each plate (Browne et al., 2013). High sensitivity CRP was analysed in single replicates with Human CRP Quantikine kit (R&D technologies, MN, USA) according to the manufacturer's protocol.

The cytokine RANTES was excluded from further analyses since 87% of the measurements were above the upper limit of detection, leaving 26 cytokines for further analysis. Ten of the cytokines had serum concentrations below the limits of detection in less than 15% of total

samples, and these values were replaced with the lowest detectable value divided by two (Hornung & Reed, 1990).

2.3 Data processing and statistical analysis

Study population characteristics were tested for normality with D'Agostino-Pearson test in GraphPad Prism 7.0 (GraphPad Software, CA, USA). Normally distributed data are reported as mean (± standard deviation), non-normal data as median (interquartile range) and categorical variables as numbers (percentages). The 26 cytokines were divided into four groups by main function; 1) inflammatory cytokines, 2) anti-inflammatory cytokines, 3) growth and colony-stimulating factors and 4) chemokines. Cytokine data were tested for normality by visual inspection of quantile-quantile plots. Outliers were identified by Grubbs test in log-transformed data and visual inspection of principal component analysis (PCA) plots including all samples. Linear mixed effects models (LMM) were used to explore time dependent development of individual cytokines and CRP. LMM allows for adjustment of the possible random effect added when analysing multiple samples from the same woman. LMM were performed with log-transformed cytokine and CRP concentration as response variables, study visit gestational age and maternal age, parity, smoking status, body mass index (BMI) and systolic blood pressure measured at GA 10 weeks as fixed effects, and individual as random effect. GA was reported as a continuous variable. The results were corrected for multiple testing using Benjamini-Hochberg false discovery rate, and $q \leq 0.05$ was considered statistically significant. LMM was performed in Stata 2017 (Stata Statistical Software: Release 15, TX, USA).

Maternal serum cytokine patterns were explored by the multivariate analyses PCA and PLS-DA. These methods can identify underlying patterns in multivariate data by defining simpler and more information-rich latent variables, and the resulting variables can be visualized with scores and loadings plots (Barker & Rayens, 2003; Wold, Esbensen, & Geladi, 1987). Multilevel PLS-DA, which resembles a multivariate paired student t-test were only patients

192 with samples from both time points of interest are included, allowed the study of the
193 individual variance with gestational age in cytokine profile (Westerhuis, van Velzen,
194 Hoefsloot, & Smilde, 2010). Loading plots provided information on which cytokines that
195 differed the most between time points. Cytokine data were autoscaled prior to multilevel
196 PLS-DA analysis. The classification models were evaluated by double cross validation,
197 where a model was built on training data (80% of the included women) and used to predict
198 independent test samples (the remaining 20%). All samples from one women were put in
199 either the training or the validation set. The optimal number of latent variables included in the
200 model was determined by cross-validation of the training data. Both the inner and outer loop
201 of validation were repeated 20 times and median sensitivity, specificity and accuracy of
202 classification was calculated. The resulting model was orthogonalized for easier
203 interpretation, and the statistical significance of the model was assessed by permutation
204 testing (n = 1000 permutations). $P \leq 0.05$ was considered statistically significant. Multivariate
205 analyses were done in Matlab v.r2017a (The Mathworks Inc., MA, USA) with PLS_toolbox
206 8.2.1 (Eigenvector Research, WA, USA).
207
208 Spearman rank correlation was used to assess interrelatedness between all cytokines and
209 CRP and the results were presented in heat maps from each gestational age. Mean
210 correlation within cytokine groups was calculated by combining data from all gestational
211 ages.
212

3. Results

3.1 Study population

The characteristics of the included pregnancies were comparable to the national average from the MBRN (Table 1). More women in the study population smoked at time of inclusion compared to the national average, but the registration of smoking status was performed during pregnancy in our study and in retrospect after pregnancy in the MBRN. Due to exclusion of women giving birth preterm, the gestational age and the birth weight of the children in the study were slightly higher than the national average (Table 1). All babies born to the included mothers had APGAR score of 9 or 10 after 10 minutes, except one baby with shoulder dystocia who had APGAR score 7 after 10 minutes. Two babies were diagnosed as large for gestational age and one as small for gestational age, otherwise all babies showed appropriate weight for gestational age (data not shown).

Table 1

Characteristics of study population (n = 110) compared to the national average from the Medical Birth Registry of Norway in 2017.

Characteristics at first study visit or before pregnancy		
	Study population	**MBRN 2017**
Gestational age (weeks)	10.7 ± 1.0[a]	nd
Age (years)	28.7 ± 4.2	29.2 ± 4.8[h]
BMI (kg/m^2)	22.9 (21.5-25.1)[b]	23.2 (21.0-26.5)[i]
Primipara n (%)	55 (56)[c]	nd
SBP (mmHg)	114 ± 11[d]	nd
DBP (mmHg)	69 ± 8[e]	nd
Smoking n (%)	11 (12.5)[a]	(3.9)[j]
Characteristics at delivery		
Gestational age (weeks)	40.0 ± 1.3[f]	39.3 ± 1.9
Birth weight (g)	3577 ± 505[g]	3489 ± 591
Birth length (cm)	49.4 ± 5.3[g]	nd
Head circumference (cm)	35.3 ± 1.4[g]	nd
Placental weight (g)	653 ± 136[g]	nd
Fetal sex n (%) male	52 (51.0)[g]	(51.5)

Continuous variables are reported as mean (± standard deviation) or median (25th and 75th percentile), categorical variables are reported as percent (%). Blood pressure was measured three times with two minutes interval after at least 10 minutes rest. Data from the MBRN includes preterm births.

Missing information for [a] 11 women, [b] 22 women, [c] 4 women, [d] 16 women, [e] 17 women, [f] 3 women and [g] 8 women; [h] average age for primigravida, [i] average pre-pregnant BMI and [j] smoking at start of pregnancy.

BMI, body mass index; DBP, diastolic blood pressure; MBRN; Medical Birth Registry of Norway; nd, no data; SBP, systolic blood pressure.

3.2 Maternal serum reference values for 26 cytokines

The serum cytokine reference values at four time points during first half of the pregnancy are presented in Table 2. Fig. 1 shows the development in median cytokine concentrations for the four cytokine groups. Changes in serum cytokine expression during the first half of pregnancy were apparent, but notable variations between individuals were evident by the relatively large interquartile ranges. There was a great span in absolute concentrations for different cytokines, with platelet-derived growth factor BB (PDGF-BB) having the highest (1622 pg/ml), and IL-5 having the lowest levels (0.6 pg/m) at GA 10 weeks (Table 2 and Fig. 1). The cytokines with the greatest variation between the four time points in pregnancy were the inflammatory cytokine IL-2 and the chemokine eotaxin. Both decreased to nearly half of their original concentration from GA 10 to 24 weeks (Table 2 and Fig. 1).

Table 2

Reference values and variation with gestational age for maternal serum CRP (µg/ml) and 26 cytokines (pg/ml) measured by Bio-Plex Pro Human Cytokine 27-Plex Assay.

	GA 10 weeks (n = 104)	GA 12 weeks (n = 96)	GA 19 weeks (n = 93)	GA 24 weeks (n = 101)	q-values from LMM
CRP	3.5 (1.4-7.2)	4.8 (2.2-7.3)	4.9 (2.6-7.9)	4.6 (1.9-8.5)	< 0.001[a]
Inflammatory cytokines					
IL-1β	1.6 (1.2-2.0)	1.6 (1.1-1.9)	1.5 (1.2-1.9)	1.5 (1.2-1.9)	0.126
IL-2	1.7 (0.6-3.4)	1.6 (0.7-2.9)	1.1 (0.4-3.0)	0.8 (0.2-2.0)	< 0.001[a]
IL-6	1.4 (0.8-2.1)	1.3 (0.8-1.8)	1.1 (0.6-1.7)	1.1 (0.7-1.8)	< 0.001[a]
IL-8 (CXCL8)	5.5 (4.5-7.0)	5.4 (4.3-6.6)	5.3 (4.1-6.3)	5.2 (4.1-6.1)	< 0.001[a]
IL-12p70	3.1 (1.8-5.5)	3.3 (2.0-5.5)	2.9 (1.9-5.9)	3.3 (1.9-6.9)	0.637
IL-15	5.0 (2.9-10.1)	5.5 (2.7-10.2)	4.6 (1.9-8.0)	4.4 (1.7-7.6)	< 0.001[a]
IL-17	41.3 (32.2-49.5)	41.3 (34.0-49.4)	37.4 (30.5-45.0)	38.9 (29.7-49.7)	0.027[a]
IFN-γ	54.6 (43.3-66.4)	53.4 (45.3-66.4)	52.2 (45.5-61.0)	52.6 (41.3-60.7)	0.067[b]
TNF-α	24.1 (20.4-29.9)	23.5 (20.4-28.7)	24.2 (20.3-29.2)	22.8 (20.6-29.3)	0.423
Anti-inflammatory cytokines					

IL-1Ra	68.1 (55.2-103.1)	69.9 (55.6-97.9)	72.3 (56.7-90.5)	66.5 (54.3-86.0)	0.068[b]
IL-4	3.0 (2.6-3.5)	3.0 (2.6-3.5)	3.0 (2.5-3.6)	3.0 (2.6-3.3)	0.091
IL-5	0.6 (0.3-2.6)	0.9 (0.3-2.6)	1.0 (0.3-2.6)	0.5 (0.3-2.5)	0.895
IL-9	54.6 (41.1-67.4)	54.4 (42.1-62.9)	49.4 (40.9-61.4)	50.2 (39.6-61.5)	0.015[a]
IL-10	1.7 (0.8-4.3)	2.0 (1.2-3.7)	1.5 (0.9-3.4)	1.6 (0.8-3.3)	0.005[a]
IL-13	2.0 (0.6-3.9)	1.8 (0.5-3.5)	1.7 (0.7-3.1)	2.2 (1.0-4.5)	0.177
Growth and colony-stimulating factors					
VEGF	6.7 (4.5-9.7)	6.9 (4.7-10.5)	7.2 (4.9-10.6)	7.9 (5.5-10.4)	0.117
FGF basic	55.0 (47.9-62.1)	55.5 (49.6-66.4)	55.5 (49.4-62.2)	54.0 (48.1-64.1)	0.117
PDGF-BB	1622 (975-2196)	1424 (1053-2011)	1394 (897-1898)	1174 (857-1710)	< 0.001[a]
G-CSF	26.2 (21.2-32.8)	27.7 (21.4-35.0)	27.6 (22.1-36.7)	26.3 (22.7-34.6)	0.214
GM-CSF	65.8 (47.8-98.7)	65.3 (48.9-108.8)	58.1 (46.8-103.3)	58.8 (47.0-84.6)	0.031[a]
IL-7	3.4 (0.6-5.5)	3.3 (1.5-5.9)	4.0 (1.9-6.3)	3.9 (2.0-6.4)	0.067[b]
Chemokines					
MCP-1 (CCL2)	31.3 (23.5-47.6)	30.1 (22.0-42.1)	30.2 (20.7-38.4)	27.3 (19.3-38.0)	< 0.001[a]
MIP-1α (CCL3)	2.1 (1.7-2.5)	2.0 (1.7-2.4)	2.0 (1.6-2.3)	2.0 (1.7-2.5)	0.895
MIP-1β (CCL4)	58.1 (42.4-7.7)	53.5 (39.2-68.9)	52.4 (36.4-66.7)	49.9 (38.6-65.7)	< 0.001[a]
Eotaxin (CCL11)	74.4 (53.7-100.2)	66.7 (50.5-85.5)	49.0 (42.5-69.0)	47.7 (41.1-59.5)	<0.001[a]
IP-10 (CXCL10)	1151 (749-1790)	1076 (733-1620)	1023 (696-1500)	1017 (687-1554)	0.171

Cytokine and CRP data are reported as median (25^{th}-75^{th} percentile).

[a] Significant before and after Benjamini-Hochberg correction for multiple testing.

[b] Significant before Benjamini-Hochberg correction for multiple testing.

CCL, CC chemokine ligand; CRP, C-reactive protein; CXCL, CXC chemokine ligand; FGF, fibroblastic growth factor; GA, gestational age; G-CSF, granulocyte colony-stimulating factor; GM, granulocyte macrophage; IFN, interferon; IL, interleukin; IP, IFN-γ-induced protein; LMM, linear mixed effects models; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; Ra, receptor antagonist; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

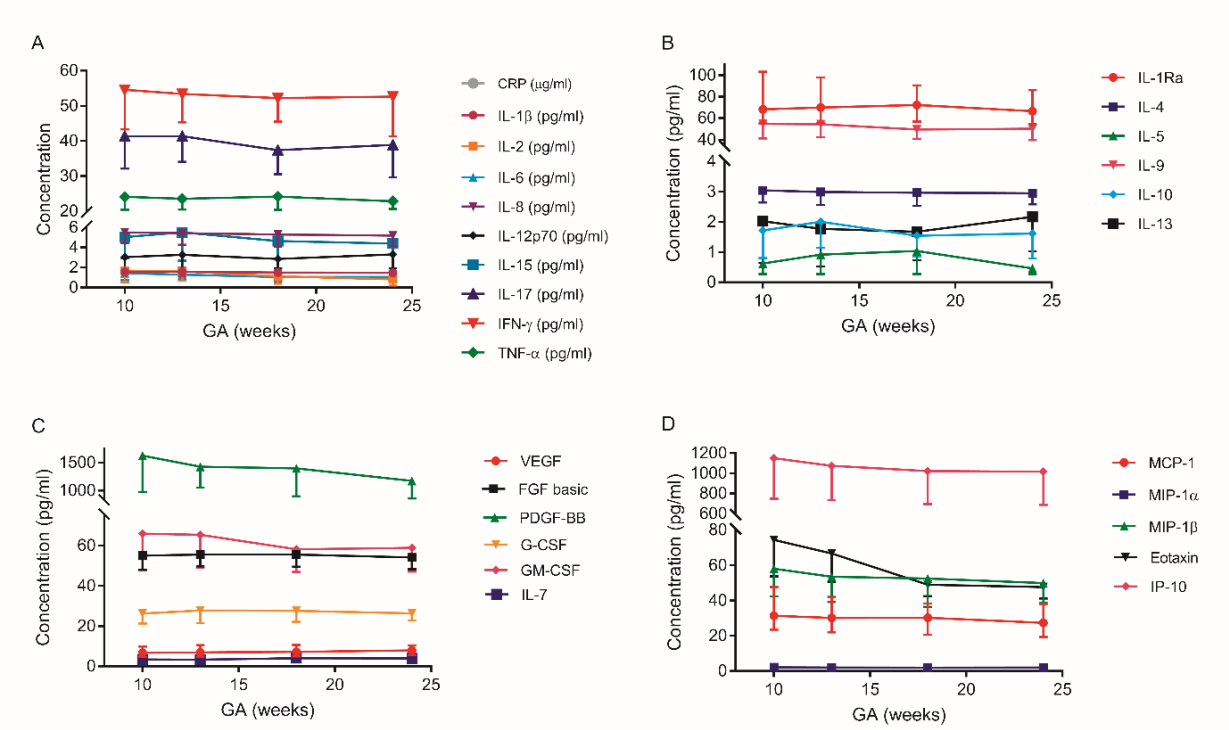


Fig. 1. Expression of maternal serum cytokines (pg/ml) and CRP (µg/ml) at GA 10, 13, 18 and 24 weeks. A) Inflammatory cytokines and CRP, B) Anti-inflammatory cytokines, C) Growth and colony-stimulating factors, D) Chemokines. Data are reported as median (25^{th}-75^{th} percentile). N=110. CRP, C-reactive protein; FGF, fibroblastic growth factor; GA, gestational age; G-CSF, granulocyte colony-stimulating factor; GM, granulocyte macrophage; IFN, interferon; IL, interleukin; IP, IFN-γ-induced protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; Ra, receptor antagonist; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

3.3 Cytokine levels from early to mid-pregnancy

To better assess the overall serum cytokine variation during pregnancy and adjust for individual basal cytokine levels, the cytokine levels were normalized to the first study visit by subtracting all the participant's measurements with the measurement at GA 10 weeks (Supplementary Table S1 and Fig. 2). A general tendency of decreasing cytokine concentrations with increasing pregnancy length was observed, and this was especially apparent for the chemokines, in addition to IL-2 and IL-15 in the inflammatory cytokine group. The change in cytokine concentration with gestational age seemed to vary mostly within the anti-inflammatory and the growth and colony-stimulating factors groups.

Supplementary table S1

Relative change (%) in 26 maternal serum cytokines and CRP normalized to GA 10 weeks.

	GA 12 weeks (n = 93)	GA 18 weeks (n = 88)	GA 24 weeks (n = 95)
CRP	11.4 (-27.2-97.6)	16.9 (-16.9-138.5)	11.4 (-27.2-97-6)
Inflammatory cytokines			
IL-1β	-2.5 (-15.2-15.9)	0.5 (-18.9-19.9)	-2.5 (-15.2-15.9)
IL-2	-40.3 (-67.7-5.9)	-17.3 (-85.0-62.0)	-40.3 (-67.7-5.9)
IL-6	-16.3 (-51.7-33.3)	-20.8 (-48.4-19.4)	-16.3 (51.7-33.3)
IL-8 (CXCL8)	-4.8 (-25.7-14.4)	-4.8 (-24.1-14.0)	-4.8 (-25.7-14.4)
IL-12p70	-5.9 (-38.8-53.7)	-2.4 (-34.8-62.1)	-5.9 (-38.8-53.7)
IL-15	-27.0 (-56.0-25.3)	-8.6 (-54.9-73.9)	-27.0 (-56.0-25.3)
IL-17	-7.1 (-23.1-14.5)	-4.6 (-25.7-12.9)	-7.1 (23.1-14.5)
IFN-γ	-9.5 (-22.8-14.9)	-6.0 (-19.1-17.0)	-9.5 (-22.8-14.9)
TNF-α	-1.3 (-12.9-8.8)	1.3 (-10.7-14.4)	-1.3 (-12.9-8.8)
Anti-inflammatory cytokines			
IL-1Ra	-3.7 (-21.4-22.3)	2.4 (-27.6-33.6)	-3.7 (-21.4-22.3)
IL-4	-5.8 (-15.6-6.9)	-3.1 (-15.0-11.7)	-5.8 (-15.6-6.9)
IL-5	0 (-29.3-54.6)	0 (-25.6-97.6)	0 (-29.5-64.64)
IL-9	-7.8 (-26.0-10.1)	-7.6 (21.2-19.5)	-7.8 (-26.0-10.1)
IL-10	-12.3 (-49.2-37.1)	-15.7 (-49.9-19.5)	-12.3 (-49.2-37.1)
IL-13	6.5 (-41.0-134.5)	0 (-45.4-67.5)	6.5 (-41.9-134.5)
Growth and colony-stimulating factors			
VEGF	8.6 (-21.3-66.2)	8.8 (-24.2-59.9)	8.6 (-21.3-66.2)
FGF basic	-2.4 (-14.4-7.9)	1.2 (-12.1-17.7)	-2.4 (-14.4-7.9)
PDGF-BB	-24.5 (-43.2-10.7)	-19.2 (36.1-23.5)	-24.5 (-43.2-10.7)
G-CSF	5.4 (-16.4-39.3)	15.6 (-14.6-44.6)	5.4 (-16.4-39.3)
GM-CSF	-7.8 (-20.7-14.4	0.7 (-23.5-33.0)	-7.8 (-20.7-15.4)
IL-7	0 (-27.4-113.5)	4.5 (-36.2-111.6)	0 (-27.4-113.5)
Chemokines			
MCP-1 (CCL2)	-15.1 (34.1-4.4)	-7.1 (-26.1-14.0)	-15.1 (-34.13-4.4)
MIP-1α (CCL3)	-4.0 (-18.1-18.8)	2.2 (-15.6-23.0)	-4.0 (-18.1-18.8)
MIP-1β (CCL4)	-11.2 (-30.5-7.2)	-3.1 (-23.7-11.5)	-24.5 (-43.2-10.7)
Eotaxin (CCL11)	-33.0 (-46.2- (-13.7))	-23.7 (-42.0 - (-7.9))	-33.0 (-46.23-(-13.7))
IP-10 (CXCL10)	-11.3 (-36.5-18.8)	-16.1 (-37.0-14.9)	-11.3 (-36.5-18.8)

Percent cytokine and CRP change are reported as median (25^{th}-75^{th} percentile). N = 110.

CCL, CC chemokine ligand; CRP, C-reactive protein; CXCL, CXC chemokine ligand; FGF, fibroblastic growth factor; GA, gestational age; G-CSF, granulocyte colony-stimulating factor; GM, granulocyte macrophage; IFN, interferon; IL, interleukin; IP, IFN-γ-induced protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; Ra, receptor antagonist; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

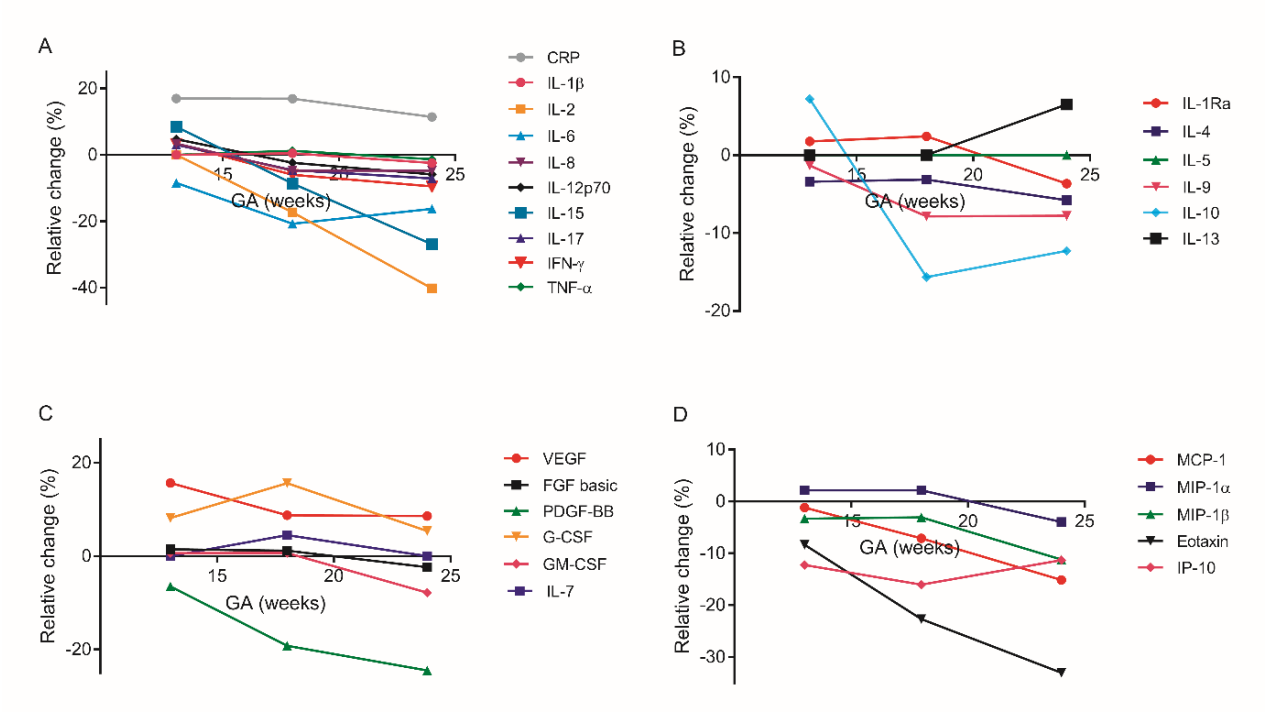


Fig. 2. Expression of maternal serum cytokines and CRP at GA 13, 18 and 24 weeks shown as relative change (%) normalized to GA 10 weeks (n = 104). A) Inflammatory cytokines and CRP, B) Anti-inflammatory cytokines, C) Growth and colony-stimulating factors, D) Chemokines. Data are reported as median.

CRP, C-reactive protein; FGF, fibroblastic growth factor; GA, gestational age; G-CSF, granulocyte colony-stimulating factor; GM, granulocyte macrophage; IFN, interferon; IL, interleukin; IP, IFN-γ-induced protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; Ra, receptor antagonist; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

LMM analysis confirmed a significant decrease with gestational age for many of the cytokines (Table 2). Three out of the five chemokines decreased significantly: monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1β and eotaxin (Table 2 and Fig. 2). More than half of the inflammatory cytokines decreased significantly from first to second trimester: IL-2, IL-6, IL-8, IL-15 and IL-17. However, the timing of the decrease in the inflammatory group varied; IL-2 showed stable levels from GA 10 to 13 weeks, followed by a total median decrease of 40% from GA 13 to 24 weeks, while IL-17 showed a more subtle median decrease of 7% from GA 10 to 24 weeks. In the anti-inflammatory cytokine group, only IL-9 and IL-10 decreased significantly with gestation, and the same was confirmed for PDGF-BB and granulocyte macrophage colony-stimulating

factor (GM-CSF) in the growth and colony-stimulating factors group. No cytokine showed significant increase with gestational age during the first half of pregnancy. The changes in cytokine pattern was independent of maternal age, parity, smoking status, BMI and systolic blood pressure measured at GA 10 weeks.

3.4 Multivariate paired data analysis and gestational variation

Multilevel orthogonalized PLS-DA explored the overall cytokine pattern and interactions between cytokines and revealed significant gestational variations in the overall serum cytokine pattern (Fig. 3). The cytokine patterns at GA 10 and 24 weeks were separated with 90% accuracy ($P < 0.001$), showing clear differences in cytokine profiles between the two different time points in pregnancy (Fig. 3A). The loading plot (Fig. 3B) showed which cytokines contributed most to the difference between time points and displayed decreased concentrations of eotaxin, MCP-1, MIP-1β and PDGF-BB between GA 10 and 24 weeks. When comparing the overall cytokine pattern of samples from GA 10 and 13 weeks, GA 13 and 18 weeks and GA 18 and 24 weeks, all time intervals could be significantly differentiated with a classification accuracy of 66.7%, 66.7% and 66.7%, respectively (all $P < 0.001$) (Supplementary Fig. S1). The cytokines most responsible for the separation between GA 10 and 13 weeks were eotaxin, interferon gamma-induced protein (IP)-10, MIP-1β and PDGF-BB (which all decreased) and vascular endothelial growth factor (VEGF) (which increased) (Supplementary Fig. S1B). The separation between GA 13 and 18 weeks was characterized by decreasing concentrations of eotaxin, IL-6 and IL-15 (Supplementary Fig. S1D). Lastly, decreased levels of IL-4, eotaxin, MIP-1β and PDGF-BB constituted the greatest variation between GA week 18 and 24 (Supplementary Fig. S1F). From these analyses of cytokine patterns at different gestations, the chemokines seemed to hold the most sensitive assessment of gestational variation (Fig. 3 and Supplementary Fig. S1).

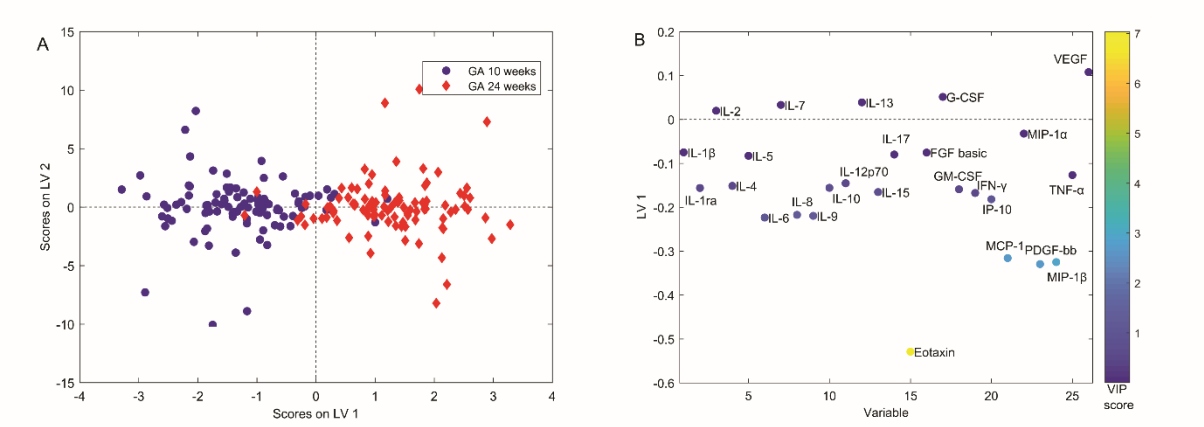
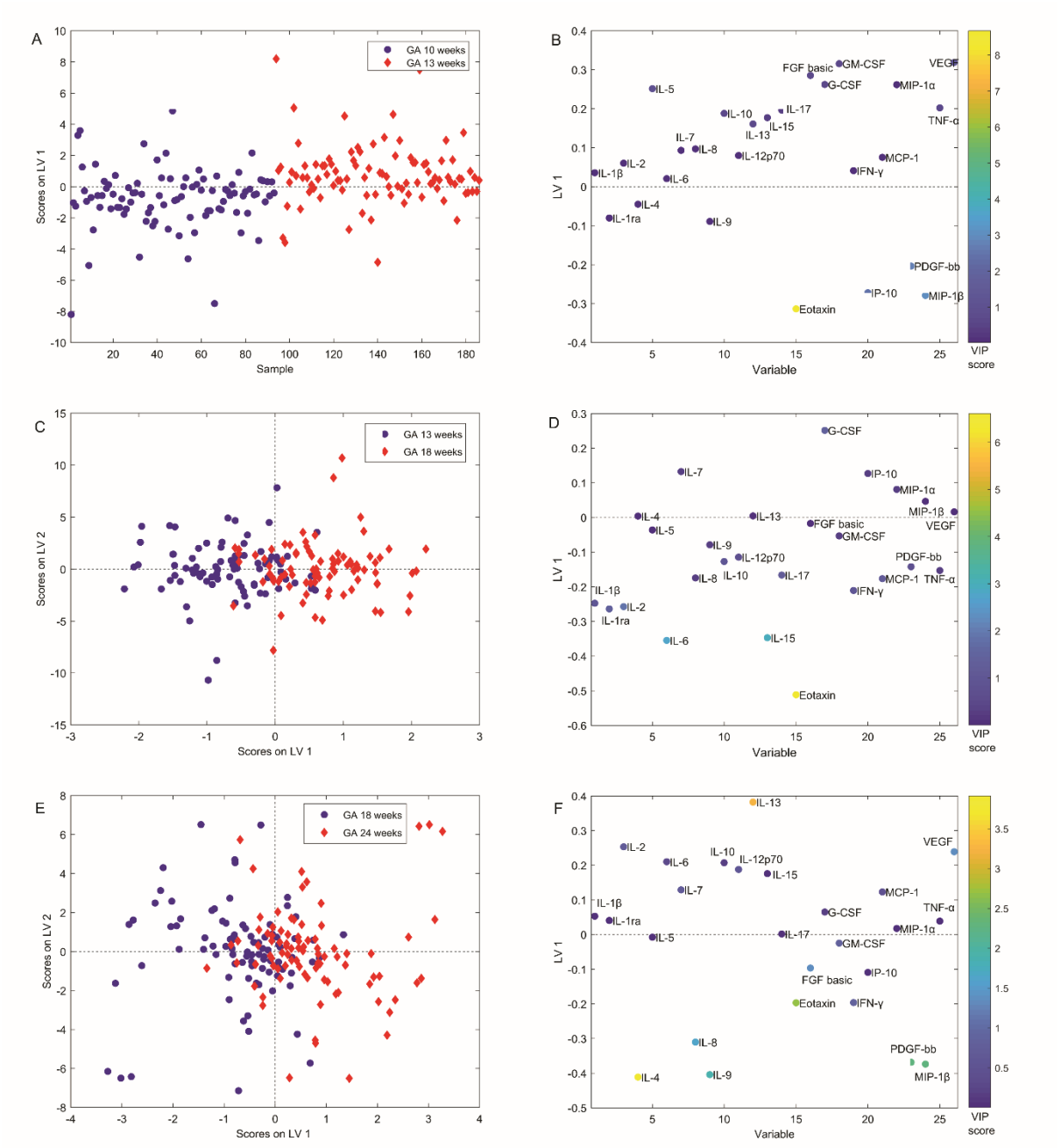


Fig. 3. Multilevel PLS-DA of serum samples from GA 10 and 24 weeks visualized as variation with gestational age in cytokine patterns in first half of normal pregnancy. The orthogonalized score plot of latent variable (LV) 1 and 2 (A) shows the clear distinction between GA 10 weeks and GA 24 weeks. The cytokines most important for the separation between the two gestational ages are shown in the corresponding loadings plot (B) of LV1, colored by variable of importance for projection (VIP) score. The higher VIP score, the more important for separation. Samples from GA 24, with high LV1 scores, have higher levels of cytokines with high LV1 loading values and lower levels of cytokines with low, negative LV1 loading values.

FGF; fibroblastic growth factor; GA, gestational age; G-CSF, granulocyte colony-stimulating factor; GM, granulocyte macrophage; IFN, interferon; IL, interleukin; IP, IFN-γ-induced protein; LV, latent variable, MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; Ra, receptor antagonist; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.



353

354 **Supplementary fig. S1:** Multilevel PLS-DA of serum samples from different time points visualize
355 gestational variation in cytokine patterns in first half of normal pregnancy. Orthogonalized score plots
356 show clear separation between (A) GA 10 and 13 (n = 93), (C) GA 13 and 18 (n = 85) and (E) GA 18
357 and 24 (n = 88). The classification accuracy in all time intervals was 67%. The cytokines most
358 important for the separation are shown in the corresponding loadings plot colored by variable of
359 importance for projection (VIP) score for (B) GA 10 and 13, (D) GA 13 and 18 and (F) GA 18 and 24.
360 Samples with high LV1 scores have higher levels of cytokines with high LV1 loading values and lower
361 levels of cytokines with low, negative LV1 loading values.
362 FGF; growth factor; GA, gestational age; G-CSF, granulocyte colony-stimulating factor; GM,
363 granulocyte macrophage; IFN, interferon; IL, interleukin; IP, IFN-γ-induced protein; LV, latent variable,
364 MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived

growth factor; Ra, receptor antagonist; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

3.5 Cytokine correlation analyses at all gestational ages

Assessment of the relationship between individual cytokines at all time points revealed a particularly high correlation in the inflammatory cytokine group (Fig. 4). When calculating mean correlation within cytokine groups combining all gestational ages, the inflammatory cytokines showed a mean correlation (ρ) of 0.46, indicating common development with gestational age. The mean correlation within the other cytokine groups was considerably lower, with ρ = 0.22 for the anti-inflammatory cytokine group, ρ = 0.35 for the growth and colony-stimulating factor group, and ρ = 0.24 for the chemokine group. Of a total of 325 possible cytokine pairs at each of the four time points, a mean of 237 pairs showed significant correlations. The cytokine pairs with the highest mean correlation were IL-2 and IL-15 (ρ = 0.76), IL-17 and fibroblastic growth factor (FGF) basic (ρ = 0.71), VEGF and FGF basic (ρ = 0.71) and IL-17 and MIP-1α (ρ = 0.71) (Fig. 4).

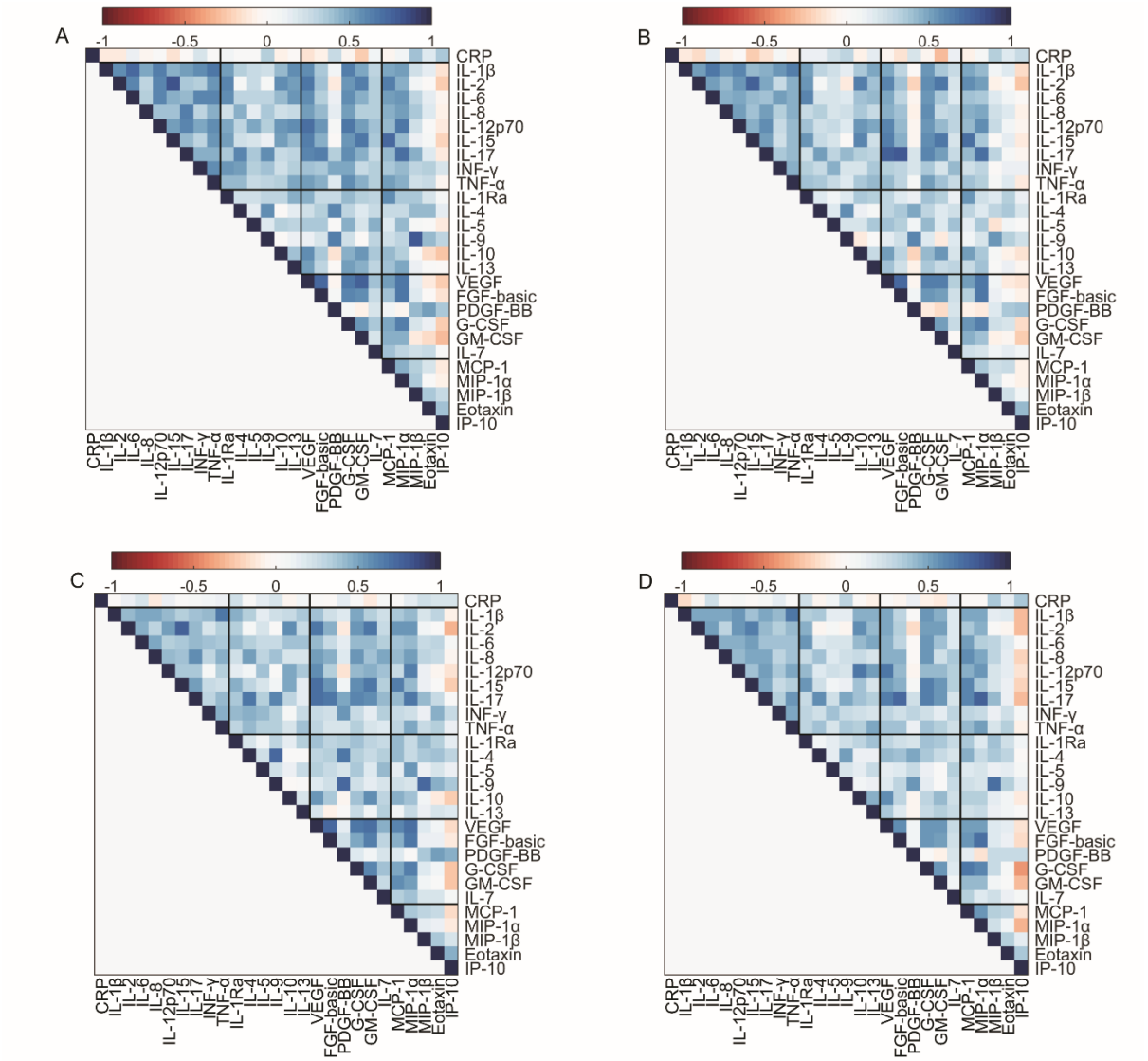


Fig. 4. Spearman rank correlation heatmaps of maternal serum cytokines and CRP (n = 110). The 26 cytokines are grouped from the top as inflammatory cytokines; anti-inflammatory cytokines; growth factors and colony-stimulating factors; and chemokines, divided by thicker black lines. GA A) 10 weeks, B) 13 weeks, C) 18 weeks and D) 24 weeks. The correlation (rho - ρ) is visualized with color intensity grade (top).

CRP; C-reactive protein, FGF, fibroblastic growth factor; GA, gestational age; G-CSF, granulocyte colony-stimulating factor; GM, granulocyte macrophage; IFN, interferon; IL, interleukin; IP, IFN-γ-induced protein; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; Ra, receptor antagonist; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

3.6 Gestational development of CRP

393 Contrary to most of the cytokines, CRP increased with pregnancy length (Table 2 and Fig. 1
394 and 2). This was confirmed with LMM (Table 2). Surprisingly, CRP did not correlate
395 significantly with any of the inflammatory cytokines at any measured gestational age (Fig. 4).
396 CRP only correlated significantly with the chemokine MIP-1β out of the 26 cytokines, with a
397 mean positive correlation of 0.33.
398

4. Discussion

The present study provides extensive serum cytokine reference values and a broad serum cytokine pattern analysis at four time points during the first half of normal pregnancy. Overall, maternal cytokine levels decreased throughout the first half of pregnancy. The chemokines provided the most sensitive measurement of variation with gestational age. The inflammatory cytokines showed the highest intra-group correlation at all time points, while CRP levels did not correlate with changes in the inflammatory cytokines.

To our knowledge, this is the first large-scale longitudinal measurement of more than 20 cytokines in normal pregnancy. Simultaneous measurements of several cytokines with multiplex magnetic bead-based immunoassays are becoming increasingly common in scientific research. Serum cytokine reference values for normal pregnancy as presented here are required to explore the clinical potential of cytokine profiling in pregnancy. Without standardized methods, a reference table must ideally be presented for each commercially available type of multiplex analysis. The absolute cytokine values in this study are valid for a Bio-Plex Assay measured on the Luminex 200 system which is commonly used for broad cytokine profiling (Bozza et al., 2007; Ormstad, Aass, Lund-Sørensen, Amthor, & Sandvik, 2011). As individual basal cytokine levels vary and must be taken into account (Biancotto et al., 2013; Kraus et al., 2010), our reference table of relative individual changes in cytokine levels provides information that has a wider application. With caution, these normalized reference values can be used for comparison of cytokine development in samples analysed with different cytokine profiling assays.

The gestational variation of chemokines reported here has not been revealed previously, but a significant decrease of MCP-1 from first to third trimester is consistent with our results (Polari, Kumar, Rautava, Salminen, & Isolauri, 2018). The common function of chemokines is directed movement of leukocytes. The first trimester is characterized by increased immune activity and leukocyte recruitment, as part of implantation and placentation (Mor & Cardenas,

2010). The decreasing chemokine levels in second trimester may accompany a physiological reduction in immune activity and leukocyte migration. Early pregnancy disease-specific differences in chemokine levels has been reported. Increased serum MIP-1α and decreased serum MCP-1 in first trimester has been associated with later development of preeclampsia (Salazar Garcia et al., 2017) or giving birth to small for gestational age babies (Grigorescu et al., 2014). In second trimester, elevated levels of MCP-1 in women experiencing preeclampsia have been detected (Cui et al., 2017). These seemingly contradictory MCP-1 observations related to preeclampsia and our findings of substantial gestational variation of chemokines in first half of normal pregnancies, points to caution for measuring a single chemokine when investigating disease-specific patterns.

This study of gestational changes in cytokine patterns in normal pregnancies with multivariate methods is novel. In studies of single immune biomarkers, inflammatory cytokines and CRP appear most frequent but with contradictory results. The gestational IL-2 and IL-8 development observed here is supported by others (Christian & Porter, 2014; Curry et al., 2008). We showed increasing levels of CRP from first to second trimester, earlier this has been shown from early to late pregnancy (Larsson, Palm, Hansson, Basu, & Axelsson, 2008), while others have reported stable CRP levels (Belo et al., 2005; Skarzynska et al., 2018). Our results reported decreasing levels of IL-6 throughout the first half of pregnancy, while others have shown either increasing (Holtan et al., 2015; Vassiliadis et al., 1998) or stable levels of IL-6 with increasing gestational age in this period (Austgulen, Lien, Liabakk, Jacobsen, & Arntzen, 1994; Christian & Porter, 2014; Curry et al., 2008). Likewise, we found decreasing levels of IL-12p70, while an increase between the first and second trimester has been reported (Curry et al., 2008). The exclusion of women with previous or current disease or pregnancy complication may differentiate this from some other studies where such women were included or the medical history was unknown (Austgulen et al., 1994; Belo et al., 2005; Curry et al., 2008; Larsson et al., 2008; Skarzynska et al., 2018; Vassiliadis et al., 1998). Diverging results may also be expected from small study populations (Christian &

Porter, 2014; Holtan et al., 2015). Serum inflammatory cytokines may reflect a wide number of infectious and non-infectious diseases, highlighting the importance of including well characterized study groups.

The nine inflammatory cytokines were strongly correlated with each other at all gestational ages, indicating a common regulation of this cytokine group during pregnancy. IL-1β, IL-6 and TNF-α has been shown to correlate positively at all trimesters (Christian & Porter, 2014). In our study, the two cytokines with the highest mean correlation were the inflammatory cytokines IL-2 and IL-15. They are both members of the 4α-helix bundle family and have similar functions (Ikemizu, Chirifu, & Davis, 2012). To our knowledge, their specific role in human pregnancy has not yet been explored. The high intra-group correlation may reflect that different inflammatory cytokines have a powerful potentiating effect on each other in normal pregnancy, and highlight the detrimental role of inflammatory cytokines when dysregulated. Surprisingly, CRP did not correlate significantly with the inflammatory cytokines during first half of pregnancy, even though the synthesis of CRP is regulated primarily by inflammatory cytokines (Pepys & Hirschfield, 2003). A positive correlation between CRP and inflammatory cytokines in pregnancy has previously been indicated in a study group with higher mean BMI (Christian & Porter, 2014), possibly affecting the results (Pendeloski, Ono, Torloni, Mattar, & Daher, 2017). We find that the inflammatory cytokine patterns in pregnancy may be more sensitive and informative than CRP in characterising the early maternal systemic immune status. Inflammatory cytokines has been connected to development of pregnancy complications. Preeclampsia is associated with increased levels of IL-6, IL-8, IL-12p70, IFN-γ and TNF-α compared to healthy pregnant controls (Kronborg et al., 2011; Molvarec, Czegle, Szijarto, & Rigo, 2015; Molvarec et al., 2011; Szarka et al., 2010). Still, the conflicting reports on predictive value of single cytokine measurements from early pregnancy clearly underlines the need for more sensitive methods (Cui et al., 2017; Mosimann, Wagner, Poon, Bansal, & Nicolaides, 2013; Salazar Garcia et al., 2017; Townsend et al., 2018). The first half of pregnancy forms the basis of many common

483 complications later in pregnancy such as intrauterine fetal growth restriction and
484 preeclampsia (Redman & Sargent, 2005; Yockey & Iwasaki, 2018), and maternal
485 inflammation during pregnancy may further have impact on cognitive development in infants
486 (Rudolph et al., 2018). This underlines the relevance of cytokine analyses in pregnancy, not
487 only for a successful pregnancy outcome, but also for future development of the child. The
488 stable intra-group correlation of inflammatory cytokines in normal pregnancies shown here,
489 combined with the inflammatory dysregulation in many diseases, provides further evidence
490 for the clinical usefulness of combined profiling of inflammatory cytokines in maternal serum.
491 This must be explored in disease-specific populations.
492
493 Multivariate models take interactions between cytokines into account and were used to
494 accurately distinguish samples from different time points in pregnancy. The distinction
495 accuracy did not increase with increasing time between the gestational time points,
496 indicating that the cytokine development does not depend on time only, but rather
497 physiological milestones during pregnancy. Despite the short time interval between GA 10
498 and 13 weeks, the model could with almost 70% accuracy distinguish the two time points,
499 indicating a substantial cytokine pattern variation. This interval represents the ending of the
500 vitelline circulation and the transition to full circulation via the placental arteries (Burton &
501 Jauniaux, 2018). VEGF is important for vascular remodelling in early pregnancy, and
502 showed the greatest increase in this time interval. So far, studies on pregnancy
503 complications using cytokine pattern analyses provides promising results (Azizieh et al.,
504 2018; Tangeras et al., 2015). The methodological simplicity and biological strength of
505 assessing combined cytokine patterns underlie our strong advice of expanded use of such
506 methods to further identify cytokine biomarkers of pregnancy complications.
507
508 This study provides evidence for the clinical usefulness of broad cytokine profiling as a
509 sensitive measurement of the gestational influence on maternal immune status in
510 pregnancy. Chemokines were shown important for revealing gestational variation in first half

of pregnancy, while the inflammatory cytokines showed high intra-group correlation in normal pregnancy. Our study shows that broad cytokine profiling and modelling of interactions between cytokines successfully reveals normal pregnancy cytokine patterns. Our results provide a fundament for normal pregnancy cytokine patterns, and cytokine profiling may be a powerful tool for future studies on pregnancy complications.

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