DISTINCT FIRST TRIMESTER CYTOKINE PROFILES FOR GESTATIONAL HYPERTENSION AND PREECLAMPSIA

Running title: Cytokines in hypertensive pregnancy disorders

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Abstract

Objective: Gestational hypertension and preeclampsia involve dysregulated maternal inflammatory responses to pregnancy, but whether such responses differ between the disorders has not been determined. We aimed to investigate disease-specific early pregnancy serum cytokine profiles of women subsequently developing gestational hypertension or preeclampsia, for new insight into the underlying pathogeneses and differences between the disorders. **Approach and Results:** The study cohort consisted of 548 pregnant Norwegian women that were either multiparous with previous gestational hypertension or preeclampsia or were nulliparous. Maternal sera at gestational weeks 11^{0}-13^{6} were assayed for 27 cytokines, C-reactive protein, total cholesterol, high-density lipoprotein, triglyceride, creatinine, calcium, uric acid and placental growth factor. Compared to normotensive women, both hypertensive conditions presented an atherogenic lipid profile at early gestation but only women later developing gestational hypertension had significantly higher serum levels of interleukin (IL)-5 and IL-12. Comparing the two hypertensive pregnancy disorders; women subsequently developing gestational hypertension had higher serum levels of IL-1β, IL-5, IL-7, IL-8, IL-13, basic fibroblast growth factor and vascular endothelial growth factor than the women subsequently developing preeclampsia. **Conclusion:** This study identifies early pregnancy differences in serum cytokine profiles for gestational hypertension and preeclampsia.

Abbreviations

BMI; body mass index; CRP, C-reactive protein; IL, interleukin; IFN, interferon; MAP, mean arterial pressure; PlGF, placental growth factor; PLS-DA, partial least squares discriminant analysis; TNF; tumor necrosis factor, UtAPI, uterine artery pulsatile index.

Introduction

Hypertensive disorders of pregnancy are major causes of maternal and fetal morbidity and mortality.[1-3] Most common is gestational hypertension, defined as new-onset hypertension after 20 weeks gestation, affecting 2-17% of pregnant women.[1, 2] Preeclampsia is characterized by de novo hypertension with proteinuria after gestational week 20 and complicates 2-7% of pregnancies.[1] The distinction between the two diagnoses is not clear-cut, with several recent international clinical guidelines defining preeclampsia as hypertension combined with any from a list of signs and symptoms of end-organ damage, including proteinuria.[4, 5] Whether gestational hypertension and preeclampsia are separate disorders with the common clinical sign of hypertension, or part of a spectrum, is an ongoing debate.[6, 7] Several risk factors, including obesity and diabetes, are shared between the two conditions.[8] Primiparity is strongly associated with risk of developing preeclampsia only and the risk of recurrence is higher for gestational hypertension, while both disorders are considered warning signs for future cardiovascular disease.[4, 8-11] Most studies group these two conditions together or focus solely on preeclampsia.[6] A recent systematic review of the global incidence of hypertensive pregnancy disorders excluded gestational hypertension from the analysis due to the lack of specific studies.[12] Further research is warranted to specifically address the underlying pathogenesis of gestational hypertension and how it interrelates with preeclampsia.

Serum biomarkers reflecting underlying disease processes of hypertensive disorders of pregnancy include heightened C-reactive protein (CRP) implicating inflammation, a dyslipidemic lipid profile suggesting ongoing atherosclerotic processes, altered creatinine and uric acid levels indicating renal dysfunction, decreased calcium levels reflecting metabolic changes, and reduced placental growth factor (PlGF) pointing to placental dysfunction.[13-18] A shared pathophysiology is indicated by dyslipidemia in early gestation and elevated CRP at clinical manifestation in both gestational hypertension and preeclampsia, while reduced PlGF levels are predominantly associated with preeclampsia.[15, 19-22] More refined biomarkers identifying distinct components and differences between gestational hypertension and preeclampsia in early pregnancy are missing.

Gestational hypertension and preeclampsia are characterized as excessive maternal inflammatory responses to pregnancy.[23] Inflammation involves a complex network of cytokines released from stressed cells and damaged tissue.[23] Essential hypertension has been associated with increased serum levels of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, and an inflammatory cytokine profile is detected prior to disease manifestation.[24-26] Regarding hypertensive disorders of pregnancy, preeclampsia has been linked to increased serum levels of pro-inflammatory cytokines, such as IL-6, IL-8 and TNF-α, accompanied by an angiogenic factor imbalance, and for gestational hypertension increased serum IL-1β, IL-10 and TNF-α has been reported.[27-32] Information is limited for early pregnancy; serum IL-1β and TNF-α has been shown elevated prior to onset of disease in women developing preeclampsia, while gestational hypertension has not been separately investigated at early gestation.[33, 34] This study aimed to compare disease-specific early gestation serum cytokine profiles of women subsequently developing gestational hypertension or preeclampsia.

Material and methods

Materials and Methods are available in the online-only Data Supplement.

Results

Characteristics of the Study Population

A flow chart describing the 640 pregnant women invited to the study and the final study population is shown in Figure 1. Of the 548 pregnant women included in the final analyses, 504 (92%) remained normotensive throughout pregnancy, 19 (3.5%) developed gestational hypertension and 25 (4.6%) developed preeclampsia. Of the 513 nulliparous women, 3.1% developed gestational hypertension and 3.9% preeclampsia, whereas of the 35 multiparous women, 8.6% developed gestational hypertension and 14.3% preeclampsia. All preeclamptic cases and 18 (95%) of the women developing gestational hypertension were classified as late onset (delivery after gestational week 34). Characteristics of the final study population are shown in Table 1 and are further described in Skråstad *et al*.[35] In the study population, 98.5% of the women were classified as white. Compared to normotensive women at gestational weeks 11^0-13^6, body mass index (BMI) was significantly higher for women who later developed gestational hypertension, while uterine artery pulsatile index (UtAPI) was higher for women who later developed preeclampsia (Table 1). Systolic and diastolic blood pressure was significantly higher in both case groups, but below the threshold for hypertension (Table 1). Only women who subsequently developed preeclampsia delivered significantly smaller babies and at an earlier gestation compared to normotensive women (Table 1).

Early Pregnancy Serum Profiles of Hypertensive Pregnancy Disorders Compared to Normotensive Pregnancies

Serum levels of cytokines and serum markers are shown in Table 2A and 2B, respectively. Women who later developed gestational hypertension showed significantly higher serum levels of IL-5 and IL-12 compared to normotensive women at gestational weeks 11^0-13^6 (Table 2A). In contrast, the serum cytokine profile of women who subsequently developed preeclampsia did not differ from normotensive women at this gestational age (Table 2A). Women who later developed either of the hypertensive pregnancy disorders showed an atherogenic serum lipid profile compared to normotensive pregnancies; significantly higher levels of serum triglyceride was associated with gestational hypertension, while lower levels of high-density lipoprotein and higher levels of low-density lipoprotein was associated with preeclampsia (Table 2B). As expected, women who later developed preeclampsia had significantly lower serum PlGF than normotensive women at gestational weeks 11^0-13^6 (Table 2B). Adjusting for BMI and mean arterial pressure (MAP) did not eliminate cytokine or serum marker differences between the three outcome groups, except for the significant difference in triglyceride levels between later gestational hypertension and normotensive pregnancies (data not shown). The significant cytokine or serum marker differences between the three outcome groups were not affected by including outlier values (data not shown). Comparisons of the two case groups with the rest of the cohort (including women with other pregnancy complications) yielded the same significant differences as when comparing to the normotensive control group (data not shown).

Early Pregnancy Serum Profiles Comparing Gestational Hypertension and Preeclampsia

The early pregnancy serum cytokine profile clearly differed between women who later developed gestational hypertension and those who developed preeclampsia, while the serum markers did not distinguish between the two disorders at 11^0-13^6 weeks

gestational age (Table 2). Women later developing gestational hypertension showed higher serum levels of IL-1β, IL-5, IL-7, IL-8, IL-13 and basic fibroblast growth factor compared to women later developing preeclampsia (Table 2A and Supplementary Figure I). When only nulliparous women were included in the analyses, IL-1β, IL-5, IL-7 and IL-8 remained significantly different and, additionally, serum levels of granulocyte colony-stimulating factor were significantly higher in women who later developed gestational hypertension compared to women who later developed preeclampsia. These cytokine values are presented in scatterplots labeled for parity for women who subsequently developed gestational hypertension or preeclampsia (Supplementary Figure II).

Multivariate analysis combines variables that are not necessarily significant on their own into combinations of variables that may be characteristic for a specific class. Partial least squares discriminant analysis (PLS-DA) compresses multiple variables into simpler and more information-rich latent variables, and the resulting PLS-DA model can be visualized with score and loading plots.[36] Variable importance in projection is a method of assessing which variables are most important to the model; a score ≥1 indicates that the variable is important in discriminating between groups.[37] PLS-DA is prone to overfitting, so to ensure that an equally good model cannot be built from randomly assigned classes, classification results are validated by permutation testing.[38] In permutation testing, classes are shuffled and models built using the same parameters. The classification error is recorded and the process repeated 1000 times, giving a distribution of errors for the null hypothesis that no difference exists between classes. The results obtained from the true classes should be outside the 95% confidence interval of the permutated result for the model to be considered valid.[38] Maternal serum cytokines, serum markers and selected population characteristics (maternal age, BMI, MAP, UtAPI and gestational age at enrolment) were further explored by multivariate analysis to uncover group differences and covariance (valid models; $P \leq 0.05$ by permutation testing). PLS-DA classified serum cytokine profiles as gestational hypertension or preeclampsia with 74% accuracy (sensitivity = 0.68 and specificity = 0.80, eight latent variables) (Table 3 and Figure 2). This confirmed that serum cytokine profiles clearly separated between the hypertensive pregnancy disorders. The score plot (Figure 2A) displays the separation between the hypertensive disorders, with pregnant women in each group represented by symbols, and the loading plot (Figure 2B) shows the magnitude of contribution for each of the 25 cytokines as indicated on the variable importance in projection color scale. Women who later developed gestational hypertension could by multivariate analysis be distinguished from later preeclamptic women by their higher serum levels of, in order of importance; vascular endothelial growth factor A, IL-5, IL-4, IL-8, interferon (IFN)-γ-induced protein 10, IL-12 and IFN-γ at 11^0-13^6 weeks gestational age. The PLS-DA models using serum markers or clinical characteristics as input were not significant, underlining that only serum cytokines contributed useful information to separate between pregnant women who later developed gestational hypertension or preeclampsia (Table 3 and Figure 2).

Discussion

This study revealed that the serum cytokine profile of women who subsequently developed gestational hypertension differed considerably from that of women who subsequently developed preeclampsia, revealing novel distinctive features of these hypertensive pregnancy disorders at early gestation. Subsequent development of gestational hypertension, but not preeclampsia, could at gestational age 11^0-13^6 weeks be distinguished from normotensive pregnancies by serum cytokines. Maternal BMI and MAP at study visit did not influence the differences in serum cytokine levels, confirming that the cytokine profiles reflected underlying early disease development. These findings clearly indicate differences in the underlying pathogeneses of gestational hypertension and preeclampsia, highlighting the importance of separately addressing the two conditions.

Serum cytokine levels were increased in women later developing gestational hypertension compared to normotensive pregnancies, with concomitant rise of pro-inflammatory IL-12 and anti-inflammatory IL-5 at early gestation. While pro-inflammatory cytokines promote inflammation, endothelial activation and elevated blood pressure,[39, 40] the simultaneous increase of their anti-inflammatory counterparts might represent an early compensatory mechanisms, possibly counteracting endothelial dysfunction.[41] Similar serum cytokine profiles of both pro- and anti-inflammatory nature have been reported at term for gestational hypertension.[28, 29] A first trimester study by Wolf *et al.* concluded that inflammatory activation, assessed by hsCRP measurements, was not associated with gestational hypertension,[42] but our somewhat contradictory findings result from a more comprehensive cytokine profiling approach. For preeclampsia, serum cytokine profiling has previously shown results ranging from elevated first trimester IL-1β levels predicting early onset disease,[34] to no cytokine differences prior to clinical manifestation of early or late onset preeclampsia.[43, 44] We did not find any such differences in our cohort of late onset preeclampsia compared to normotensive pregnancies.

The increased serum levels of cytokines IL-1β and IL-8 identified in early detection of late-stage gestational hypertension in comparison to preeclampsia point to systemic stress and inflammation.[45, 46] Higher levels of IL-1β have been detected at term for gestational hypertension compared to preeclampsia.[28] The anti-inflammatory cytokines IL-5 and IL-13 and pro-angiogenic basic fibroblast growth factor and vascular endothelial growth factor A shown elevated in this early detection of late-stage gestational hypertension in comparison to preeclampsia, could reflect a vascular rescue mechanism.[41, 47] The first stage of the preeclampsia pathogenesis is characterized by placental dysfunction, which in this study was evident at gestational weeks 11^0-13^6 by reduced PlGF levels and higher UtAPI compared to normotensive women.[21, 22] The initial placental insufficiency eventually manifests systemically closer to term,[48] but based on our data the maternal serum cytokine response is not initiated at this early gestation in late onset preeclamptic pregnancies. For late onset gestational hypertension our data indicate that systemic inflammatory activation is initiated in early pregnancy, but is subdued by compensatory mechanisms. This implies that cytokine profiling at gestational age 11^0-13^6 weeks represents a novel tool to characterize early maternal responses in gestational hypertension, while preeclampsia is better identified by markers like PlGF, reflecting the local placental disease at this early stage. For both hypertensive pregnancy conditions dyslipidemia manifested in early gestation, reflecting their previously reported atherogenic

nature,[19, 20] but unlike serum cytokines, lipid profiling could not distinguish between the hypertensive pregnancy conditions. Interestingly, the distinct cytokine profile for gestational hypertension at gestational age 11^{0}-13^{6} weeks resembles allergic responses characterized by elevated levels of IL-4, IL-5, and IL-13,[49] but the incidence of self-reported allergic or asthmatic disease or medication did not differ between the groups in this cohort, and such a link could therefore not be substantiated.

The serum cytokine profiles reported here are based on a cross-sectional study design, which prevents comparison of cytokine kinetics throughout pregnancy for the two disorders, but the importance of revealing the distinct characteristics of gestational hypertension at this early gestation remains. A limitation of this study is the relatively small number of cases, but we find these numbers to be comparable to previous case-control studies investigating serum cytokines in hypertensive pregnancy disorders,[33, 34, 50] and our study includes a larger control group. Since our medium- to high-risk cohort predominantly included women developing late onset gestational hypertension or preeclampsia, the findings presented here should be followed-up in a general pregnant population with larger groups including early and late onset disease. Since maternal ethnicity is known to affect the levels of first trimester serum biomarkers such as PlGF and cytokines,[51, 52] these findings should be investigated in study populations of other ethnical origins. The issue of whether gestational hypertension and preeclampsia are separate disorders with the common clinical sign of hypertension, or part of a spectrum, has not been settled.[6] The findings of this study support the former, with marked differences in early pregnancy cytokine profiles for gestational hypertension and preeclampsia reflecting different underlying pathogeneses. Our findings strongly suggest that these hypertensive pregnancy disorders should be addressed separately in future studies.

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Disclosures
None.

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Significance

This study identified early pregnancy differences in serum cytokine profiles for gestational hypertension and preeclampsia, and revealed a state of inflammatory activation for gestational hypertension at gestational age 11^{0}-13^{6} weeks. Broad serum cytokine profiling provides a novel tool for early detection of late-stage gestational hypertension and for distinguishing the two hypertensive pregnancy disorders. The distinct serum cytokine profiles identified in this study reflect differences in mechanisms underlying development of gestational hypertension and preeclampsia and warrants separating between these two pregnancy disorders.

Table 1. Characteristics of Study Population.

Variables	Gestational hypertension (n = 19)	Preeclampsia (n = 25)	Normotensive (n = 504)
Characteristics at study visit			
Age, years	25.9 (±3.9)	26.4 (±5.2)	27.6 (±3.9)
BMI, kg/m^2	27 (23-31)*	25 (21-27)	24 (22-26)
Smoking, n (%)	2 (11)	3 (12)	61 (12)
Nullipara, n (%)	16 (84)	20 (80)†	477 (95)
Multipara, n (%)	3 (16)	5 (20) †	27 (5)
SBP, mm Hg	118 (115-128)‡	113 (109-123)†	109 (104-114)
DBP, mm Hg	80 (74-84)‡	76 (69-80)*	70 (66-74)
UtAPI	1.49 (1.26-1.66)	1.75 (1.41-2.11)†	1.44 (1.22-1.75)
GA, weeks	13 (13-13)	13 (13-13)	13 (12-13)
Characteristics at birth			
GA, weeks	40 (39-40)	38 (37-40)‡	40 (39-41)
Birth weight, g	3408 (±654)	3139 (±619)‡	3543 (±424)
Fetal sex, n (%) male	13 (68)	11 (44)	246 (49)

Continuous variables are reported as mean (±standard deviation) or median (25th-75th percentile). BMI indicates body mass index; DBP, diastolic blood pressure; GA, gestational age, SBP, systolic blood pressure; UtAPI, uterine artery pulsatile index. Comparisons between outcome groups (ANOVA and Tukey's test or Kruskal-Wallis and Dunn's test for continuous variables, Chi-square test for categorical variables). *P<0.05, †P<0.01, ‡P<0.001 vs normotensive controls.

Table 2A. Maternal Serum Cytokine Levels (pg/mL) at Gestational Weeks 11^0-13^6.

Cytokines	Gestational hypertension (n = 19)	Preeclampsia (n = 25)	Normotensive (n = 504)
IL-1β	2.81 (2.66-3.27)†	2.41 (2.08-2.89)	2.59 (2.24-3.04)
IL-1Ra	210 (189-264)	190 (169-237)	198 (166-233)
IL-2	18.6 (15.9-24.3)	18.7 (15.6-22.9)	18.3 (16.1-21.2)
IL-4	5.22 (4.68-6.15)	5.03 (4.49-5.62)	5.14 (4.64-5.69)
IL-5	2.89 (2.67-3.86)*,†	2.18 (1.69-2.74)	2.53 (1.98-3.13)
IL-6	9.88 (7.68-11.09)	9.40 (8.36-12.53)	9.28 (7.98-10.88)
IL-7	17.4 (15.3-23.1)†	14.9 (11.0-18.7)	15.8 (13.2-18.6)
IL-8/CXCL8	19.3 (17.5-23.3)†	16.6 (14.3-19.3)	18.0 (15.6-21.5)
IL-9	30.7 (22.8-36.3)	26.6 (22.7-34.5)	26.9 (22.2-33.2)
IL-10	4.89 (3.80-5.94)	3.83 (2.60-4.99)	3.83 (2.88-5.21)
IL-12p70	38.6 (24.7-46.1)*	25.8 (20.7-31.6)	26.5 (20.8-34.8)
IL-13	6.87 (4.85-8.57)†	4.88 (3.73-5.76)	5.64 (4.38-7.15)
IL-15	7.87 (6.84-10.5)	7.59 (6.51-8.85)	7.73 (6.68-9.18)
IL-17A	111.1 (89.6-162.5)	95.4 (79.0-120.1)	97.9 (80.2-123.0)
Eotaxin/CCL11	188 (137-207)	153 (128-192)	158 (134-188)
bFGF	124 (111-143)†	108 (97-122)	113 (100-131)
G-CSF	89.0 (78.6-114.8)	75.6 (70.3-90.3)	85.9 (74.5-99.9)
GM-CSF	93.5 (83.5-115.2)	85.3 (72.3-96.2)	88.4 (79.8-99.1)
IFN-γ	475 (400-532)	415 (334-493)	434 (371-501)
IP-10/CXCL10	987 (775-1442)	874 (627-1102)	860 (681-1098)
MCP-1/CCL2	15.9 (12.2-20.9)	15.7 (14.0-18.3)	16.2 (13.5-19.2)
MIP-1α/CCL3	4.80 (4.24-6.28)	3.97 (3.65-5.00)	4.33 (3.62-5.18)

MIP-1β/CCL4	98.0 (80.3-114.2)	95.8 (72.6-118.8)	89.3 (71.4-110.2)
TNF-α	65.3 (58.8-80.51)	60.9 (54.9-69.2)	65.5 (57.1-76.4)
VEGF-A	38.0 (31.7-51.7)	33.9 (26.5-41.9)	35.5 (29.8-42.5)

Data are reported as median (25th-75th percentile). bFGF indicates basic fibroblast growth factor; 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; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor. Comparisons between outcome groups (Kruskal-Wallis and Dunn's test): **P*<0.05 vs normotensive controls; †*P*<0.05 vs preeclampsia.

Table 2B. Maternal Serum Marker Levels at Gestational Weeks 11^{0}-13^{6}.

Serum markers	Gestational hypertension (n = 19)	Preeclampsia (n = 25)	Normotensive (n = 504)
hsCRP, µg/mL	6.23 (3.01-14.6)	3.16 (1.95-6.32)	3.84 (2.05-6.67)
Total cholesterol, mmol/L	5.1 (4.3-5.5)	4.9 (4.4-5.3)	4.5 (4.1-5.0)
HDL, mmol/L	1.73 (1.37-1.99)	1.62 (1.35-1.85)*	1.80 (1.56-2.04)
Triglyceride, mmol/L	1.37 (0.98-1.64)*	1.13 (0.91-1.36)	0.96 (0.75-1.21)
LDL†, mmol/L	2.61 (1.95-3.22)	2.80 (2.10-3.20)*	2.23 (1.82-2.65)
Creatinine, µmol/L	48 (42-52)	49 (45-52)	50 (45-53)
Calcium, mmol/L	2.35 (±0.09)	2.36 (±0.10)	2.36 (±0.10)
Uric acid, µmol/L	198 (172-220)	186 (172-218)	183 (161-207)
PlGF MoM‡	0.74 (0.58-0.84)	0.64 (0.53-0.85)*	0.79 (0.64-0.98)

Data are reported as mean (±standard deviation) or median (25th-75th percentile). HDL indicates high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; MoM, multiple of the median; PlGF, placental growth factor. Comparisons between outcome groups (ANOVA and Tukey's test or Kruskal-Wallis and Dunn's test): *P<0.05 vs normotensive controls. †LDL was calculated based on the Friedewald equation.[53] ‡MoM calculations were performed by Perkin Elmer, based on median values from a large reference population.[35, 54]

Table 3. PLS-DA Classification of Samples as Preeclampsia or Gestational Hypertension.

Variables in model	Variables contributing substantially to separation (VIP > 1)	LVs	Classification accuracy	Sensitivity	Specificity	*P*
Cytokines*	VEGF-A, IL-5, IL-4, IL-8, IP-10, IL-12, IFN-γ	8	74 %	0.7	0.8	0.001
Serum markers†	hsCRP	1	62 %	0.5	0.7	0.076
Clinical characteristics‡	MAP, BMI	1	65 %	0.6	0.7	0.056

The sensitivity is for detecting a preeclampsia sample using PLS-DA. Classification accuracy, sensitivity and specificity are from the leave-one-out cross validation, *P*-values are from permutation testing the model with 1000 repeats. BMI indicates body mass index; hsCRP, high-sensitivity; C-reactive protein; IL, interleukin; IP, interferon (IFN)-γ-induced protein; LVs, latent variables; MAP, mean arterial pressure; PLS-DA, partial least squares discriminant analysis; UtAPI, uterine artery pulsatile index; VIP, variable importance in projection. *Includes 25 cytokines (Human Cytokine Group I multiplex panel). †Includes hsCRP, total cholesterol, high-density and low-density lipoprotein, triglyceride, creatinine, uric acid, calcium and placental growth factor. ‡Includes maternal age, BMI, MAP, UtAPI and gestational age at enrolment.

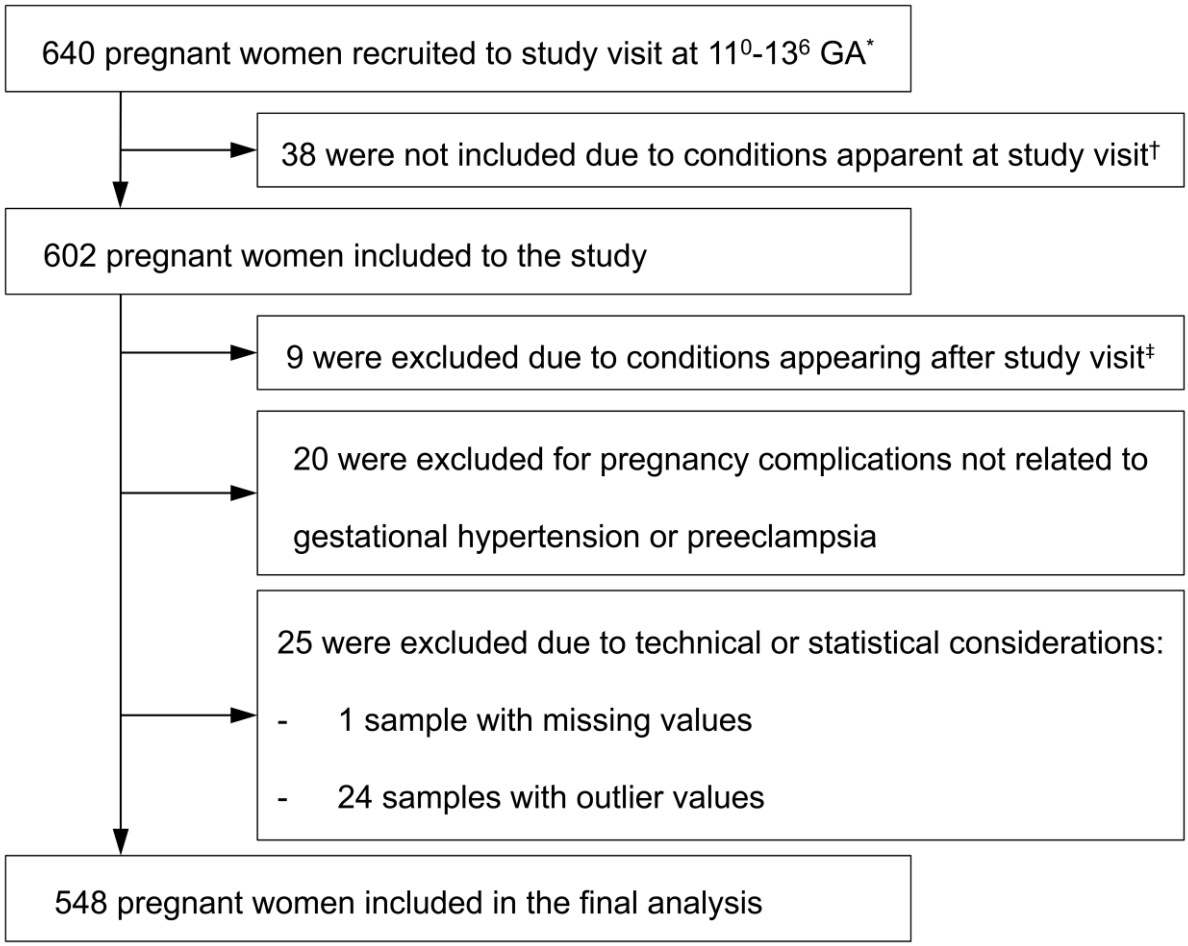

Figure 1: Flowchart of the study population of pregnant women. GA indicates gestational age. *Corresponding to fetal crown-rump length of 45-84 mm. †Conditions apparent at study visit resulting in exclusion included self-reported chronic hypertension, twin pregnancy, missed abortion, crown-rump length > 84 mm, abnormal fetal anatomy, use of acetyl-salicylic acid or low-molecular-weight heparin during pregnancy, and multiparity without a history of gestational hypertension or preeclampsia in a previous pregnancy. ‡Conditions appearing after study visit resulting in exclusion included termination of pregnancy, spontaneous abortion, and women that could not be venepunctured.[35]

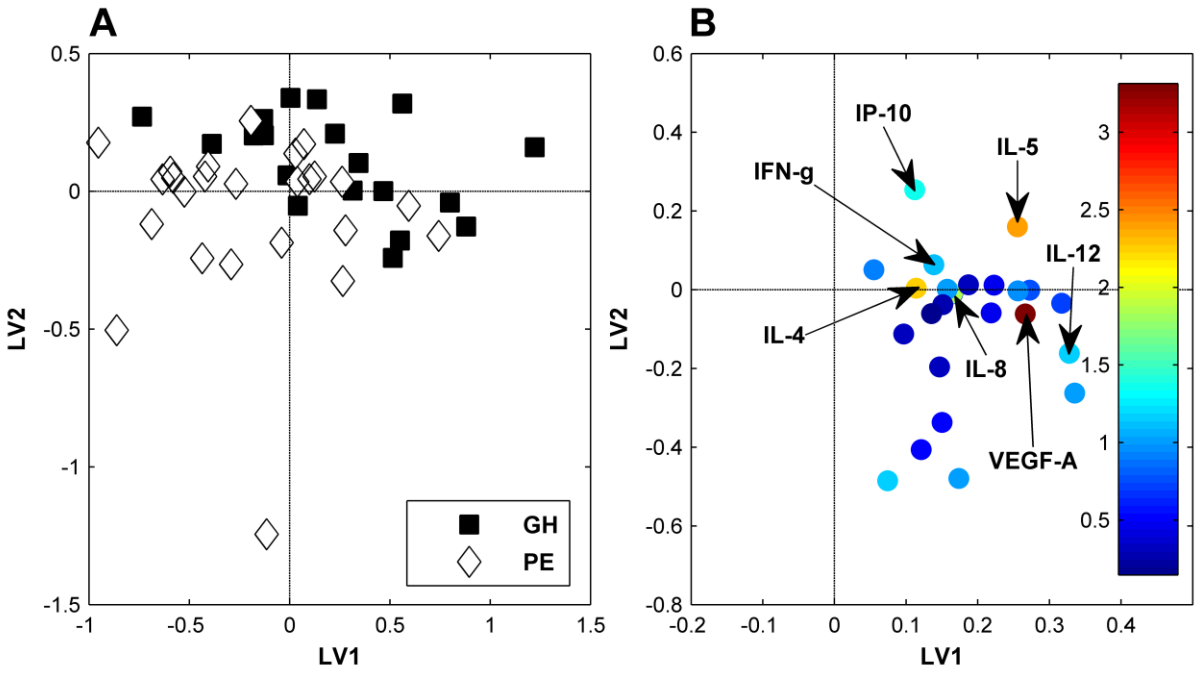

Figure 2: Partial least squares discriminant analysis (PLS-DA) classification of serum samples at gestational weeks 11^0-13^6 from women later developing gestational hypertension (GH; n = 19) or preeclampsia (PE; n = 25). PLS-DA compresses multiple variables into simpler latent variables which contain most of the variation in the data set, and the resulting PLS-DA model can be visualized with score and loading plots. The score plot shows each pregnant woman as an object in the latent variable space and the loading plot shows each cytokine's contribution to defining latent variables. Comparing the score plot to the loading plot gives information about which individual cytokines are reduced or increased when women later developing gestational hypertension are compared to women later developing preeclampsia. **A)** 2D-Score plot for model using serum cytokine profiles, **B)** 2D-Loading plot for model using serum cytokine profiles, showing variable importance in projection (VIP)-score grading of variables by color intensity. The score and loading plots for the first two LVs are shown, with additional clustering information found in the subsequent LVs. Only variables with VIP scores ≥ 1 were considered important to the model.[37] IL indicates interleukin; IP, interferon (IFN)-g-induced protein; LV, latent variable; VEGF, vascular endothelial growth factor.

Materials and methods

Study Population

The study was approved by the Regional Committee for Medical Research Ethics in mid-Norway, entries REK 2010/102 and 2013/386. All women gave written informed consent. The study population consisted of pregnant women who were nulliparous or had preeclampsia or gestational hypertension in a previous pregnancy, invited to attend an examination at 11^0-13^6 weeks of gestation, as previously described.[1, 2] Women were not eligible if they were parous without previous preeclampsia or gestational hypertension, or if they used acetylsalicylic acid or low-molecular-weight heparin in the current pregnancy. At the study visit participants were interviewed about maternal age, chronic diseases, medication, ethnical origin, smoking status, method of conception, any previous pregnancies affected by preeclampsia or gestational hypertension, family history of preeclampsia and present height. Participants were weighed on a scale and body mass index (BMI) was calculated in kg/m^2. Blood pressure was measured with a CAS 740 MAX NIBP automated device (CAS Medical Systems Inc, CT, USA; http://www.casmed.com), calibrated prior to and once during the study period. The procedure was identical to that recommended by the European Society of Hypertension.[3] The woman was positioned in a chair with her upper arms on armrests. The cuff-size was adapted to the overarm circumference, and the cuff was placed at the level of the heart. The woman rested for at least 10 min before blood pressure was measured three times with an approximately one-minute interval for both arms. The first blood pressure taken on each arm was discarded, and the average mean arterial blood pressure (MAP) from the last two recordings on each arm was calculated.[4] The robustness of the MAP measurement resulted in the selection of MAP as the parameter for blood pressure entered into the linear regression and multivariate analysis.[5] The MAP from the arm with the highest MAP was used. The blood pressure measurements were conducted by a physician, medical secretary or staff engineer, who had received specific training. Participants were examined with transabdominal ultrasound using a Siemens ACUSON Antares™ machine (Siemens Medical Solutions USA Inc, CA, USA). Missed abortions, multiple pregnancies and severe congenital anomalies were excluded. Fetal crown-rump length was used to estimate gestational age, and women were included if this measured between 45 and 84 mm. The uterine artery pulsatility index (UtAPI) was measured according to the method described by Khalil and Nicolaides.[6] The average of three PI measurements on each side was calculated, to correct for intra-observer variability. The average of the PI from the right and left uterine artery was calculated and used. All scans were carried out by specialized trained midwifes certified by the Fetal Medicine Foundation (http://www.fetalmedicine.com). Participants were asked to fast for one hour before their visit. Maternal venous blood was drawn into non-heparinized tubes and centrifuged at 1800G for 10 minutes. A serum sample (0.8 mL) was separated and stored at -80°C, thawed once and aliquots were stored at -80°C until further analysis. Data on pregnancy outcomes were collected from hospital records.

We used the Norwegian Association for Obstetricians and Gynecologists´ definitions of hypertensive pregnancy disorders,[7] which are slightly modified versions of the guidelines of the American Congress of Obstetricians and Gynecologists.[8] Gestational hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg occurring after gestational week 20. Preeclampsia was defined by the same hypertension criteria as for gestational

hypertension in combination with proteinuria ≥ 0.3g per 24 hours measured twice within 4-6 hours, occurring after gestational week 20. Women in the study population who remained normotensive throughout pregnancy were eligible as controls, while women with self-reported chronic hypertension, preterm birth before 37 weeks or small-for-gestational age babies (birth weight mean ≤ –22%) were excluded.[9] None of the women in the cohort had chronic diseases known to increase the risk of gestational hypertension or preeclampsia,[10] except for the two women with pre-existing hypertension who were excluded.

Serum Measurements

Laboratory analyses were performed blinded to pregnancy outcomes after all women had delivered. Serum levels of 27 cytokines (Human Cytokine Group I multiplex panel) were measured using Luminex xMAP Technology on a Bio-Plex 200 system (Bio-Rad Laboratories, CA, USA). Two of the 27 cytokines analyzed (PDGF-BB and RANTES) were excluded from further analyses, since more than half of the measurements were above the upper detection limit. The serum samples were measured in a single replicate, whereas cytokine standards and blank samples were measured in duplicate on each plate. To minimize technical variation when comparing samples analyzed on different plates, a quality control sample of two pooled sera was run in replicates on each plate, and between-run coefficients of variation used for adjustment of cytokine measurements.[11] The serum markers high sensitivity C-reactive protein (turbidimetric assay, Modular P analyzer, Roche, Burgess Hill, UK), total cholesterol, high-density lipoprotein, triglyceride, creatinine, uric acid and calcium (enzymatic colorimetric assays, Modular P analyzer), were measured at the Department of Clinical Chemistry at St. Olavs Hospital. The serum levels of low-density lipoprotein were calculated using the Friedewald equation.[12] Placental growth factor was measured, as previously described in Skråstad *et al.*,[1, 2] on the 6000 DELFIA Xpress clinical random access screening platform (Perkin Elmer Life and Analytical Sciences, Turku, Finland).

Statistical Analyses

Statistical analyses were done in GraphPad Prism v.5.0 (GraphPad Software, CA, USA), Matlab v.r2013b (The Mathworks Inc, MA, USA) and SPSS v.21.0 (SPSS Inc, IL, USA). Data were tested for normality using the D'Agostino-Pearson test.[13] Non-normal data was reported as median (interquartile range), normally distributed data as mean (± standard deviation), and categorical variables as number (percentages). Outliers were identified by Grubbs' test, for which non-normal data was logarithmically transformed before 24 outliers (4%, total of 602 samples) were identified.[14] Graphical presentations of the data were inspected to verify the statistical identification of outliers. Samples with at least one outlier value were removed from all analyses prior to final significance testing. Non-normal data was analyzed by Kruskal-Wallis test and Dunn's test for pairwise comparisons, normal data by ANOVA and Tukey's test for pairwise comparisons, and categorical variables by Chi-square test. To adjust for the possible confounding effects of maternal BMI and MAP, linear regression models with serum measurements as the dependent variable and group, BMI and MAP as independent variables were generated. The interaction between group and BMI or MAP was included in the model.

Multivariate analysis was used to further compare women who later developed gestational hypertension or preeclampsia, by analyzing the measured cytokines,

maternal serum markers, or clinical characteristics as a set. Prior to partial least squares discriminant analysis,[15] all data were mean centered and cytokine and serum marker data was additionally logarithmically transformed.[16] Multivariate models comparing women later developing preeclampsia with women later developing gestational hypertension were constructed using PLS Toolbox 7.3.1 (Eigenvector Research, WA, USA). The classification models were evaluated by leave-one-out cross validation, and mean sensitivity, specificity and accuracy of cross validated classification was calculated. The classification results were validated using 1000 permutation tests.[17] For all statistical analyses $P \leq 0.05$ was considered statistically significant.

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SUPPLEMENTAL MATERIAL

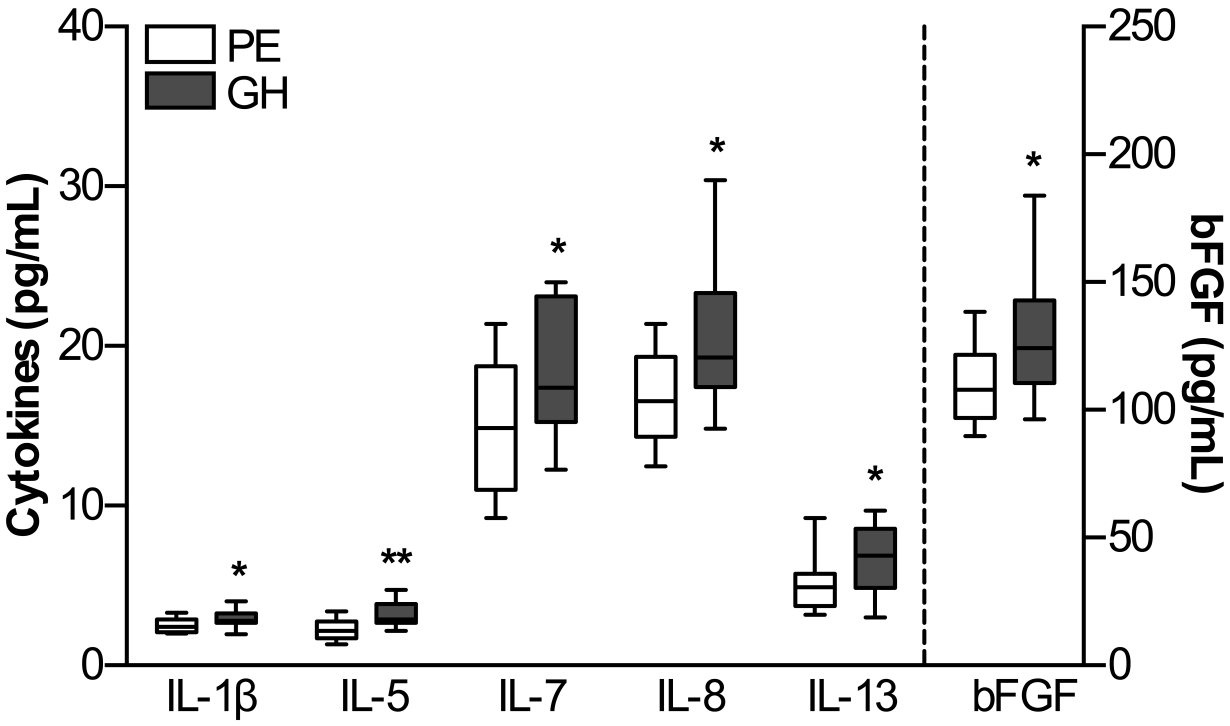

Figure I: Significant differences in serum cytokine profiles at gestational age 11^0-13^6 weeks between women who later developed gestational hypertension (GH; n = 19) or preeclampsia (PE; n = 25). bFGF indicates basic fibroblast growth factor; IL, interleukin. *P<0.05, **P<0.01 vs PE.

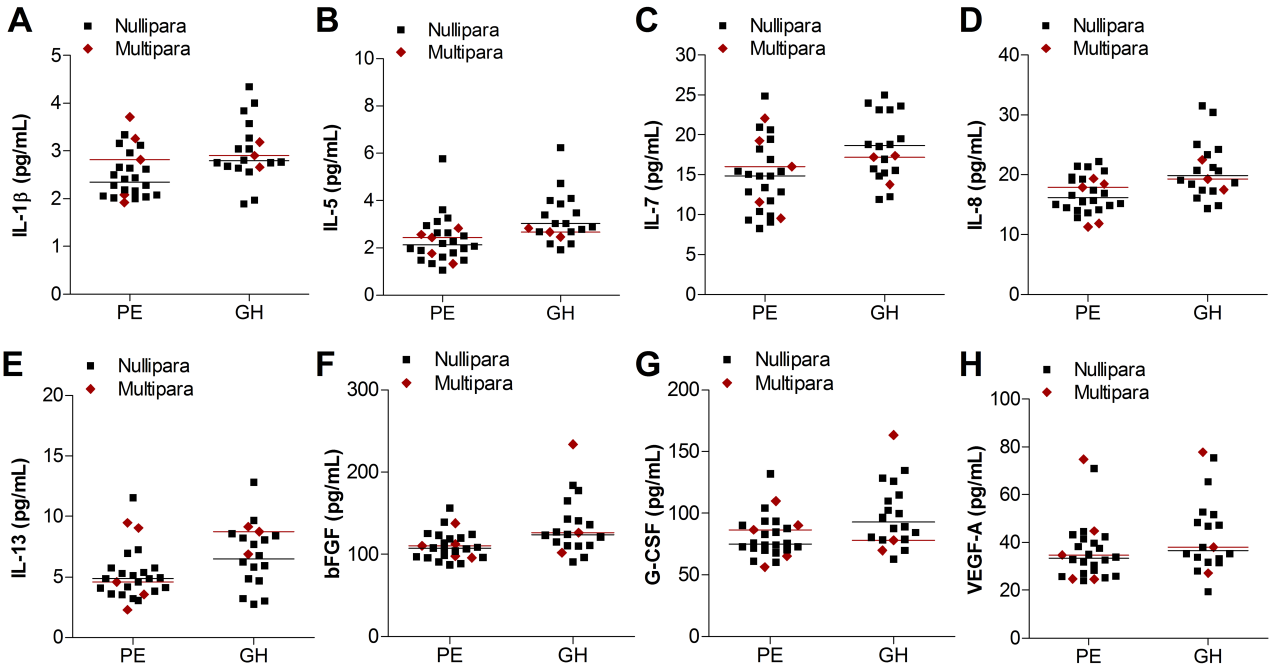

Figure II: Serum cytokine profiles at gestational age 11^0-13^6 weeks for nulliparous (black squares) and multiparous (red diamonds) women who later developed gestational hypertension (GH; n = 19) or preeclampsia (PE; n = 25). bFGF indicates basic fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; VEGF-A, vascular endothelial growth factor A.