Quantification of Calcyclin and Heat Shock Protein 90 in Sera from Women with and without Preeclampsia by Mass Spectrometry

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Purpose: The objective of present study is to determine serum levels and placental distribution of two interacting proteins calcyclin and heat shock protein 90 in preeclampsia.

Experimental design: Maternal serum levels of calcyclin and heat shock protein 90 are compared throughout pregnancy from the first trimester till term among women with preeclampsia (n = 43) and age-matched normotensive pregnant controls (n = 46). A serum-based 2D LC-MS assay using Parallel Reaction Monitoring is applied to quantify both calcyclin and heat shock protein 90.

Results: Serum levels of calcyclin are significantly lower in patients with preeclampsia in the second trimester of pregnancy as compared to controls (p < 0.05). Serum levels of heat shock protein 90 are significantly higher in patients with preeclampsia in the third trimester as compared to controls (p < 0.001).

Conclusion and clinical relevance: Both interacting proteins calcyclin and heat shock protein 90 are notably changed in preeclamptic patients compared to controls. Calcyclin is already decreased before the onset of preeclampsia in the second trimester and HSP90 is strongly increased in the third trimester. This suggests that these proteins may play a role in the pathogenesis of preeclampsia and ought to be investigated in large cohort studies as molecular biomarkers.

1. Introduction

Preeclampsia (PE) is a pregnancy-specific multi-organ disorder that is diagnosed by new-onset hypertension and proteinuria after 20 weeks of gestation.[3] It affects 2–8% of all pregnancies and is one of the leading causes of maternal mortality worldwide.[1,2] Also, perinatal mortality is five times higher in women with PE.[3] The exact cause of PE remains unknown but most likely is the result of an abnormal placentation during the first trimester of pregnancy usually observed in early-onset PE. During normal pregnancies, cytotrophoblast cells promote arteriolar dilation by invading the uterine spiral arteries. In PE, cytotrophoblast cells do not invade the spiral arteries adequately, resulting in reduced placental blood flow leading to excessive placental oxidative stress.[3] Signs of pending PE may be expected to be notable early in pregnancy before the onset of the clinical disease. Studying serum markers may lead to an understanding of the pathogenesis of PE at a protein level. The latter may contribute to better screening, monitoring, and possible prevention of this disorder.

Previously, we reported significantly discriminating peptide patterns between trophoblast and stroma cells by laser capture microdissection (LCM).[4] We also demonstrated by immunohistochemistry that S100A6 was significantly more abundant in placentas of preeclamptic women as compared to controls.[5] By multiple reaction monitoring (also known as SRM) we showed significantly elevated levels of S100A6 in formalin-fixed paraffin-embedded (FFPE) preeclamptic placentas compared to controls by LCM.[6] Concentrations of serum S100A6 in healthy nonpregnant persons were known to be low (≈2–8 ng mL−1).[7] S100 proteins, including S100A6, interact with the tetratricopeptide repeat (TPR) domains of the HSP70 and HSP90 (HSP70/HSP90)-organizing protein (Hop) in a Ca2+-dependent manner. After interacting with S100A6, HSP70, and HSP90 dissociates from Hop-HSP70 and Hop-HSP90 complex and blocks binding to other partner proteins.[8]
Based on our previous research on placental tissue and on the hypothesis, that $S100A6$ activates HSP90 via the HOP protein as a result of oxidative stress, it is suggested that HSP90 might also play a role in the pathophysiology of PE. For this purpose, a cross-sectional study was performed with hard to acquire set of maternal serum samples transversally collected during the first, second, and third trimesters of pregnancy. We investigated whether these two proteins behave differently in patients with PE as compared to pregnant normotensive controls. A serum-based 2D LC/MS PRM assay\(^9\) was used to quantify both proteins. In addition, we developed a double immunofluorescence staining with antibodies against $S100A6$ and HSP90 to show that both proteins $S100A6$ and HSP90 are present in trophoblast cells.

2. Experimental Section

2.1. Study Design

A case-control study was conducted at the Department of Obstetrics and Gynecology of the Erasmus MC, Rotterdam, the Netherlands. Serum samples from two different studies were used: a nested case-control study embedded in the Rotterdam Periconceptional Cohort (Predict study),\(^10\) a prospective tertiary hospital-based study, and the Lepra Study,\(^11\) a retrospective tertiary hospital-based case-control study focused on brain involvement during PE. Both studies were approved by the local Medical Ethical and Institutional Review Board of Erasmus MC (MEC-2004-227 and MEC 2007–086, respectively). At admission, participants gave written informed consent for participation. Serum samples were available of 43 patients with PE, consisting of 20 early-onset and 23 late-onset PE, and 46 normotensive controls. Sixty-three percent of the samples were collected in the first trimester (<14 weeks), 17 percent in the second trimester (14–27 weeks) and 20 percent in the third trimester (>28 weeks) of pregnancy. These percentages did not differ between cases and controls. Cases and controls were matched for maternal age, geographic origin, parity and gestational age at sampling, PE was defined as systolic blood pressure $\geq 140$ mmHg or diastolic blood pressure $\geq 90$ mmHg on at least two occasions 4 h apart after 20 weeks of gestation with proteinuria (protein/creatinine ratio of $\geq 30$ mg mmol$^{-1}$) following the ISSHP guidelines. Clinical data were obtained from questionnaires and medical records. An independent set consisting of ten placental tissues that were provided by the department of Pathology, Erasmus MC, were used for immunofluorescence studies. Five were obtained from women who experienced early-onset PE and five from women with spontaneous preterm delivery without hypertension.

2.2. Sample Preparation by SCX Chromatography

Seven microliters of each serum sample was diluted 47 times in 0.01% RapiGest (Waters, Milford, MA) dissolved in 50 mm ammonium bicarbonate, reduced using 15 mm DTT followed by alkylation using 15 mm iodoacetamide (IA) and subsequently enzymatically digested by adding 30 $\mu$L trypsin (100 $\mu$g mL$^{-1}$ dissolved in 3 mm Tris-HCl pH 8.8) (Gold, Mass Spectrometry Grade, Promega, Madison, WI) at 37 °C overnight. The enzymatic reaction was stopped by adding 50% of formic acid (FA) to reach a final concentration of 0.5–1.0% FA. All digested sera were spiked with 40 fmol of both $S100A6$ and HSP90 stable isotope-labeled (SIL) peptides (Thermo Fisher Scientific, Bremen, Germany; purity of $>97\%$ as stated by the manufacturer (Ultimate-grade)), followed by desalting by Solid Phase Extraction (Discovery DSC-18 SPE 96-well Plate, Sigma-Aldrich, the Netherlands). Subsequently digested samples were fractionated by SCX chromatography to measure relatively low levels (ng mL$^{-1}$) of $S100A6$ and HSP90 in sera from PE patients. All samples were off-line fractionated with a Luna 5 $\mu$m, 150 $\times$ 2 mm SCX column (Phenomenex, Torrance, CA) that was connected to a nano-LC system (Thermo Fisher Scientific, Gernerning, Germany) using the following conditions: buffer A (14 mm KH$_2$PO$_4$, 24 mm H$_3$PO$_4$, pH 2.5, adjusted with 37% w/w HCl) in 25% v/v acetonitrile (HPLC grade; Biosolve, Valkenswaard, the Netherlands) in Milli-Q water; buffer B (buffer A containing 350 mm KCl); linear gradient from 100% buffer A to 40% buffer B in 40 min, followed by a wash with 100% buffer B until 45 min at a flow rate of 200 $\mu$L min$^{-1}$ and equilibration of the column in buffer A for 17 min. All chemicals used for SCX fractionation were purchased from Sigma-Aldrich (St Louis, MO). As shown previously,\(^9\) 50 $\mu$L fractions (180 fractions in total for each serum sample) were automatically collected in 384-well plates (VWR, Amsterdam, the Netherlands) and sealed with an adhesive aluminum foil (VWR, Amsterdam, the Netherlands). Fractions were dried down in SpeedVac concentrator (RVT4104, Scientific Savant, San Jose, CA) and subsequently stored at $-20$ °C until further analysis. Only fractions containing the two peptides (on average eight) were reconstituted in 0.1% FA prior PRM measurements.

Clinical Relevance

It is previously shown that calcyclin ($S100A6$) is significantly elevated in placental tissue of patients with preeclampsia. The presence of $S100A6$ in relation to an interacting heat shock protein 90 (HSP90) binding was studied in serum of preeclamptic patients. Maternal serum samples were collected in the first, second, and third trimesters of pregnancy in 43 patients with preeclampsia and 46 normotensive pregnant controls. A serum-based 2D LC-MS assay on Parallel Reaction Monitoring mode using a mass spectrometer was used to quantify both calcyclin and heat shock protein 90. Localization of both proteins in placental tissue was determined by immunofluorescence. Both proteins were notably changed in serum of preeclamptic patients as compared to controls. $S100A6$ was already significantly decreased before the onset of preeclampsia in the second trimester of pregnancy and HSP90 was strongly (significant) increased in the third trimester. This suggests that these two proteins may play a role in the pathogenesis of preeclampsia and could have clinical and diagnostic implications in the treatment of this disease. There is a medical need to find molecules that can predict preeclampsia in an early stage. Until now only molecules have been described that are expressed if the disease is already clinical manifest.
2.3. PRM in Serum and Placental Tissue

A PRM assay was developed for quantitative measurements of S100A6 and HSP90 levels in serum using SIL peptides serving as internal standards, that is, LQDAEIAR (13C615N4) for S100A6 and YIDQEEELNK (13C615N4) related to both isomers HSP90α and HSP90β. HSP90β was not analyzed, because it is known from literature that very low levels (∼1–2 ng mL⁻¹) of HSP90β were usually measured in serum.[32]

For S100A6, a relatively low molecular weight protein (∼10 kDa) only the single signature peptide LQDAEIAR was used because other tryptic S100A6 peptides contain amino acids that are prone to oxidation (methionine, cysteine), acetylation, phosphorylation or are too long, that is, more than 20 amino-acids. The single signature peptide YIDQEEELNK was used for HSP90 measurements, because almost identical results were obtained for another HSP90 peptide DQ-VANSAPFEV as explained in our previous paper.[33] In this study, an agreement between ELISA and PRM results for HSP90 was shown.

PRM signals for S100A6 were recorded for doubly-charged endogenous and SIL peptide LQDAEIAR precursor ions with m/z of 458.25 and m/z 463.25, respectively. For HSP90, m/z 576.28 and m/z 580.29 were taken for YIDQEEELNK, respectively. PRM measurements were carried out on a nano-LC system (Thermo Fisher Scientific, Germering, Germany) online coupled to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA, US). Three microliters of each SCX-fractionated sample were loaded on to a trap column (PepMap C18, 300 μm ID × 5 mm length, 5 μm particle size, 100 Å pore size; Thermo Fisher Scientific) and desalted for 5 min in using 0.1% TFA in water as loading solvent. The trap column was then switched in-line with the analytical column (PepMap C18, 75 μm ID × 250 mm, 2 μm particle and 100 Å pore size, Thermo Fisher Scientific). Peptides were eluted in a binary gradient from 12% to 25% solvent B for 14.7 min, where solvent A consisted of 0.1% FA in water, and solvent B consisted of 80% acetonitrile and 0.08% FA in water. The column flow rate was set to 250 nL min⁻¹ and oven temperature to 40 °C. All LC solvents were UHPLC grade and purchased at Biosolve, Valkenswaard, the Netherlands. For electrospray ionization, nano ESI emitters (New Objective, Woburn, MA) were used and a spray voltage of 1.8 kV was applied. For PRM of the doubly-charged precursor ions of LQDAEIAR and YIDQEEELNK (endogenous and SIL), the targeted MS/MS mode set up as follows was used: isolation width 1.4 Da, HCD fragmentation at a normalized collision energy of 25%, ion injection time was set to 512 ms (by setting the AGC target to 500 000 ions), Orbitrap resolution of 240 000. Selection of the precursor ions was time scheduled and each duty cycle consisted of two targeted MS/MS scans (endogenous and SIL form of a peptide) yielding a scan rate of approximately 0.83 Hz. Fluoranthene (202.0777 Da) was infused as lock mass (Easy IC option active).

The PRM data have been deposited to the ProteomeXchange Consortium via the PRIDE[13] partner repository with the dataset identifier PXD009025.

Placental abundances of S100A6 and HSP90; whole tissue lysates collected from placentas of six preeclamptic patients, five age-matched preterm controls and four term controls were determined. Collected tissue pieces were collected in 200 μL 0.1% Rapigest SF detergent, sonicated for 3 min using a horn sonifier bath (Ultrasonic Disruptor Sonifier II, Bransons Ultrasonics, Danbury, CT, USA) at 85% amplitude and heated for 5 min at 99 °C for protein denaturation. Fifty out of the 200 μL tissue lysates were each spiked with 1 fmol of S100A6 and HSP90 SIL peptides prior to enzymatic digestion. Samples were digested by adding 2 μg trypsin at 37 °C overnight. PRM measurements were carried out on an Orbitrap Lumos (Thermo Fisher Scientific, Germering, Germany) instrument as described above with an adjustment of the gradient condition (from 4% to 38% solvent B for 30 min) to quantify protein levels of HSP90, and S100A6. Measured data were normalized based on UV data obtained during online LC-MS measurements of the tissue taken for each sample.

2.4. Data Analysis

The PRM signals were integrated and analyzed using Skyline.[14] The linearity, LOD and LOQ of the assay were determined for S100A6 and HSP90 peptides according to our previous work[34] by spiking five different concentrations in triplicate into an SCX-separated serum digest. For each protein, the linear regression data including reproducibility of the serial dilutions of SIL peptide standards (expressed as %CV), LOD and LOQ are represented in Supporting Information, Excel data 1.

The SCX-fractionated serum digests were measured in a single run whereas ratios between endogenous and SIL peptides of S100A6 and HSP90 were calculated to determine the concentrations. Only fractions containing the highest S100A6 or HSP90 concentration were considered for further analysis. Statistical differences between serum levels of S100A6 and HSP90 in the total PE group, in the subgroups of early- and late-onset, and control group during the first, second, and third trimesters were calculated with an unpaired t-test. A probability below 0.05 was considered to be significant. Serum levels were tested for normality using Kolmogorov–Smirnoff and Shapiro–Wilk tests. Natural log (ln)-transformed values were used for statistical testing if data were not normally distributed. To evaluate the repeatability and reproducibility of the PRM assay, for both peptides three technical (three PRM measurements of an identical sample (quality control) throughout the assay run) and three methodological (three independently prepared replicates of an SCX-fractionated serum sample) replicates were measured and CVs as percentages calculated, respectively.

For statistical analysis of clinical characteristics independent students’ t-tests (normal distributed data) or Mann–Whitney U tests (non-normal distributed data) were used for continuous variables and Chi-square tests or Fisher’s exact tests were used for categorical variables.

Unsupervised hierarchical cluster analysis was performed to illustrate whether the proteins S100A6 and HSP90 were correlated with various metadata (Supporting Information, Excel data 2) of the investigated subjects. The following parameters were set using PermutMatrix 1.9.3. (http://www.atgc-montpellier.fr/permutexmatrix): Pearson distance for dissimilarity, Ward’s method, as a cluster method and Bipolarization seriation.[15,16]
2.5. Double Immunofluorescence Microscopy

FFPE placental material from an independent set consisting of five preeclamptic women and five age-matched preterm delivered controls (26–32 weeks; used for routine procedures according to our department of Pathology and “Code of Conduct for Responsible use” by the FEDERA, http://www.federa.org) were used for immunofluorescence with antibodies against S100A6 and HSP90. Tissues were cut in 4 μm sections and routinely processed. Immunofluorescence assays were performed on a VENTANA BenchMark Discovery automated staining instrument (Ventana Medical Systems), using VENTANA reagents except as noted, according to the manufacturer’s instructions. Slides were de-paraffinized using EZ Prep solution (cat # 950–102) for 16 min at 72 °C. Epitope retrieval was accomplished with CC1 solution (cat # 950–224) at high temperature 97 °C for a period of time 36 min. Mouse-anti-S100A6 (clone CACY-100, Sigma-Aldrich) was manually applied for 1 h and 4 min at 37 °C followed by second antibody UMAP anti-mouse conjugated with horseradish peroxidase for 12 min followed by 0.01% H2O2 and Red 610-tyramide detection (cat # 760-245). Next, antibody denature step was done at 97 °C for 12 min with CC2 solution (cat # 950-223). Subsequently mouse-anti-HSP90 (clone D7a, Abcam) was manually applied for 1 h and 4 min at 37 °C followed by second antibody UMAP anti-mouse with horseradish peroxidase for 12 min followed by the application of 0.01% H2O2 and FAM-tyramide detection (cat # 760-243). The slides were counterstained with DAPI Vectashield (Vector Laboratories, Burlingame, CA) and imaged using the LSM 700 (Carl Zeiss, Germany) laser scanning confocal microscope.

3. Results

3.1. Clinical Characteristics of Study Groups

Clinical characteristics (Table 1) did not statistically differ between the study groups, except for “chronic hypertension” and “PE in a previous pregnancy,” which was present extremely more often in the cases. Women with PE had higher blood pressure and lower birth weight of the neonate. Women with PE reported more often complaints of headache, visual disturbances, upper abdominal pain, nausea, and general discomfort.

3.2. Serum S100A6 and HSP90 Levels

Table 2 shows the results of comparisons between patients with PE and controls at different gestational ages for S100A6 and HSP90 measured by PRM. Serum levels were not normally distributed and for this reason the In-transformed values were used for statistical testing. A complete overview of S100A6 and HSP90 levels measured in PE and control is illustrated in Figure 1 by plotting each protein level against the trimester group. Serum levels of S100A6 were overall lower in patients with PE compared to controls (Table 2, 59.8 ng mL$^{-1}$ vs 0.6 ng mL$^{-1}$, $p < 0.05$) analyzed for all three trimesters together. When analyzing the trimesters separately, it was observed that during the second trimester S100A6 was significantly lower in patients with PE (Table 2, 41.7 ng mL$^{-1}$ versus 75.6 ng mL$^{-1}$, $p < 0.05$). In the other two trimesters, an increasing not significant trend was observed.

Serum levels of HSP90 were overall significantly higher in patients with PE compared to controls (Table 2, 103.3 ng mL$^{-1}$ vs 93.7 ng mL$^{-1}$, $p < 0.05$). When analyzing the trimesters separately HSP90 was significantly higher in serum of cases only in the third trimester (Table 2, 194.2 ng mL$^{-1}$ vs 62.6 ng mL$^{-1}$, $p < 0.001$).

The LOD and LOQ for S100A6 that was based on peptide LQDAEIAR was 0.93 ng mL$^{-1}$ and 3.6% respectively. The LOD and LOQ for HSP90 was 2.8 and 8.6 ng mL$^{-1}$ respectively. The CV for repeatability and reproducibility calculated for the S100A6 peptide LQDAEIAR was 3.6% and 20.0%, respectively. In the case of HSP90, the CV was 2.1% and 6.8% for YIDQEELNK, respectively.

Sensitivity analysis of the S100A6 and HSP90 serum levels individually analyzed per trimester and for all three trimesters together did not reveal any significant difference between early- and late-onset PE. Furthermore, we investigated by supervised hierarchical clustering that various parameters were correlated with S100A6 and HSP90. The patient and control group were separated completely (Figure S1, Supporting Information).

3.3. The Abundances of S100A6 and HSP90 in Placental Tissue Measured by PRM

Results showed that S100A6 (Figure S2A, Supporting Information) was significantly different between preeclamptics and term controls ($p = 0.0381$). Comparing the preeclampsia group with the preterm control group was not significant ($p = 0.7619$). For HSP90 (Figure S2B, Supporting Information), we did not find significant differences between preeclampsia and term controls ($p = 0.9307$). Comparing the preeclampsia group with the preterm control group was also not significant ($p = 0.0519$) (borderline).

3.4. Colocalization of S100A6 and HSP90 in Placentas by Immunofluorescence Staining

Using immunofluorescence staining, it was observed that S100A6 and HSP90 were partially colocalized in cytoplasm of trophoblast cells from placental tissue of preeclamptic patients ($n = 5$) and preterm matched controls ($n = 5$). Figure 2 is an example of immunofluorescence trophoblast staining for S100A6 and HSP90 in placenta of a PE case and age-matched preterm control. The immunofluorescence results showed that often overlap was observed between S100A6 and HSP90 in the trophoblast cells. At the stromal part of the chorion villi, no staining or at least no overlap was observed.
Table 1. Clinical characteristics.

| General characteristics a | PE (n = 43) | Normotensive control (n = 46) | p-value g |
|---------------------------|-------------|------------------------------|-----------|
| Maternal age, years       | 33 (5)      | 33 (5)                       | 0.820     |
| Geographical origin       |             |                              | 0.800     |
| Western                   | 29 (67.4%)  | 28 (62.2%)                   |           |
| Non-Western               | 14 (32.6%)  | 17 (37.8%)                   |           |
| Study cohort              |             |                              |           |
| Predict study             | 30 (70%)    | 22 (48%)                     |           |
| Lepra study               | 13 (30%)    | 24 (52%)                     |           |
| Index pregnancy           |             |                              |           |
| Nulliparous               | 23 (53.5%)  | 24 (52.2%)                   | 0.901     |
| Preconcept BMI, kg m⁻²    | 27 (5)      | 25 (6)                       | 0.237     |
| Smoking (during pregnancy)| 3 (7.5%)    | 0 (0%)                       | 0.215     |
| Highest systolic blood pressure, mmHg | 155 (17) | 126 (10) | <0.001 |
| Highest diastolic blood pressure, mmHg | 100 (9) | 77 (6) | <0.001 |
| Proteinuria (gram per 24 h) | 0.54     | NA                           |           |
| Protein/creatinine-ratio  | 63          | NA                           |           |
| Gestational diabetes      | 1 (2.3%)    | 2 (4.3%)                     | 1.000     |
| Twin pregnancy            | 3 (7%)      | 3 (6.5%)                     | 1.000     |
| Serum sampling            |             |                              |           |
| Gestational age in weeks  | 12 (6–31)   | 10 (6–31)                    | 0.608     |
| Gestational age in days   | 87 (46–117) | 75 (45–117)                  | 0.464     |
| Trimester comparison      |             |                              | 0.982     |
| Number of samples         |             |                              |           |
| First trimester           | 27 (62.8%)  | 29 (63.0%)                   |           |
| Second trimester          | 7 (16.3%)   | 8 (17.4%)                    |           |
| Third trimester           | 9 (20.9%)   | 9 (19.6%)                    |           |
| Medical history           |             |                              |           |
| Chronic hypertension      | 13 (30.2%)  | 0 (0%)                       | <0.001    |
| Insulin Dependent Diabetes Mellitus | 1 (2.3%) | 1 (2.2%) | 1.000 |
| Obstetric history         |             |                              |           |
| Recurrent miscarriages    | 0 (0.0%)    | 5 (10.9)                     | 0.056     |
| PE in previous pregnancy  | 13 (30.2%)  | 2 (4.3%)                     | <0.05     |
| Previous Caesarean delivery | 8 (18.6%) | 10 (21.7%)                  | 0.713     |
| Phenotypes preeclampsia   |             |                              |           |
| HELLP                     | 13 (30.2%)  | NA                           | NA        |
| Early-onset PE b          | 20 (8e, 4f, 8f) (47.6%) | NA | NA |
| Late-onset PE b           | 23 (19e, 3e, 1f) (52.4%) | NA | NA |
| Severe PE b               | 21 (50%)    | NA                           | NA        |
| Clinical symptoms         |             |                              |           |
| Headache                  | 21 (51.2%)  | 2 (4.3%)                     | <0.001    |
| Visual complaints          | 10 (24.4%)  | 2 (4.3%)                     | <0.05     |
| Upper abdominal pain       | 15 (36.6%)  | 1 (2.2%)                     | <0.001    |
| Nausea                    | 11 (26.8%)  | 2 (4.3%)                     | <0.05     |
| General discomfort         | 6 (14.6%)   | 0 (0%)                       | <0.05     |
| Dyspnea                   | 3 (7.3%)    | 1 (2.2%)                     | 0.339     |
| Neonatal characteristics c |             |                              |           |
| Birth weight, gram        | 2335 (1410–2845) | 3310 (2940–3675) | <0.001 |
| Birth weight < 10th percentile | 9 (20%)    | 2 (4.2%)                     | <0.05     |
| Male gender               | 25 (54.3%)  | 20 (41.7%)                   | 0.219     |

a) Data are presented as n (%), mean with standard deviation (SD) or median with range. b) These definitions are according to the ISSHP guidelines, see reference. c) Due to five twin pregnancies with living children (n = 94). d) number of samples in the first trimester, e) second trimester, f) third trimester. g) For comparisons between groups, independent students’ t-tests, Mann–Whitney U tests, Chi-square tests and Fisher's exact tests were used. NA = not applicable.
### Table 2. S100A6 and HSP90 serum levels measured by PRM of women with PE and pregnant normotensive controls from the first (1st), second (2nd), and third (3rd) trimesters of pregnancy. Statistical comparisons were made by using an unpaired t-test. Serum levels were not normally distributed and therefore an ln-transformation was used to normalize the data. A probability value below 0.05 was considered to be significant.

| S100A6 trimester | PE (n = 43) concentration in ng mL\(^{-1}\) (median; IQR) | CO (n = 46) concentration in ng mL\(^{-1}\) (median; IQR) | p-value |
|------------------|----------------------------------------------------------|----------------------------------------------------------|---------|
| 1st (n = 56)     | 59.3 (34.0–68.6)                                         | 63.2 (47.3–92.0)                                         | NS      |
| 2nd (n = 15)     | 41.7 (36.6–47.2)                                         | 75.6 (54.5–143.4)                                        | <0.05   |
| 3rd (n = 18)     | 113.2 (68.7–191.2)                                       | 171.8 (63.4–781.7)                                       | NS      |
| all (n = 89)     | 59.8 (36.8–78.0)                                         | 80.6 (49.7–102.2)                                        | <0.05   |

| HSP90 trimester  | PE (n = 43) concentration in ng mL\(^{-1}\) (median; IQR) | CO (n = 46) concentration in ng mL\(^{-1}\) (median; IQR) | p-value |
|------------------|----------------------------------------------------------|----------------------------------------------------------|---------|
| 1st (n = 56)     | 100.1 (80.8–118.9)                                       | 96.7 (76.9–140.7)                                        | NS      |
| 2nd (n = 15)     | 91.9 (66.0–101.3)                                        | 105.4 (63.9–132.5)                                       | NS      |
| 3rd (n = 18)     | 194.2 (116.8–412.4)                                      | 62.6 (53.7–79.0)                                         | <0.001  |
| all (n = 89)     | 103.3 (83.0–157.6)                                       | 93.7 (65.0–125.8)                                        | <0.05   |

PE = preeclampsia; CO = pregnant normotensive control; IQR = interquartile range; NS = not significant.

### Figure 1. Serum levels of A) S100A6 and B) HSP90 were measured (for individual concentrations per trimester, see Table 2) for each PE (red) and control (blue) group obtained from the first, second, and third trimesters. In the second trimester S100A6 was significantly lower in serum of patients with PE compared to controls. Serum levels of HSP90 were significantly elevated in the third trimester.

### 4. Discussion

Serum S100A6 and HSP90 levels of matched cases and controls could be measured in a sensitive and high-quality manner. PRM results of S100A6, based on endogenous peptide LQDAEIAR, showed a significantly lower serum concentration in the second trimester of pregnancy in women with PE compared to normotensive controls. PRM results of HSP90, based on endogenous peptide YIDQEELNK, showed a significantly higher serum concentration in third trimester of pregnant women with PE compared to normotensive controls, but not in the first and second trimesters.

The finding that a significant decrease was observed of S100A6 levels in the second trimester of PE patients was remarkable and suggests that S100A6 is involved in the pathophysiology of PE as it was already observed previously in placental tissue.\(^5\) In placental tissue, we observed that S100A6 was higher abundant in patients with PE. However, the expression in serum of this protein may be more complex and different from the expression in placental tissue. So, the idea that elevated levels in tissue would also automatically result in higher levels in serum could be not true because S100A6 might have a different clearance rate from the blood or have a different degradation process in serum or in the originating tissue. The precise biological or cellular function of S100A6 remains unclear.\(^2\) It is known that S100A6 plays a role in cellular stress response; there is an upregulation of S100A6 in cells which are exposed to oxidative stress. Placental oxidative stress is known to precede the development of PE.\(^1\) Placenta from preeclamptic women have reduced antioxidant capacity showing lower levels of antioxidants in blood.\(^1\) S100A6 is a member of the S100 subfamily which consists of two EF-hand calcium binding motifs that act as a signal-transducer in intracellular processes.\(^1\) Intracellularly, these proteins function as Ca\(^{2+}\)-signaling and Ca\(^{2+}\)-buffering proteins. Calcium induces a conformational change of the protein structure. By this change an interaction site of S100 proteins with their target proteins is exposed.\(^3\) Extracellularly, the S100 proteins (including S100A6) are known to be interacting with the Receptor for Advanced Glycation End products, known as RAGE-receptors.\(^4\) These proteins have a cytokine-like function and could for example act...
as chemotactic molecules during inflammation. Recent evidence suggests RAGE to play a separate role in inflammatory and vascular autoimmune diseases. RAGE proteins are expressed in several cells in vasculature tissue such as endothelial cells, infiltrating inflammatory cells, cardiomyocytes, and fibroblasts.\cite{22} Placental cells from PE patients were found to have increased levels of RAGE proteins\cite{23} suggesting an involvement of S100A6 protein in the pathophysiology of PE.

Because of the limited power of the study it was not possible to establish significant differences between early- and late-onset PE per trimester. Most likely HSP90 increases also as a consequence of secondary mechanisms (e.g., inflammation) and not necessarily as a cause or as an early event that leads to the clinical onset of PE. S100A6 and HSP90 are both expressed in trophoblast cells as confirmed by double immunofluorescence staining (Figure 2). S100A6 is partially located in nuclei and cytoplasm while HSP90 was only found in cytoplasm of trophoblast cells although these proteins have also been found outside cells.\cite{24} A similar expression is described in “The Human Protein Atlas” (http://www.proteinatlas.org). S100A6 interacts with heat shock proteins such as HSP70/HSP90 complexes and may promote endothelial cell-cycle progression by this interaction. HSP90 is the most abundant heat shock protein in eukaryotic cells. It consists of two isoforms with largely similar

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**Figure 2.** Example of double immunofluorescence staining. It shows trophoblast staining for both proteins S100A6 and HSP90 in a) preeclamptic and B) age-matched preterm delivered control (placental tissue at 40 × magnification. For S100A6 (red) both cytoplasm and nucleus (partially) of trophoblast cells were positive, while for HSP90 (green) only cytoplasm was stained of trophoblast cells. Colocalization of both proteins is indicated with a merged color. Red blood cells showed autofluorescence for S100A6. For nuclear counterstain the slides were stained with DAPI.
functions known as HSP90α and HSP90β. In this study, we made no distinction between these two protein isoforms. HSP90 is a chaperone protein that plays a role in the folding, stabilization, and activation of denatured and synthesized proteins which are involved in various cellular processes. HSP90 plays an important role in stress responses and maintaining cellular homeostasis. HSP90 proteins interact with other heat shock proteins regulated by S100A6.[25] Recent research showed that S100A6-binding protein and Siah-1 interacting protein (CacyBP/SIP) interact directly with HSP90.[26] There are a few studies about HSP90 levels in PE. Although not comparable with this study, Hromoadnikova et al. found decreased HSP90 mRNA in whole peripheral blood of mothers with PE. On the other hand, they found a significant upregulation of HSP90 in placental tissue in patients with mild PE while there was no difference in severe PE.[27]

The immunofluorescence results of S100A6 and HSP90 (Figure 2) showed partially colocalization in trophoblast cells. The numbers of placenta tissue analyzed in this study (n = 5 in each group) were too small to report information whether HSP90 was upregulated in PE. In case of S100A6, we reported previously that S100A6 was elevated in a larger cohort (n = 138).[25]

Heme oxygenase plays a role in the protective mechanism against oxidative and nitrosative stress. Ekambaram et al.[28] showed increased levels of HSP90 in umbilical cord blood RBC (red blood cells) and decreased heme oxygenase-2 in PE. HSP90 is thought to play a role in this mechanism. Higher hemoglobin concentrations have been associated with inadequate plasma volume expansion, which may be part of the preeclamptic syndrome.[29] An alternative explanation for the higher HSP90 level in PE in the third trimester of pregnancy might be that patients with PE frequently show hemolysis. In order to investigate this, we tested the correlation of HSP90 levels in the third trimester of pregnancy with haptoglobin and lactate dehydrogenase concentrations, which were available in the PE group. We did not find correlation between HSP90 and these parameters of hemolysis (data not shown). However, this might have been due to the small size of this group (n = 9). By unsupervised hierarchical clustering of the metadata the preeclamptic and control group were completely separated. S100A6 and HSP90 contributed to the hierarchical clustering.

Serum levels of S100A6 and HSP90 were measured to our knowledge for the first time in a relatively large group of women with PE and normotensive controls although for an significant statistical analysis still low in number. Moreover, measuring these samples cross-sectionally during all trimesters of pregnancy is unique. Although EU activities in that direction to get access to unique. Although EU activities in that direction to get access to FFPE, HSP90, immunofluorescence, placenta, preeclampsia, PRM, S100A6, serum

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest
The authors declare no conflict of interest

Keywords
FPPE, HSP90, immunofluorescence, placenta, preeclampsia, PRM, S100A6, serum

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