Quantification of calcyclin and heat shock protein 90 in sera from women with and without pre-eclampsia by mass spectrometry

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ABSTRACT

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 pre-eclampsia.

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 pre-eclampsia (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 pre-eclampsia 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 pre-eclamptic patients compared to controls. Calcyclin is already decreased before the onset of pre-eclampsia 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 pre-eclampsia and ought to be investigated in large cohort studies as molecular biomarkers.

INTRODUCTION

Pre-eclampsia (PE) is a pregnancy-specific multi-organ disorder that is diagnosed by new-onset hypertension and proteinuria after 20 weeks of gestation (1). It affects 2-8% of all pregnancies and is one of the leading causes of maternal mortality worldwide (1, 87). Also, perinatal mortality is five times higher in women with PE (87). 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 (88, 89). 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) (90, 91). We also demonstrated by immunohistochemistry that *S100A6* was significantly more abundant in placentas of pre-eclamptic women as compared to controls (92). By Multiple Reaction Monitoring (also known as Selected Reaction Monitoring) we showed significantly elevated levels of *S100A6* in formalin-fixed paraffin-embedded (FFPE) pre-eclamptic placentas compared to controls by LCM (93). Concentrations of serum *S100A6* in healthy non-pregnant persons were known to be low (~2-8 ng/mL) (94, 95). S100 proteins, including *S100A6*, interact with the tetratricopeptide repeat (TPR) domains of the heat shock protein 70 (*HSP70*) and *HSP90* (*HSP70*/*HSP90*)-organizing protein (*Hop*) in a Ca^{2+}-dependent manner. After interacting with *S100A6*, *HSP70* and *HSP90* dissociates from *Hop-HSP70* and *Hop-HSP90* complex and blocks binding to other partner proteins (96, 97).

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, we performed a cross-sectional study with serum sampling during the first, second and third trimester of pregnancy to investigate if these two proteins behave differently in patients with PE as compared to pregnant normotensive controls. A serum based 2D LC-MS Parallel Reaction Monitoring (PRM) assay (98) was used to quantify both proteins. Also, 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.

EXPERIMENTAL SECTION

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) (99), a prospective tertiary hospital-based study, and the Lepra Study (100), 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). 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 ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg on at least two occasions four hours apart after 20 weeks of gestation with proteinuria (protein/creatinine ratio of ≥ 30 mg/mmol) 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.

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 dithiothreitol (DTT) followed by alkylation using 15 mM iodoacetamide (IA) and subsequently enzymatically digested by adding 30 µL trypsin (100 µg/mL 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 strong cation exchange (SCX) chromatography to measure relatively low levels (ng/mL) of *S100A6* and *HSP90* in sera from PE patients. All samples were off-line fractionated with a Luna 5 µm, 150 × 2 mm SCX

column (Phenomenex, Torrance, CA) that was connected to a nano LC sytem (Thermo Fisher Scientific, Germering, Germany) using the following conditions: buffer A (14 mM KH_2PO_4, 24 mM H_3PO_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 minutes, followed by a wash with 100% buffer B until 45 minutes at a flow rate of 200 µL/min and equilibration of the column in buffer A for 17 minutes. All chemicals used for SCX fractionation were purchased from Sigma-Aldrich (St Louis, MO). As shown previously, 50 μl 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 aluminium 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.

PRM

A PRM assay was developed for quantitative measurements of *S100A6* and *HSP90* levels in serum using SIL peptides serving as internal standards, i.e. LQDAEIAR ($^{13}C_6{}^{15}N_4$) for *S100A6* and YIDQEELNK ($^{13}C_6{}^{15}N_2$) related to both isoforms *HSP90a* and *HSP90b. HSP90b* was not analyzed, because it is known from literature that very low levels (~1-2 ng/mL) of *HSP90b* are usually measured in serum (101, 102).

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 YIDQEELNK was used for *HSP90* measurements, because almost identical results were obtained for another HSP90 peptide DQVANSAFVER as explained in our previous paper. In this study, an agreement between ELISA and PRM results for *HSP90* was shown.(98)

PRM signals for *S100A6* were recorded for doubly-charged endogenous and SIL peptide LQDAEIAR precursor ions with mass-to-charge ratio (*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 YIDQEELNK, 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). Samples 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), washed and desalted for five minutes using 0.1% trifluoroacetic acid (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 with a binary gradient from 12 to 25% solvent B in 14.7 minutes,

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 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 YIDQEELNK (endogenous and SIL), we used the targeted MS/MS mode set up as follows: 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 partner repository with the dataset identifier PXD009025.(103)

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 Utrasonics, 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.

Data analysis

The PRM signals were integrated and analyzed using Skyline (104). The linearity, limit of detection (LOD) and limit of quantification (LOQ) of the assay were determined for *S100A6* and *HSP90* peptides according to our previous work (98) 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 LOD 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 weremeasured 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/permutmatrix): Pearson distance for dissimilarity, Wards' method, as a cluster method and Bipolarization seriation.(105, 106).

Double immunofluorescence staining

FFPE placental material from an independent set consisting of five pre-eclamptic 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 hour and 4 minutes at 37 °C followed by second antibody UMAP anti-mouse conjugated with horseradish peroxidase for 12 minutes followed by 0.01% H_2O_2 and Red 610-tyramide detection (cat # 760-245). Next, antibody denature step was done at 97 °C for 12 minutes with CC2 solution (cat # 950-223). Next, mouse-anti-HSP90 (clone D7a, Abcam) was manually applied and incubated for 40 minutes at 37 °C followed by 2nd antibody UMAP anti-mouse conjugated with horseradish peroxidase for 12 minutes followed by the application of 0.01% H_2O_2 and FAM-tyramide detection (cat #760-243). The slides were counterstained with DAPI Vectashield (Vector Laboratories, Burlingame, CA) and imaged using the LSM700 (Carl Zeiss, Germany) laser scanning confocal microscope.

RESULTS

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 expectedly 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.

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 ln-transformed values were used for statistical testing.

Table 1. Clinical characteristics

General characteristics	PE (n = 43)	Normotensive control (n = 46)	p-value*
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
Preconception 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/24 hours)	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%)	

Table 1. Clinical characteristics (continued)

General characteristics	PE (n = 43)	Normotensive control (n = 46)	p-value*
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 pre-eclampsia			
HELLP	13 (30.2%)	NA	NA
Early-onset PE [b]	20 (8^{d}, 4^{e}, 8^{f}) (47.6%)	NA	NA
Late-onset PE [b]	23 (19^{d}, 3^{e}, 1^{f}) (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 < 10^{th} 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.(107) [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. * 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.*

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 versus 80.6 ng/mL, $p < 0.05$) 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 versus 75.6 ng/mL, $p < 0.05$). 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 versus 93.7 ng/mL, $p < 0.05$). When analyzing the trimesters

separately *HSP90* was significantly higher in serum of cases only in the third trimester (Table 2, 194.2 ng/mL versus 62.6 ng/mL, $p < 0.001$).

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) trimester of pregnancy. Statistical comparisons were made by using an unpaired t-test. Serum levels were not normally distributed, a ln-transformation was used to normalize the data, $p < 0.05$ was statistically significant.

S100A6 **trimester**	**PE (n = 43) concentration in ng/mL (median; IQR)**	**CO (n = 46) concentration in ng/mL (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			
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 = pre-eclampsia; CO = pregnant normotensive controls; IQR = interquartile range; NS = not significant.

The LOD and LOQ for *S100A6* that was based on peptide LQDAEIAR was 0.3 ng/mL and 0.8 ng/mL, respectively. The LOD and LOQ for *HSP90* was 2.8 and 8.6 ng/mL, respectively.

The CV for repeatability and reproducibility calculated for the *S100A6* peptide LQDAEIAR was 3.6% and 2.0%, 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 unsupervised hierarchical clustering that various parameters were correlated with *S100A6* and *HSP90*. The patient and control group were separated completely (Figure S1, Supporting Information).

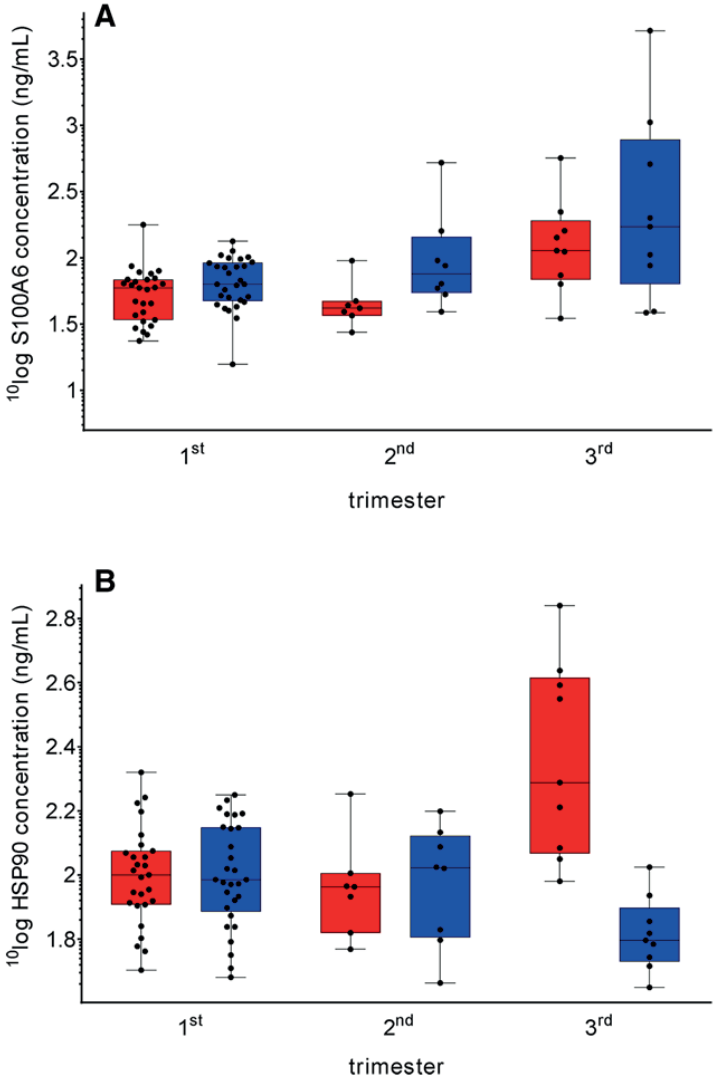
The Abundances of S100A6 and HSP90 in Placental Tissue Measured by PRM

Results showed that *S100A6* (Figure S2A, Supporting Information) was significantly different between preeclamptics and term controls ($p = 0.0381$). Comparing the pre-eclampsia 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 preeclamptics and term controls ($p = 0.9307$). Comparing the pre-eclampsia group with the preterm control group was also not significant ($p = 0.0519$) (borderline).

Co-localization of S100A6 and HSP90 in placentas by immunofluorescence staining

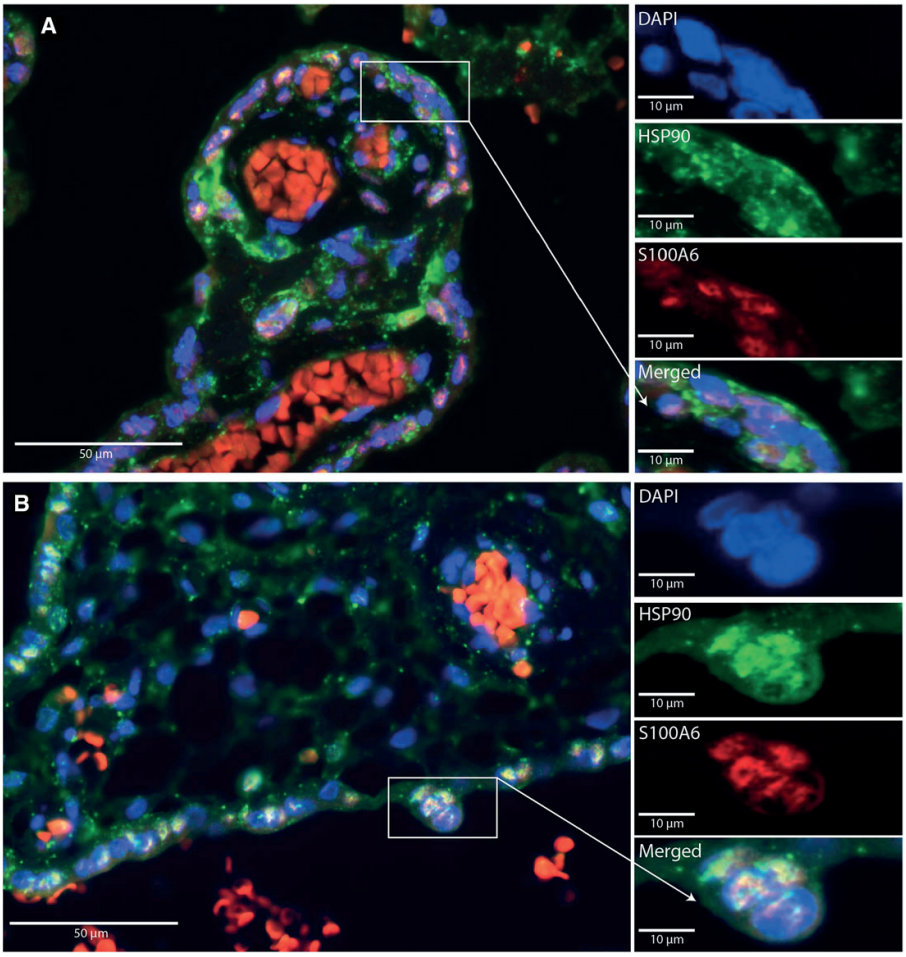
Using immunofluorescence staining, it was observed that *S100A6* and *HSP90* were co-localized in cytoplasm of trophoblast cells from placental tissue of pre-eclamptic patients and preterm matched controls. Figure 2 is an example of immunofluorescence trophoblast staining for *S100A6* and *HSP90* in placenta of a PE case and age-matched preterm control.

Figure 1. Serum levels of S100A6 (A) and HSP90 (B) were measured for each PE (red) and control (blue) group obtained from the first, second and third trimester. 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.



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.

Figure 2. Example of double immunofluorescence staining. It shows trophoblast staining for both proteins S100A6 and HSP90 in a pre-eclamptic (A) and age-matched preterm delivered control (B) 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. Co-localization of both proteins is indicated with a merged color. Red blood cells showed autofluorescence for S100A6. For nuclear counterstain the slides were counterstained with DAPI.



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 was already observed previously in placental tissue (92). 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 (108). 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 (109). Placentas from pre-eclamptic women have reduced antioxidant capacity showing lower levels of antioxidants in blood (109). *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 (110). 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 (111). Extracellularly, the S100 proteins (including *S100A6*) are known to be interacting with the Receptor for Advanced Glycation End products, known as RAGE-receptors (112). 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 (113). Placental cells from PE patients were found to have increased levels of RAGE proteins (114) 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. 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 functions known as *HSP90a* and *HSP90b*. 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* (115-117). Recent research showed that *S100A6*-binding protein and Siah-1 interacting protein (CacyBP/SIP) interact directly with *HSP90* (118). 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 (119).

The immunofluorescence results of *S100A6* and *HSP90* (Figure 2) showed partially overlapping expression in trophoblast cells. The numbers of placenta tissue analyzed in this study (n = 5 in each group) is too small to report information about agreement with the results of Hromoadnikova et al. We previous reported that S100A6 is elevated in a larger cohort (n = 138) (92).

Heme oxygenase plays a role in the protective mechanism against oxidative and nitrosative stress. Ekambaram et al. (120) 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 haemoglobin concentrations have been associated with inadequate plasma volume expansion, which may be part of the pre-eclamptic syndrome (121). 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. 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 elaborate 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 larger numbers of PE and control serum samples are ongoing (the IMPROvED programme, http://www.fp7-improved.eu), the uniqueness of collecting these samples hampers the finding of reliable biomarkers for PE. Another limitation of this study is that samples were collected in a tertiary hospital setting in which some patients suffered

from other chronic diseases. For example, *S100A6* levels were notably high in patients with diabetes mellitus and gestational diabetes (122). This may be related to the fact that the RAGE receptor plays an important role in diabetes. However, excluding the five patients with this diagnosis from analysis did not change the results.

In the last decades, many inventories have been made to find good predictive biomarkers for PE. However, still low predictive values with markers have been reached, for both single and multiple protein markers (123). To get more insight in the pathophysiological process during impaired placentation and PE will lead to more targeted ways for searching predictive markers. We showed that *S100A6* and *HSP90* are proteins that need further validation as candidate biomarkers in dedicated large cohort study initiatives for PE as the IMPROvED programme mentioned above.

In conclusion, we developed a PRM method to measure concentrations of *S100A6* and *HSP90* that could play a role during the pathophysiological process in PE, in a unique set of maternal serum samples collected transversally in all trimesters of pregnancy from women with PE and age-matched normotensive controls.