

# Preeclampsia

## Multiplex Analysis of Circulating Maternal Cardiovascular Biomarkers Comparing Preeclampsia Subtypes

Tove Lekva, Meryam Sugulle, Kjartan Moe, Chris Redman, Ralf Dechend, Anne Cathrine Staff

**Abstract**—Preeclampsia, a hypertensive pregnancy disorder, links to increased long-term maternal cardiovascular disease (CVD). The risk is further increased with early-onset preeclampsia (EPE) and delivery of a growth-restricted child. We hypothesized that circulating biomarkers associated with CVD risk differed between preeclampsia subtypes and controls. We compared EPE; n=37, delivery <week 34, late-onset preeclampsia (LPE); n=29, delivery ≥week 34, and normotensive controls (n=49) using Olink Proseek multiplex CVD I assay (targeting 92 biomarkers). We stratified analysis to uteroplacental spiral artery acute atherosclerosis presence in preeclampsia patients, sharing morphological similarities with atherosclerosis. We found 47 CVD-related biomarkers differing between the groups, 42 markers between normotensive controls and EPE, 28 markers between normotensive controls and LPE, and 9 markers between EPE and LPE. Among these 9 markers, ST2 (ST2 protein), MMP (matrix metalloproteinase) 1, MMP3, and fractalkine (CX3CL1) were uniquely dysregulated in EPE. Principal component (PC) analysis of the differing markers identified 4 clusters (named PC1–PC4) that largely separated the preeclampsia and control groups as well as pregnancies with low and high circulating PIGF (placental growth factor). The combination of the single markers PIGF, ST2, MMP1, MMP3, and CX3CL1 had a high discriminatory property to differentiate between EPE and LPE. Preeclampsia with acute atherosclerosis or with fetal growth restriction could be differentiated by Olink biomarkers as compared with preeclampsia without these features. We identified specific CVD-related biomarkers in pregnancy depending on preeclampsia subtypes and uteroplacental acute atherosclerosis. Assessment of these pregnancy measured biomarkers' relation to long-term cardiovascular dysfunction and hard end points is warranted. (*Hypertension*. 2020;75:1513-1522. DOI: 10.1161/HYPERTENSIONAHA.119.14580.)

• [Data Supplement](#)

**Key Words:** biomarkers ■ cardiovascular disease ■ matrix metalloproteinase  
■ placental growth factor ■ preeclampsia

Preeclampsia is a severe pregnancy complication, defined by new-onset hypertension and another sign of organ dysfunction after gestational week 20 (eg, proteinuria or elevated liver enzymes), affecting 3% to 5% of all pregnancies and representing a major cause of maternal and neonatal morbidity and mortality.<sup>1</sup> Women with previous preeclampsia have a 2-fold increased risk for future major maternal cardiovascular disease (CVD; eg, stroke, myocardial infarction) and a 4-fold risk of hypertension and heart failure with preserved ejection fraction.<sup>2-4</sup> The CVD risk is increased 7- to 8-fold when preeclampsia is complicated by premature delivery before week 34, early-onset preeclampsia (EPE), and fetal growth restriction (FGR).<sup>4-6</sup> The mechanisms for these associations are not fully understood. Presence of prepregnancy CVD risk factors in women later developing preeclampsia does only partly explain the association between preeclampsia and long-term

CVD risk,<sup>7,8</sup> indicating that pregnancy, and especially preeclampsia, may promote or induce additional permanent maternal cardiovascular changes.<sup>2</sup> Women with preeclampsia, and especially EPE, present with signs of endothelial dysfunction, hypertension, and subclinical cardiac changes, both during pregnancy and postpartum.<sup>4,9,10</sup> Studies have measured levels of a single or a few CVD-related risk markers in preeclampsia during pregnancy, and some biomarkers have been stratified relative to preeclampsia subtype (eg, EPE versus late-onset preeclampsia [LPE]).<sup>11,12</sup> Differences in circulating cardiovascular biomarkers between preeclampsia subtypes and normotensive controls in pregnancy could reflect subtype-specific cardiovascular changes, reflecting different CVD risks.<sup>13,14</sup> This biomarker phenotyping could be utilized to target women at highest risk of CVD, to tailor cardiovascular follow-up and preventive measures,<sup>15</sup> as suggested by us.<sup>2</sup>

Received December 18, 2019; first decision January 19, 2020; revision accepted March 17, 2020.

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**The Data Supplement is available with this article at <https://www.ahajournals.org/doi/suppl/10.1161/HYPERTENSIONAHA.119.14580>.**

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*Hypertension* is available at <https://www.ahajournals.org/journal/hyp>

DOI: 10.1161/HYPERTENSIONAHA.119.14580

Increased maternal CVD risk is seen after preeclampsia but also after other subtypes of placental dysfunction, such as in FGR without preeclampsia.<sup>2</sup> Uteroplacental spiral artery acute atherosclerosis is a pregnancy-specific lesion, resulting in downstream placental dysfunction and sharing morphological features of early atherosclerosis.<sup>16</sup> We have proposed that acute atherosclerosis represents a marker of increased CVD risk<sup>17</sup> and have identified an association to classical CVD risk markers in older pregnant women.<sup>18</sup>

In this study, we assessed whether a multiplex panel of 92 circulating CVD-risk biomarkers differed between women with EPE, LPE, and normotensive pregnancies (normal controls [NC]). Furthermore, we stratified this biomarker analysis to the presence or absence of acute atherosclerosis, FGR, and the lowest quartile of circulating placental growth factor (PlGF), the latter suggested by us to represent a marker of placental dysfunction and placental cellular (syncytiotrophoblast) stress.<sup>19</sup>

## Methods

Reasonable requests to access part of the anonymous data set can be sent to corresponding author, but access is restricted because of ongoing clinical follow-up, the sensitive nature of the data collected for this study, and the patient informed consents (and thereby ethical body approval). All procedures were in accordance with institutional guidelines.

## Patient Recruitment and Blood Sampling

Pregnant women scheduled for elective cesarean section were recruited from 2001 to 2014 to the Oslo Pregnancy Biobank. The Regional Committee for Medical and Health Research Ethics in South-Eastern Norway approved the study. All patients signed informed consent. Preeclampsia was defined as new-onset hypertension (blood pressure  $\geq 140/90$  mm Hg) and proteinuria ( $\geq 1+$  on

dipstick or  $\geq 30$  total protein/creatinine ratio) at  $\geq 20$  weeks gestation.<sup>20</sup> EPE was defined as delivery before gestational week 34, LPE as delivery  $\geq 34$  weeks. Clinical characteristics of the patient groups are presented in Table 1. None of the patients had pregestational or gestational diabetes mellitus. Further clinical details,<sup>21,22</sup> including blood sampling<sup>23</sup> and blood pressure measurement devices, are presented in the [Data Supplement](#).

## Decidua Basalis Tissue Evaluation for Acute Atherosclerosis

In a subset of the cohort (n=79), the immunohistological diagnosis of decidua basalis (endometrium of pregnancy) acute atherosclerosis was available.<sup>24</sup> Sampling of decidua basalis tissue and immunohistochemical acute atherosclerosis identification was described previously.<sup>24</sup>

## Multiplex Biomarker Analysis

EDTA plasma samples from NC (n=49), EPE (n=37), and LPE (n=29) were analyzed using the Proseek multiplex CVD I assay at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala, Sweden, that had developed the proximity assay technology, targeting 92 CVD linked biomarkers.<sup>25</sup> Analytical details and acronyms/full names of markers are presented in Table S1 in the [Data Supplement](#). Plasma from 108 of the 115 pregnancies analyzed with Olink CVD biomarkers were also analyzed for serum protein PlGF concentration with immunochemiluminescence assay (Roche, Elecsys 2010 Modular) and showed excellent correlation, as shown in Figure S1;  $r=0.97$  (95% CI, 0.91–1.01).

## Statistics and Multivariate Data Analysis

Statistical analyses were performed using SPSS Statistics 25.0 (IBM). For continuous variables, a nonparametric Kruskal-Wallis test adjusting for multiple testing (false discovery rate) was used to identify markers that were significantly different between the 3 study groups. Dunn's Bonferroni post hoc test was applied to the significant different markers to determine significant differences between groups. Unadjusted and adjusted logistic regression was performed on standardized variables using forced entry, adjusting for body mass index (BMI), age, systolic (SBP) and diastolic

**Table 1. Clinical Characteristics of the Patient Groups During Pregnancy**

| Clinical Variables                                 | NC (n=49)         | LPE (n=29)                      | EPE (n=37)                     | Between Groups (P) |
|--|-------------------|---------------------------------|--------------------------------|--------------------|
| Maternal age at delivery, y                        | 33 (4)            | 34 (5)                          | 32 (5)                         | 0.147              |
| Body mass index early pregnancy, kg/m <sup>2</sup> | 22.3 (20.6, 23.7) | 24.1 (21.0, 30.3)*              | 24.1 (22.0, 29.8) <sup>†</sup> | 0.002              |
| Systolic blood pressure <20 wk, mm Hg              | 110 (103, 117)    | 115 (110, 123)                  | 117 (108, 126) <sup>†</sup>    | 0.008              |
| Diastolic blood pressure <20 wk, mm Hg             | 67 (60, 71)       | 75 (67, 80)*                    | 72 (65, 78)*                   | 0.003              |
| Systolic blood pressure at delivery, mm Hg         | 121 (115, 132)    | 160 (158, 173) <sup>‡</sup>     | 162 (155, 180) <sup>‡</sup>    | <0.001             |
| Diastolic blood pressure at delivery, mm Hg        | 71 (65, 80)       | 100 (96, 108) <sup>‡</sup>      | 100 (95, 108) <sup>‡</sup>     | <0.001             |
| Gestational age at delivery and sampling, wk       | 39.1 (38.9, 39.3) | 35.9 (34.4, 38.1) <sup>†¶</sup> | 30.3 (27.9, 32.9) <sup>‡</sup> | <0.001             |
| Ever smoked during pregnancy, n (%)                | 3 (6.1)           | 4 (13.8)                        | 3 (8.3)                        | 0.509              |
| Primipara, n (%)                                   | 25 (51.0)         | 18 (62.1)                       | 22 (59.5)                      | 0.917              |
| Small for gestational age, n (%)                   | 1 (2.0)           | 13 (44.8) <sup>†¶</sup>         | 34 (91.9) <sup>‡</sup>         | <0.001             |
| Fetal growth restriction, n (%)                    | 0 (0)             | 9 (31.0) <sup>†¶</sup>          | 27 (75.0) <sup>‡</sup>         | <0.001             |
| Newborn weight, g                                  | 3399 (299)        | 2603 (819) <sup>¶</sup>         | 1212 (439) <sup>‡</sup>        | <0.001             |
| Newborn weight percentile                          | 52.1 (28.3, 74.5) | 32.5 (1.78, 50.5) <sup>¶</sup>  | 0.10 (0.01, 3.54) <sup>‡</sup> | <0.001             |
| Newborn sex (girl/boy)                             | 21/28 (42.9/57.1) | 13/16 (44.8/55.2) <sup>§</sup>  | 26/11 (70.3/29.7)*             | 0.027              |
| Acute atherosclerosis (all/yes), n (%)             | 36/3 (8.3)        | 19/5 (26.3)                     | 24/10 (41.7) <sup>†</sup>      | 0.010              |

Data given as mean (SD) when normally distributed and median (25<sup>th</sup>, 75<sup>th</sup>) when skewed distributed. EPE indicates early-onset preeclampsia (delivery before gestational week 34); LPE, late-onset preeclampsia (delivery from gestational week 34); and NC, normotensive controls.

\* $P < 0.05$ , <sup>†</sup> $P < 0.01$ , <sup>‡</sup> $P < 0.001$  compared with controls.

<sup>§</sup> $P < 0.05$ , <sup>¶</sup> $P < 0.01$ , <sup>†¶</sup> $P < 0.001$  between LPE and EPE.

blood pressure (DBP), all measured before 20 weeks gestation. Principal component (PC) analysis was performed on the markers significantly different between study groups in the initial Kruskal-Wallis test (diagnostic groups) or Mann-Whitney test (low versus high PIGF). The PC was thereafter compared and associated to diagnostic groups or low versus high PIGF. A  $P < 0.05$  was considered significant.

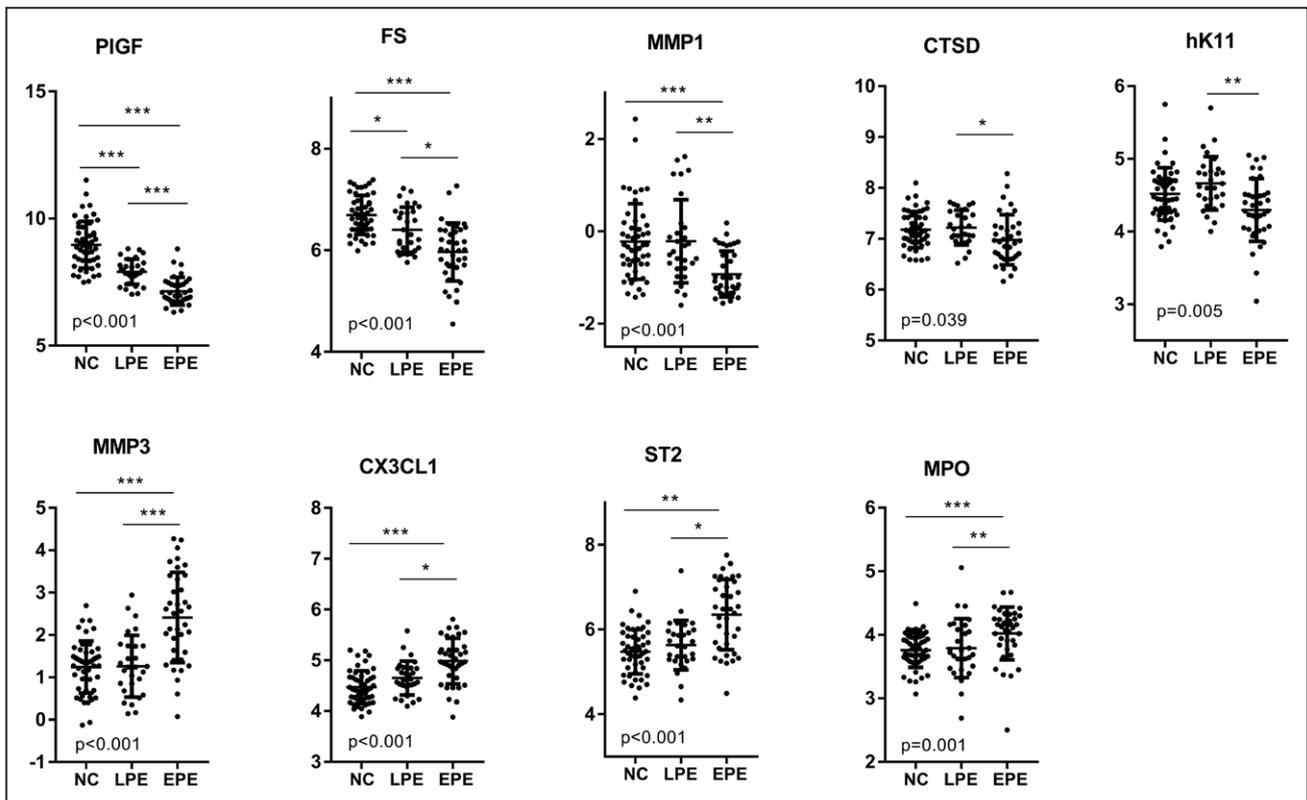
**Results**

**Single Olink Biomarker Analysis**

Among the 92 Olink biomarkers, 89 were satisfactorily identified in the pregnant women. IL4 (interleukin-4), ITGB1BP2 (melusin), and BNP (natriuretic peptides B) were excluded from further analysis as the 2 former were not detected in measurable amounts. BNP was only measurable in one NC and half of the preeclampsia group samples, the former in line with previous Olink reports of nonpregnant populations.<sup>26</sup> NT-proBNP (N-terminal pro-B-type natriuretic peptide) was, however, measurable in all samples, in line with its higher concentration than BNP in plasma. We identified 47 of the 89 detected biomarkers as significantly different between the 3 pregnancy outcome groups (Table S1).

Following the post hoc test, 42 biomarkers differed significantly between EPE and NC and 28 differed between LPE and NC (Figure S2). Three biomarkers were lower both in EPE and LPE groups (PIGF, TIE2 [angiopoietin-1 receptor], and FS [follistatin]), and 22 biomarkers were significantly higher

for the EPE and LPE groups relative to NC (Table S2 [summarize previous findings<sup>27-70</sup>] and Figure S2): VEGFA (vascular endothelial growth factor A), IL6 (interleukin-6), TNFR1 (tumor necrosis factor receptor 1), TRAILR2 (TNF-related apoptosis-inducing ligand receptor 2), GH (growth hormone), PTX3 (pentraxin-related protein PTX3), CHI3L1 (chitinase-3-like protein 1), TIM (TIM-1 [T-cell immunoglobulin domain and mucin domain]), IL16 (interleukin-16), UPAR (urokinase plasminogen activator surface receptor), RAGE (receptor for advanced glycosylation end products), MMP7 (matrix metalloproteinase-7), CD40 (tumor necrosis factor receptor superfamily member 5), tPA (tissue-type plasminogen activator), HBEGF (heparin-binding EGF-like growth factor), ESM1 (endothelial cell-specific molecule 1), SPON1 (spondin-1), CASP8 (caspase-8), FABP4 (fatty acid-binding protein, adipocyte), LEP (leptin), CCL20 (C-C motif chemokine 20), and NT-proBNP. The EPE and the LPE group differed for 9 biomarkers; 5 biomarkers were lower in the EPE group (PIGF, hK11 [kallikrein-11], MMP1, CTSD [cathepsin D], and FS) and 4 were higher (MMP3, MPO [myeloperoxidase], ST2 [ST2 protein], CX3CL1 [fractalkine]; Figure 1). Among these, 5 markers were altered in the EPE group relative to both the NC and LPE groups (MMP3, MPO, ST2, CX3CL1, and MMP1). Two of the 9 markers were not different compared with NC (hK11 and CTSD), and the last 2 markers were different between all the 3 groups (PIGF and FS); lowest for the EPE group, intermediate value for the LPE group, and the highest values in the control group.



**Figure 1.** Individual levels of the 9 circulating Olink biomarkers differing significantly between early-onset preeclampsia (EPE) and late-onset preeclampsia (LPE) groups (delivery before or from gestational week 34, respectively). Data are given as normalized protein expression (NPX) log 2.  $P$  represents the overall group effect (false discovery rate [FDR] adjusted Kruskal-Wallis). Dunn's Bonferroni post hoc test are used to compare groups \* $P < 0.05$ , \*\* $P < 0.01$  \*\*\* $P < 0.001$ . Horizontal bars represent mean  $\pm$  SD. CTSD indicates cathepsin D; CX3CL1, fractalkine; hK11, kallikrein-11; FS, follistatin; MMP1, matrix metalloproteinase-1; MMP3, matrix metalloproteinase-3; MPO, myeloperoxidase; NC, normal controls; PIGF, placental growth factor; and ST2, ST2 protein.

To correct for potential confounding factors between the 9 markers different between EPE and LPE such as age (odds ratio [OR], 0.64;  $P=0.087$ ), BMI (OR, 1.06;  $P=0.087$ ), and blood pressure (SBP: OR, 1.43;  $P=0.286$ ; DBP: OR, 0.89;  $P=0.720$ ), we performed a logistic regression analysis correcting for these factors when comparing the study groups. After adjusting, we found that CX3CL1, ST2, MMP3, FS, CTSD, MMP1, hK11, and PIGF remained significantly different between the EPE and LPE group (Figure 2). When adjusting for smoking, or newborn sex, our conclusions remained the same (data not shown).

We also investigated whether the 9 markers dysregulated only in EPE were different in EPE with FGR ( $n=27$ ) versus without FGR ( $n=9$ ) using logistic regression adjusting for age, BMI, SBP, and DBP and found MMP3 to be higher (OR, 3.29; 95% CI, [1.02–10.6];  $P=0.046$ ) and CTSD lower (OR, 0.08; 95% CI [0.01–0.54];  $P=0.009$ ) in the EPE FGR group.

### Multivariate Data Analysis

We performed multivariate principal component analysis of the 47 markers differing between the diagnosis groups (NC, EPE, and LPE) to identify combinations of biomarkers contributing to best grouping of the dataset. We found 4 main principal

components (PC1–PC4), explaining 46.8% of the variance in the Olink biomarker set. The relative contribution of the different markers to each PC is presented in Figure 3A. These PCs also identified clusters of the diagnosis groups (NC, EPE, and LPE; Figure 3B). To compare the PC between the diagnostic groups, we performed a logistic regression adjusting for the confounding factors maternal BMI, age, SBP, and DBP (Figure 3C). Comparing EPE versus LPE group, we identified PC2, PC3, and PC4 as most discriminatory of EPE. For the LPE group, PC1, PC2, and PC3 were the best biomarker discriminators, while all PC groups (PC1–PC4) were significantly associated with EPE compared with NC (Figure 3C). To investigate if some of the PCs predicted EPE compared to LPE better than any single markers, we performed a receiver operating characteristic analysis, indicating that PIGF displayed the best accuracy for identifying EPE, followed by PC3 and MMP3 (Figure 4). When testing individual markers (without PC groups), we found the best discrimination between EPE and LPE with the combination of the individual markers  $PIGF*ST2*MMP1*MMP3*CX3CL1$ , area under the curve (95% CI), 0.98 (0.95–1.00);  $P<0.001$  (Figure 4).

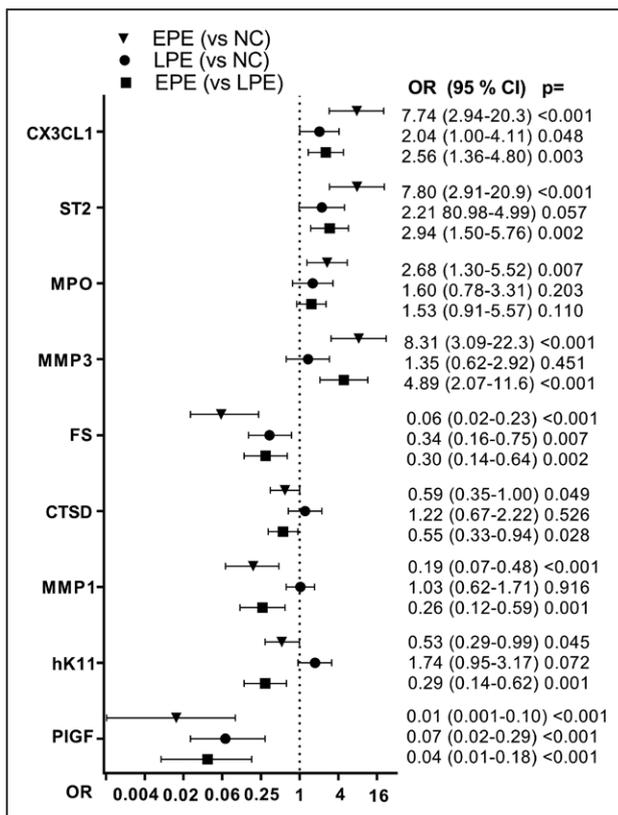
### Olink Biomarker Analysis Versus Placenta Function Biomarkers

We wanted to investigate if circulating PIGF also subclassified the Olink cardiovascular biomarkers, as low PIGF represents a marker of placenta dysfunction,<sup>19</sup> which can result in many clinical syndromes that individually associate with premature maternal CVD.<sup>2</sup> We therefore removed the Olink PIGF data from the initial PCA analysis and performed a new PC analysis on the 37 markers differently regulated between women with the lowest quartile of circulating PIGF protein compared with the remaining higher PIGF protein quartiles. As hypothesized, PCs also differed between the pregnant women with the lowest quartile of PIGF (quartile 1) and the women with the higher PIGF quartiles (quartile 2–4; Figure S3A through S3C).

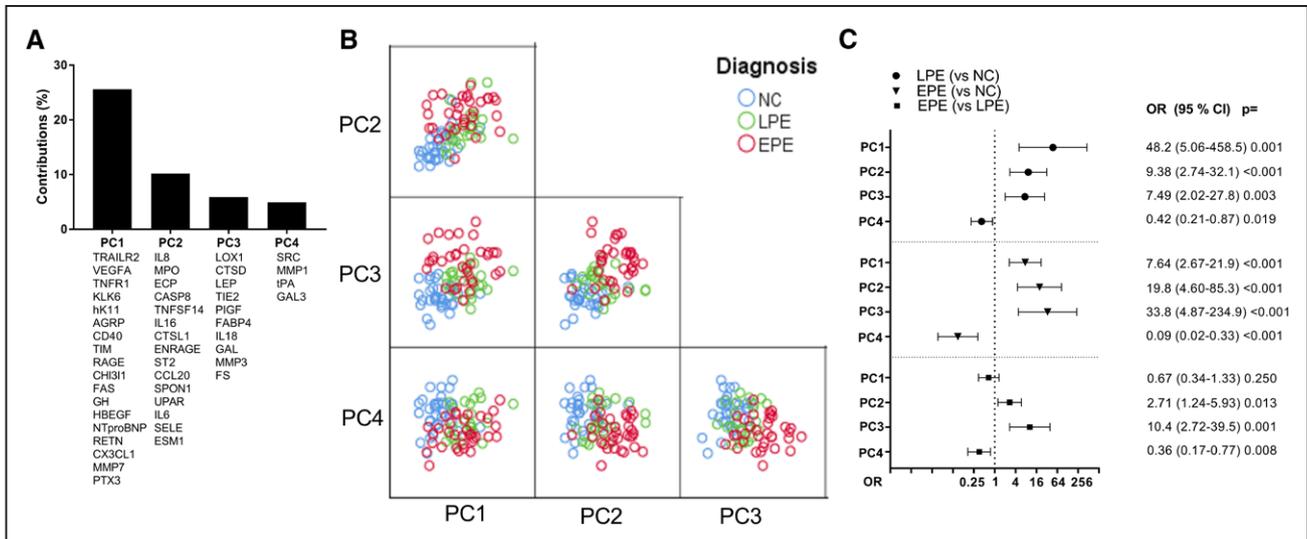
We also subclassified the total Olink dataset to uteroplacental presence of acute atherosclerosis, another biomarker of placental function. Acute atherosclerosis was as previously reported<sup>24</sup> significantly most prevalent in the EPE group (Table 1), and controls positive for acute atherosclerosis were too few for sensible analysis of an association to Olink biomarker patterns. We found 3 markers significantly (unadjusted  $P$ ) different between preeclampsia with acute atherosclerosis compared to without (FAS [tumor necrosis factor receptor superfamily member 6], TM [thrombomodulin], and MMP10; Figure 5). After adjusting for confounders in logistic regression (BMI, age, gestational age at delivery, SBP, and DBP), only FAS ( $P=0.017$ ) and TM ( $P=0.019$ ) remained significantly associated to acute atherosclerosis presence.

### Discussion

Our findings suggest a dysregulation of both single and clusters of CVD-related circulating biomarkers in pregnancy according to pregnancy phenotype. A CVD-related biomarker dysregulation was found both comparing early- and late-onset preeclampsia and normotensive pregnancies, as well as when phenotyping on circulating biomarker of placental



**Figure 2.** Logistic regression analysis showing adjusted associations between the Olink-identified biomarkers different between study groups. The figure shows the odds ratio (OR) (95% CIs) of the biomarkers associated with early-onset preeclampsia (EPE; delivery before gestational week 34) compared to normal controls (NC), late-onset preeclampsia (LPE; delivery from gestational week 34) compared to NC, and EPE compared to LPE, using standardized values. CTSD indicates cathepsin D; CX3CL1, fractalkine; hK11, kallikrein-11; FS, follistatin; MMP1, matrix metalloproteinase-1; MMP3, matrix metalloproteinase-3; MPO, myeloperoxidase; PIGF, placental growth factor; and ST2, ST2 protein.

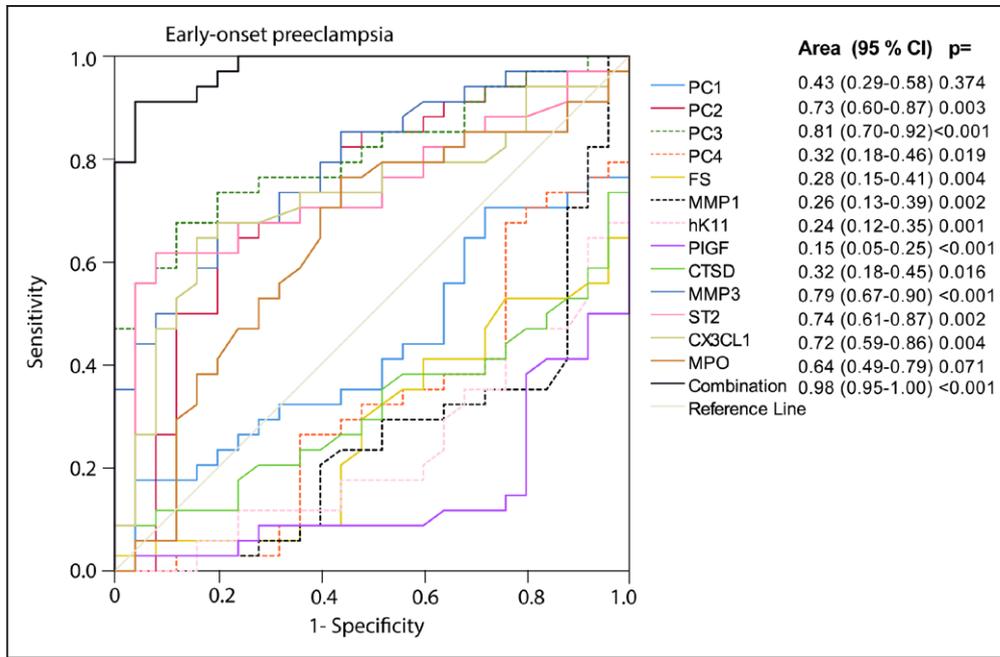


**Figure 3.** Principal component (PC) analysis. **A**, Markers contributing to each of the 4 principal components (PC1–PC4) from the initial 47 markers differently regulated between diagnostic groups. **B**, Correlation plots between the different PC showing diagnostic groups in different colors. NC (blue; online), normotensive controls; LPE (green; online), late-onset preeclampsia (delivery from gestational week 34); EPE (red; online), early-onset preeclampsia (delivery before gestational week 34). **C**, Logistic regression analysis showing adjusted associations between the different PCs and diagnostic groups (acronyms as in Figure 3B). AGRP indicates agouti-related protein; CASP8, caspase-8; CCL20, C-C motif chemokine 20; CHI3L1, chitinase-3-like protein 1; CD40, tumor necrosis factor receptor superfamily member 5; CTSD, cathepsin D; CTSL1, cathepsin L1; CX3CL1, fractalkine; ECP, eosinophil cationic protein; ENRAGE, protein S100-A12; ESM1, endothelial cell-specific molecule 1; FABP4, fatty acid-binding protein, adipocyte; FAS, tumor necrosis factor receptor superfamily member 6; FS, follistatin; GAL, galanin peptides; GAL3, galectin-3; GH, growth hormone; HBEGF, heparin-binding EGF-like growth factor; hK11, kallikrein-11; IL6, interleukin-6; IL8, interleukin-8; IL16, interleukin-16; IL18, interleukin-18; KLK6, kallikrein-6; LEP, leptin; LOX1, lectin-like oxidized LDL receptor 1; MMP1, matrix metalloproteinase-1; MMP3, matrix metalloproteinase-3; MMP7, matrix metalloproteinase-7; MPO, myeloperoxidase; NT-proBNP, N-terminal pro-B-type natriuretic peptide; OR, odds ratio; PIGF, placental growth factor; PTX3, pentraxin-related protein PTX3; RAGE, receptor for advanced glycosylation end products; RETN, resistin; SELE, E-selectin; SPON1, spondin-1; SRC, proto-oncogene tyrosine-protein kinase Src; ST2, ST2 protein; TIE2, angiotensin-1 receptor; TIM, TIM-1 (T-cell immunoglobulin domain and mucin domain); TNFR1, tumor necrosis factor receptor 1; TNFSF14, tumor necrosis factor ligand superfamily member 14; tPA, tissue-type plasminogen activator; TRAILR2, TNF-related apoptosis-inducing ligand receptor 2; UPAR, urokinase plasminogen activator surface receptor; and VEGFA, vascular endothelial growth factor A.

dysfunction (low PIGF)<sup>19</sup> or presence of uteroplacental acute atherosclerosis. The CVD biomarker dysregulation was as hypothesized most prominent in the EPE group, which epidemiologically has the highest increased risk of future CVD. Among 92 CVD-associated markers, we identified (1) 47 markers different between the NC, EPE, and LPE groups, (2) 9 markers specifically different between the EPE and LPE group, (3) 4 PCs providing segregation of the 3 clinical outcome groups, (4) the combination of PIGF, ST2, MMP1, MMP3, and CX3CL1, followed by PIGF, PC3 and MMP3 to have the best diagnostic performance for EPE versus LPE comparing single or multivariate analysis, and (5) 2 markers different for preeclampsia pregnancies with acute atherosclerosis compared with preeclampsia without this maternal uteroplacental artery lesion.

We are unaware of previous analysis of pregnancy cohorts using this Olink CVD-linked multiplex biomarker analysis. Previous studies of nonpregnant cohorts have found Olink biomarker association with atherosclerosis in 70 year olds,<sup>71</sup> with dyslipidemia in the general population<sup>26</sup> and in relation to prediction of cardiovascular mortality in end-stage renal disease.<sup>72</sup> Our results support our previous findings of a preeclampsia cardiovascular-related biomarker phenotype differing from NC (eg, midregional pro-atrial natriuretic peptide [MR-proANP]<sup>73</sup> and midregional pro-adrenomedullin [MR-proADM]).<sup>22</sup> Other studies have identified that particularly women with EPE have altered cardiac structure and function, along with increased circulating levels of the well-established cardiovascular biomarker NT-proBNP.<sup>56,74,75</sup>

The dysregulated CVD biomarkers identified in the preeclamptic pregnancies likely reflect heterogeneous physiological and pathological processes linked to the pregnancy stress of preeclampsia, and particularly to EPE, which is also linked to the highest degree of placental cellular stress (eg, endoplasmic reticulum stress).<sup>76</sup> Whether these biomarkers also can be used for targeting women at highest risk for long-term CVD, and therefore for intervention trials and personalized medicine, is an exciting possibility to explore in longitudinal studies. During pregnancy, the circulating biomarkers are likely stemming from cardiovascular (myocardial and vascular) and other (eg, placenta) sources. Eight markers were uniquely altered in EPE compared to LPE, pointing toward specific processes relevant for this preeclampsia subtype possibly connected to the highest future maternal CVD risk, which has been shown both for CVD-related deaths, as well as for major cardiovascular events in population-based studies from Norway,<sup>6,77</sup> as well as in other countries.<sup>2</sup> Among the dysregulated single markers, (low) PIGF followed by (high) MMP3 were most strongly associated with EPE compared with LPE. The PIGF finding is in line with previous angiogenic biomarker findings over the last 15 years, where low PIGF is shown to more precisely diagnose and predict EPE as compared with LPE.<sup>29,78</sup> As we have argued previously, low PIGF in pregnancy is not a marker of preeclampsia per se but represents a marker of syncytiotrophoblast dysfunction and placental cellular stress.<sup>19</sup> During pregnancy, placenta is the main source for circulating PIGF. The Olink biomarker panel was designed to identify high PIGF outside pregnancy,

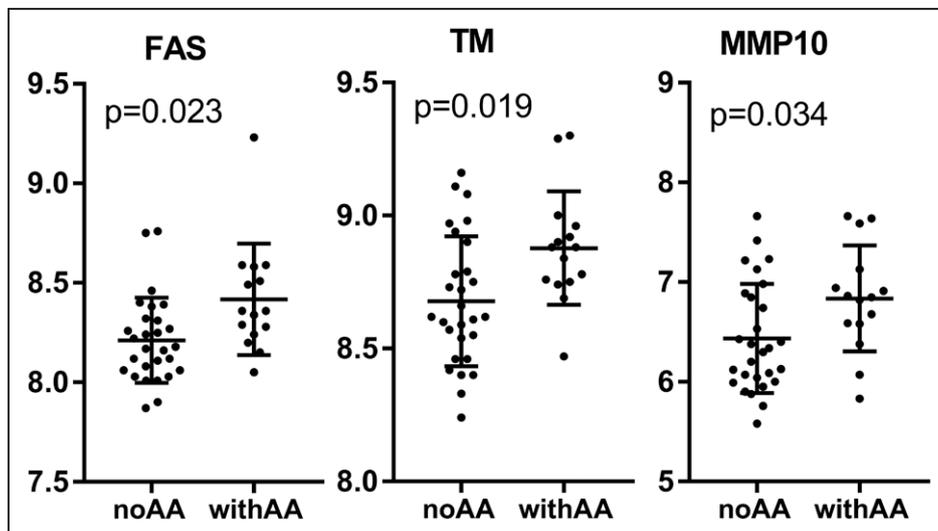


**Figure 4.** Receiver operating characteristic curves for predicting early-onset preeclampsia (EPE; delivery before gestational week 34) compared with late-onset preeclampsia (LPE; delivery from gestational week 34) by Olink multimarker (principal component [PC]1–PC4) and single markers (FS [follistatin], MMP1 [matrix metalloproteinase-1], hK11 [kallikrein-11], PIGF [placental growth factor], CTSD [cathepsin D], MMP3 [matrix metalloproteinase-3], ST2 [ST2 protein], CX3CL1, MPO [myeloperoxidase]), and combination (PIGF, ST2, MMP1, MMP3, and CX3CL1) approach.

identifying persons with increased vascular inflammation, as vascular endothelium is the main source for PIGF outside pregnancy.<sup>79</sup> In patients with non ST-segment-elevation acute coronary syndrome, elevated circulating PIGF predicts an independent increased risk for all-cause mortality or myocardial infarction.<sup>31</sup> We have suggested<sup>2</sup> that a (low) PIGF level in pregnancy is a very interesting biomarker to test for prediction of CVD end points, representing a link between placental dysfunctions and high risk of maternal CVD. This concept was recently confirmed by Benschop et al, demonstrating that low midpregnancy circulating PIGF is an independent risk marker of future cardiovascular structure and function 6 to 9 years after

pregnancy, independent of preeclampsia or hypertension,<sup>80</sup> confirming the potential for low PIGF for individualized follow-up and CVD prevention strategies. The other marker especially associated with EPE in our study was MMP3, which is a risk factor of vascular disorder and coronary heart disease,<sup>81</sup> and that has recently been reported as elevated in EPE.<sup>82</sup>

Another interesting finding was the low levels of FS in EPE, in line with previous reports of lower levels in preeclampsia and an increase with gestational age in uneventful pregnancies.<sup>28,83</sup> Through neutralization of the TGF-β (transforming growth factor-β) superfamily members, FS participates in various processes like cell growth, development, and



**Figure 5.** Levels of the circulating markers tumor necrosis factor receptor superfamily member 6 (FAS), thrombomodulin (TM), and matrix metalloproteinase 10 (MMP10) that were different between women with the presence of decidual acute atherosclerosis (with AA; n=28) and those without acute atherosclerosis (no AA; n=15) in the preeclampsia patients using logistic regression. Data are given as normalized protein expression (NPX) log<sub>2</sub>. P represent unadjusted models. Horizontal bars represent mean±SD.

differentiation,<sup>84</sup> and members of the FS family are suggested involved in CVD pathophysiology outside pregnancy.<sup>30</sup>

Biomarkers specifically dysregulated for EPE were ST2, CX3CL1, and MMP3 (all elevated), as well as MMP1 (lower). All these markers are previously described as dysregulated in preeclampsia and specific for the maternal-fetal interface and trophoblast dysfunction. Combining these markers and PIGF displayed the highest discriminatory properties of EPE versus LPE (Figure 4). Table S3 summarizes findings in preeclampsia and CVD pathophysiology.<sup>81,82,85–98</sup> The supplemental discussion text summarizes these biomarkers' potential role in the preeclampsia and CVD pathophysiology and their expression levels in different tissues summarized in Figure S4.

In line with the epidemiological evidence of further increase in maternal cardiovascular risk in EPE complicated with FGR, we identified higher MMP3 and lower CTSD in the EPE group with FGR as compared to without FGR. MMP3 and CTSD play important roles in physiological remodeling processes and trophoblast invasion.<sup>85</sup> Reduced MMP3 expression in the extravillous trophoblast at the fetal-maternal interface and higher CTSD expression in the placenta have been reported in preeclampsia.<sup>59,99</sup> These opposite EPE findings in the circulating versus placental tissue markers may reflect some of the pathological processes of abnormal placentation and compensatory mechanism in early preeclampsia.<sup>82</sup>

In the multimarker approach, we identified 4 PC clusters. PC3 was the cluster most discriminating EPE from LPE, consisting of biomarkers important for growth (CTSD, PIGF, FS), angiogenesis (TIE2, MMP3), cholesterol metabolism (LOX1 [lectin-like oxidized LDL receptor 1], LEP, FABP4, GAL [galanin peptides]), and inflammation (IL18 [interleukin-18]). PC1 did not distinguish between EPE and LPE, and these markers were mostly elevated in preeclampsia regardless of the onset-type and consisted of markers involved in TNF (tumor necrosis factor) signaling (eg, TRAILR2, TNFR1, CD40, FAS). The markers that predicted EPE best compared to LPE, comparing both single and multivariate analysis, was as expected (low) PIGF, followed by PC3 and MMP3. Reflecting the individual biomarker findings, PIGF and MMP3 were both part of PC3.

Markers of inflammation, endothelial activation, and lipids have been associated with the promotion of atherosclerosis lesion formation. We and others have shown higher presence of acute atherosclerosis in the uteroplacental arteries from preeclampsia women,<sup>24,100</sup> and here we identified higher levels of circulating FAS and TM in the presence of acute atherosclerosis in preeclampsia; however, these biomarkers did not differ in EPE compared to LPE. Interestingly, acute atherosclerosis predisposes to spiral artery thrombosis, which may lead to placental infarcts.<sup>17</sup> These results are in line with our previous results showing few associations between classical circulating CVD risk factors (eg, dyslipidemia) and presence of acute atherosclerosis.<sup>18</sup>

The major strength of this study is the clinically well characterized cohort with extensive pregnancy data and the wide range of markers included in the analysis enabling a multivariate approach. The numbers of pregnancies are, however, low relative to the number of markers. Further, the Olink biomarker levels are relative and not absolute values, but the excellent correlation identified between Olink and

immunochemiluminescence assay for PIGF protein levels and concentrations (Figure S1), across clinical diagnosis groups, are reassuring for the interpretation of the remaining Olink biomarker findings. Gestational age differed between the 3 study groups and might possibly have affected the levels of circulating markers. However, ST2, CX3CL1, MMP1, and MMP3 levels did not associate significantly with gestational age in the study groups. PIGF levels decrease significantly in the last trimester for all pregnancies, but earlier in the EPE than LPE and normal controls. Gestational age-matched blood samples would have identified a greater difference in maternal PIGF concentrations between the groups than in the current study. Storage time differs in our samples before analysis, but importantly, no difference between the study groups. However, different proteins may have different stability over time, which may have impacted our results.

To conclude, we found circulating ST2, CX3CL1, MMP1, and MMP3 levels significantly different between EPE and LPE. Few markers were uniquely changed in LPE that were not also changed in EPE, supporting that LPE does not represent biomarkerwise a specific type of preeclampsia but likely a less extreme variant of this heterogeneous syndrome. The uniquely EPE dysregulated markers may reflect differences in early placentation pathophysiology, as well as possibly associate with differences in long-term CVD risk. Further work will benefit from exploring timing of the biomarker alterations in pregnancy as well as identifying their relation to surrogate and hard cardiovascular end points.

## Perspectives

The findings of altered circulating levels of CVD risk-associated markers in preeclampsia at term are in line with epidemiological findings of the increased CVD risk after preeclampsia. Particularly, ST2, CX3CL1, MMP1, and MMP3 are interesting to pursue in longitudinal studies because these were altered in the EPE group only, which has the highest future CVD risk. Another longitudinal biomarker option to assess is NT-proBNP, which is readily available in the clinical routine today as a biomarker of CVD, and that was found elevated across both EPE and LPE groups, as well as in the group with the lowest PIGF. The identified biomarkers may alone or in clusters provide useful information for targeting women for intensified prevention of CVD, as well as provide ideas for novel intervention strategies.

## Acknowledgments

We are grateful for the laboratory assistance and patient recruitment by Lise Øhra Levy, as well as for patient recruitment by previous PhD students, including Kristin Brække, Nina K. Harsem, and Patji Alnaes-Katjavivi (all Oslo University Hospital), the latter also for acute atherosclerosis immunohistochemistry diagnosis. The contribution of previous postdoc Gro Leite Størvold to the study planning and data analysis is greatly appreciated. We are grateful for the Roche Norway in-kind donation of reagents for the PIGF protein biomarker analyses and to Nils Bolstad at the Oslo University Hospital, Norwegian Radium Hospital, for performing these angiogenic biomarker analyses. The Olink assay image in the graphical abstract is used with image courtesy of Olink Proteomics AB.

## Sources of Funding

The Research Council of Norway funded part of this work (the PATH study: Pregnancy acute atherosclerosis and future cardiovascular disease, grant number ref. 230652) as did South-Eastern Norway Regional Health Authority (the HAPPY PATH study, grant number ref. 2014026). Oslo University Hospital provided further research support.

## Disclosures

A.C. Staff has received recompensation from Roche (Norway) for advisory board service in 2018. R. Norway provided in-kind reagents for the PIGF biomarker analyses but had no impact on planning, performance, or other aspects of the study. The other authors report no conflicts.

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## Novelty and Significance

### What Is New?

- Multiplex plasma analysis of cardiovascular disease (CVD) risk-associated markers phenotyping of preeclampsia subtypes has not been published before.

### What Is Relevant?

- Several CVD risk-associated markers differed in pregnancy plasma between preeclampsia subtypes and normotensive controls and between groups defined by levels of placenta dysfunction markers.
- Four individual CVD risk-associated markers ST2 (ST2 protein), CX3CL1 (fractalkine), MMP1 (matrix metalloproteinase 1), and MMP3 (matrix metalloproteinase 3) were altered specifically for the early-onset pre-

eclampsia subtype, potentially linked to the high risk of future CVD for this group.

### Summary

Our data are consistent with a placental-cardiovascular axis in pregnancy, as tissue-based and circulating placental dysfunction markers associate with circulating CVD biomarker patterns.

The dysregulated cardiovascular biomarkers identified at delivery may contribute to improved targeting of women for intensified prevention of CVD, as well as provide ideas for novel intervention strategies