RESEARCH ARTICLE

Elevated levels of protein AMBP in cerebrospinal fluid of women with preeclampsia compared to normotensive pregnant women

Caroline B. van den Berg[1], Johannes J. Duvekot[1], Coşkun Güzel[2], Stefan R. Hansson[3], Thomas G. de Leeuw[4], Eric A. P. Steegers[1], Johannes Versendaal[1], Theo M. Luider[2] and Marcel P. Stoop[2]*

[1] Department of Obstetrics and Gynecology, Division of Obstetrics and Prenatal Medicine, Erasmus University Medical Centre, Rotterdam, The Netherlands
[2] Department of Neurology, Neuro-Oncology, Clinical and Cancer Proteomics Laboratory, Erasmus University Medical Centre, Rotterdam, The Netherlands
[3] Department of Obstetrics and Gynecology, Clinical Sciences, Lund University, Lund, Sweden
[4] Department of Anaesthesiology, Erasmus University Medical Centre, Rotterdam, The Netherlands

Purpose: To investigate the cerebrospinal fluid (CSF) proteome of patients with preeclampsia (PE) and normotensive pregnant women, in order to provide a better understanding of brain involvement in PE.

Experimental design: Ninety-eight CSF samples (43 women with PE and 55 normotensive controls) were analyzed by LC–MS/MS proteome profiling. CSF was obtained during the spinal puncture before caesarean delivery.

Results: Eight proteins were higher abundant and 17 proteins were lower abundant in patients with PE. The most significantly differentially abundant protein was protein AMBP (alpha-1-microglobulin/bikunin precursor). This finding was validated by performing an ELISA experiment ($p = 0.002$).

Conclusions and clinical relevance: The current study showed a clear difference between the protein profiles of CSF from patients with PE and normotensive pregnant women. Protein AMBP is a precursor of a heme-binding protein that counteracts the damaging effects of free hemoglobin, which may be related to the presence of free hemoglobin in CSF. Protein levels showed correlations with clinical symptoms during pregnancy and postpartum. To our knowledge, this is the first LC–MS/MS proteome profiling study on a unique set of CSF samples from (severe) preeclamptic patients and normotensive pregnant women.

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1 Introduction

Worldwide, 2–8% of all pregnancies are complicated by preeclampsia (PE), a major cause of maternal and fetal morbidity and mortality [1–3]. The disease presents as general endothelial damage and hence a multiorgan disorder, affecting the placenta, the mother's liver, kidneys, clotting system,

Correspondence: Dr. Marcel P. Stoop, Wytemaweg 80, Room Ae-305, PO Box 2040, 3000 CA, Rotterdam, The Netherlands
E-mail: m.stoop@erasmusmc.nl
Fax: +3110-704-4521

Abbreviations: **A1M**, alpha-1-microglobulin; **AMBP**, alpha-1-microglobulin/bikunin precursor; **BBB**, blood–brain barrier; **CSF**, cerebrospinal fluid; **EOPE**, early-onset preeclampsia; **HbF**, fetal hemoglobin; **PE**, preeclampsia

*Current address: Department of Obstetrics and Gynecology, Maasstad Hospital, Maasstadweg 21, 3079 DZ, Rotterdam, The Netherlands

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Clinical Relevance

This LC–MS/MS proteome profiling study on a unique set of cerebrospinal fluid (CSF) samples from (severe) patients with preeclampsia (PE) and normotensive pregnant women. We found a clear difference between the protein profiles of CSF from patients with PE and normotensive pregnant controls, of which protein AMBP (alpha-1-microglobulin/bikunin precursor) was the most significantly differentially abundant protein. This finding may be related to the presence of free hemoglobin in the CSF of both normotensive and hypertensive women and is suggested to be related to an altered BBB permeability during pregnancy. However, the function of the BBB is complex and how this exactly functions during human pregnancy and PE needs to be further elucidated.

and heart. In severe cases of PE the maternal CNS can be involved as well, which may result in major cerebrovascular complications such as eclampsia and stroke. Interestingly, PE may also lead to long-term brain pathology such as cognitive impairment and stroke [4–6]. Brain complications of PE are suggested to be the result of a disturbed cerebral autoregulatory response to the increased blood pressure in combination with endothelial cell dysfunction [7]. Due to disruption of the blood–brain barrier (BBB), inflammatory cells and fluid penetrate the brain, causing oedema and cell death [8–11]. The endothelial cells of the BBB differ from those in peripheral tissues. They have a low rate of endocytosis and are coupled by tight junctions to restrict the amount of paracellular fluid. These functions ensure that not all blood constituents can pass freely into the extracellular space in the CNS [12].

We hypothesize that PE is associated with an abnormal adaptation of the BBB to the changed physiological and immunological state of pregnancy. The aim of this study was to investigate the cerebrospinal fluid (CSF) proteome of patients with PE and normotensive pregnant women by using high-resolution MS.

2 Material and methods

Pregnant women of 18 years and older who underwent a caesarean delivery were recruited. Approval for the study was obtained from the Erasmus University Medical Centre Research Ethics Board (MEC 2007–086). After written informed consent a CSF sample (1 mL) was collected during the spinal anaesthesia procedure. The needle used during the spinal puncture was a 25 Gauge atraumatic needle. The CSF sample was collected before anaesthetics were administered. Within 1 h the CSF samples were centrifuged and cells and debris were discarded. The CSF was aliquoted and stored immediately at −20°C and within 8 h at −80°C. Cases were defined as patients with PE who were admitted on the antenatal ward and in need of a Caesarean delivery. PE was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg on at least two occasions 4 h apart after 20 wk of gestation or postpartum in combination with proteinuria (≥300 mg/24 h or protein/creatinine ratio ≥ 0.3 mg) [13, 14]. Controls were pregnant women without PE in need for a Caesarean delivery. Clinical data were obtained from the medical records of the study participants. General characteristics were statistically compared between study groups by using Mann–Whitney *U* tests data for (not normally distributed) continuous data and Chi-square tests or Fisher's exact test for categorical data. Clinical postnatal data were available up to 1 year postpartum. Neurological symptoms postpartum were defined as complaints of headache, visual disturbances, concentration problems, and other cognitive complaints.

Samples were processed in two batches, using the CSF digestion and MS measurement protocol previously published [15]. In brief, from each CSF sample, 20 μL was added to 20 μL of 0.2% Rapigest (Waters, Milford, MA) in 50 mM ammonium bicarbonate buffer. After 30 min incubation periods with 1,4-dithiothreitol (60°C) and, subsequently, iodoacetamide (37°C), 6 μL 0.1 μg/μL gold-grade trypsin (Promega, Madison, WI)/3 mM Tris-HCl (pH 8.0) was added to each sample. The samples were incubated overnight at 37°C. To adjust the pH of the digest to pH <2, TFA was added to the mixture prior to the final incubation step at 37°C for a duration of 45 min to stop the enzymatic digestion reaction. Subsequently, the samples were measured alternating between PE and control samples.

MS measurements were performed on a Ultimate 3000 nano LC system (Dionex, Germering, Germany) online coupled to a hybrid linear ion trap/Orbitrap MS (LTQ Orbitrap XL; Thermo Fisher Scientific, Germany). One microliter digest (i.e. 0.4 μL CSF) was loaded on to a C18 trap column (C18 PepMap, 300 μm id × 5 mm, 5 μm particle size, 100 Å pore size; Dionex, The Netherlands) and desalted for 10 min using a flow rate of 20 μL/min 0.1% TFA. Then the trap column was switched online with the analytical column (PepMap C18, 75 μm id × 150 mm, 3 μm particle and 100 Å pore size; Dionex, The Netherlands) and peptides were eluted with following binary gradient: 0–25% solvent B in 120 min and 25–50% solvent B in further 60 min, where solvent A consist of 2% ACN and 0.1% formic in water and solvent B consists of 80% ACN and 0.08% formic acid in water. Column flow rate was set to 300 nL/min. For MS

detection a data-dependent acquisition method was used: high-resolution survey scan from 400 to 1800 Th. was performed in an Orbitrap (value of target of automatic gain control AGC 10^6, resolution 30 000 at 400 *m/z*; lock mass was set to 445.120025 u (protonated $(Si(CH_3)_2O)_6$)). Based on this survey scan the five most intensive ions were consecutively isolated (AGC target set to 10^4 ions) and fragmented by collision-activated dissociation applying 35% normalized collision energy in the linear ion trap. After precursors were selected for MS/MS, they were excluded for further MS/MS spectra for 3 min.

The raw data were preprocessed using the Progenesis LC-MS software package (version 4.0, Nonlinear Dynamics, Newcastle-upon-Tyne, UK). Peptides were identified and assigned to proteins by exporting features, for which MS/MS spectra were recorded, using the Bioworks software package (version 3.2; Thermo Fisher Scientific; peak picking by Extract_msn, default settings). The resulting .mgf file was submitted to Mascot (version 2, Matrix Science, London, UK) for identification to interrogate the UniProt database (release 2013_07; taxonomy: *Homo sapiens*, containing 20 265 sequences). Only ions with charge states between +2 and +8 were considered and only proteins with at least two unique peptides (Mascot ions sore >25, (i.e. a peptide probability cut off value of 0.01)) assigned to them were accepted as true identifications. Modifications: carbamidomethylation of cysteine was set as fixed and oxidation of methionine as variable modification, allowing a maximum of two missed cleavages. Mass tolerance for precursor ions was set to 10 ppm and for fragment ions at 0.5 Da. The Mascot search results were imported into the Progenesis software to link the identified peptides to the detected abundances of these peptides. The peptide abundances were normalized to the total ion current to compensate for experimental variations using an algorithm available in the analysis software. Subsequently the data were exported in Excel format.

The raw abundances of all identified peptides were compared between the groups of samples by performing an unpaired two-tailed *t*-test on all individual peptides. Proteins were deemed to be significantly differentially abundant between the groups if they passed a set of three separate criteria: (1) 50% or more of the peptides of the protein had a low *p*-value ($p < 0.05$); (2) 30% of the peptides of the protein had a very low *p*-value ($p < 0.01$); (3) 75% or more of the peptides of the protein must be altered in the same direction between the groups [15–17]. We determined the statistical background level by performing a permutation test using all samples and all identified peptides. The random permutation test, on the dataset with randomized sample group assignment, was repeated 1000 times through which the resulting thresholds were saved.

Following the results of this experiment three validation experiments were performed. A commercially available ELISA for in vitro quantitative measurements of fetal hemoglobin (HbF; Cloud-clone Corp., USA) and an ELISA for in vitro quantitative measurement of protein AMBP (alpha-1-microglobulin/bikunin precursor, LifeSpan BioSciences, Inc., USA) were performed. The AMBP concentrations were calculated by generating a four-parameter logistic standard curve (CurveExpert v1.3, 2013, Supporting Information Fig. 1).

Another validation experiment for A1M (alpha-1-microglobulin), a derivative protein of AMBP, was performed. A1M was measured with an in-house RIA method, as previously described by Olsson et al. [18]. Briefly, the analysis was performed by mixing goat antiserum against human A1M (diluted 1:6000) with ^{125}I-labeled A1M (≈0.05 pg/mL) and unknown patient samples or calibrator A1M concentrations. After incubating the samples overnight at RT, antibody-bound antigen was precipitated, after which the ^{125}I-activity of the pellets was measured in a Wallac Wizard 1470 gamma counter (Perkin Elmer Life Sciences). Statistical comparison of AMBP and A1M values was performed by using the Student's *t*-test and ANOVA. Not normally distributed data (AMBP) were log transformed before testing. Correlations for gestational age at sampling and with neurological symptoms were tested by using Pearson's correlation test.

3 Results

3.1 LC–MS/MS proteome profiling

CSF samples from 43 patients with PE and 55 normotensive pregnant women were obtained for proteomics analysis. Most general characteristics did not significantly differ between the groups, except for parity, gestational age at sampling, PE in a previous pregnancy, and a caesarean delivery in the obstetric history (Table 1). As a result of the definition of PE, women with PE had a higher blood pressure (median 170/110 mm Hg) than normotensive controls (median 122/77 mm Hg). More detailed clinical information about the cases, divided by early-onset PE (EOPE) and late-onset PE is available in Supporting Information Table 1.

A total of 3473 peptides were identified, corresponding to 457 unique proteins. Of the 3473 peptides 317 peptides had *p*-values below 0.01. The number of background hits obtained by permutation was 49.6 ± 51.3, indicating that the number of peptides found with low *p*-values in the true analysis was well above expected background levels (Supporting Information Fig. 2).

Of the 457 proteins, 25 were differentially abundant between cases and controls by using the above mentioned selection criteria. Eight proteins were higher abundant and 17 proteins were lower abundant in the CSF from patients with PE (Table 2). Subtype analyses for HELLP syndrome (where HELLP is hemolysis, elevated liver enzymes and low platelet count), severe PE, and EOPE did not result in additional differentially abundant proteins.

Table 1. General characteristics[a)]

	Preeclampsia ($n = 52$)	Normotensive controls ($n = 58$)	p-value*
Maternal age (years)	32 (29–36)	34 (31–36)	0.318
Ethnicity			0.511
Dutch	38 (73.1%)	38 (66.7%)	
Other Western	0 (0%)	2 (3.5%)	
Non Western	14 (26.9%)	17 (29.8%)	
Current pregnancy			
Nulliparous	32 (61.5%)	21 (36.2%)	<0.05
Gestational age sampling (days)	215 (196–237)	270 (264–272)	<0.05
Preconception BMI (kg/m^2)	25.5 (21.6–29.4)	23.8 (21.1–29.1)	0.245
Smoking (during pregnancy)	2 (5.0%)	3 (5.4%)	0.302
Highest systolic blood pressure (mm Hg)	170 (160–180)	120 (118–134)	<0.05
Highest diastolic blood pressure (mm Hg)	110 (100–110)	77 (70–80)	<0.05
Gestational diabetes	1 (1.9%)	3 (5.2%)	0.620
Twin pregnancy	3 (5.8%)	1 (1.7%)	0.342
Medical history			
Chronic hypertension	13 (25%)	1 (1.8%)	<0.05
Diabetes mellitus type 1	2 (3.8%)	1 (1.7%)	0.602
Diabetes mellitus type 2	1 (1.9%)	0	0.473
Obstetric history			
Recurrent miscarriages	2 (3.8%)	1 (1.7%)	0.602
PE in previous pregnancy	12 (23.1%)	5 (8.6%)	<0.05
Previous caesarean delivery	10 (19.2%)	26 (44.8%)	<0.05
Phenotypes preeclampsia			
HELLP	15 (28.8%)	N/A	
Early-onset preeclampsia	40 (76.9%)	N/A	
Severe preeclampsia	45 (86.5 %)	N/A	
Clinical symptoms			
Headache	30 (58.8%)	7 (12.1)	<0.05
Visual complaints	10 (20.0%)	3 (5.2%)	<0.05
Upper abdominal pain	24 (48.0%)	3 (5.2%)	<0.05
Nausea	5 (9.8%)	2 (3.4%)	0.249
General discomfort	8 (15.7%)	8 (13.8%)	0.780
Dyspnea	8 (15.7%)	2 (3.4%)	<0.05
Child characteristics[b)]			
Birth weight (g)	1190 (900–1860)	3390 (2875–3690)	<0.05
Birth weight percentile			<0.05
<10th percentile	14 (25.5%)	4 (6.9%)	
10th–90th percentile	40 (72.7%)	40 (69.0%)	
>90th percentile	1 (1.8%)	14 (24.1%)	
Fetal gender ratio (male:female)	0.67	0.66	0.970
Neonatal death	3 (2.7%)	3 (2.7%)	1.000
Intrauterine fetal death	0	1 (0.9%)	1.000

[a)] Data are presented as n (%) or median with IQR.
[b)] Due to two twin pregnancies $n = 113$.
*For comparisons between groups Mann–Whitney U tests, Chi-square tests, and Fisher's exact tests were used.

3.2 Validation experiments

The most significantly differentially abundant protein was protein AMBP. A validation experiment (ELISA) in 19 randomly chosen normotensive pregnant controls and 20 patients with PE of the same study group was performed. The level of protein AMBP was significantly higher in patients with PE versus normotensive controls (15.24 (interquartile range (IQR) 11.42–23.76)) ng/mL versus 10.88 (IQR 7.74–12.92) ng/mL, $p = 0.002$, Table 3 and Fig. 1). When we subdivided the cases in EOPE and late-onset PE, a significant difference was found in the AMBP level of patients with EOPE and normotensive women (17.92 (IQR 12.21–3.17) ng/mL versus 10.88 (IQR 7.74–12.92) ng/mL, $p = 0.001$).

Protein AMBP is a precursor of A1M and bikunin. We additionally quantified the A1M level in 47 CSF samples of patients with PE and 52 CSF samples of normotensive controls from the same study group (in-house RIA method). A1M was significantly higher in PE compared to controls (0.35 (±0.1) ng/mL versus 0.27 (±0.14) ng/mL, $p = 0.012$). We found no correlation of A1M levels with gestational age at sampling.

Table 2. Differentially abundant proteins in CSF of patients with preeclampsia[a)]

Higher abundant proteins (accession number)		Peptides (*n*)	*p*-value[b)] <0.01 (%)	*p*-value[b)] <0.05 (%)	Peptides changed in the same direction (%)	Fold change (PE/CO)
1.	Protein AMBP (P02760)	10	100	100	100	1.80
2.	Alpha-1-acid glycoprotein 1 (P02763)	12	58	67	83	1.54
3.	Alpha-1-antichymotrypsin (P01011)	17	53	71	93	1.44
4.	Retinol-binding protein 4 (P02753)	11	45	55	89	1.44
5.	Insulin-like growth factor 2 (P01344)	3	33	100	100	1.47
6.	Lumican (P51884)	3	33	67	100	1.54
7.	Histidine-rich glycoprotein (P04196)	9	33	56	91	2.10
8.	Alpha-1B-glycoprotein (P04217)	13	31	69	82	1.27
Lower abundant proteins (accession number)		**Peptides (*n*)**	***p*-value[b)] <0.01 (%)**	***p*-value[b)] <0.05 (%)**	**Peptides changed in the same direction (%)**	**Fold change (PE/CO)**
1.	Brain acid soluble protein 1 (P80723)	7	86	86	100	0.64
2.	Cadherin-13 (P55290)	6	83	83	100	0.68
3.	Glucosidase 2 subunit beta (P14314)	3	67	67	100	0.80
4.	Neurosecretory protein VGF (O15240)	24	63	71	100	0.67
5.	Ephrin type-A receptor 4 (P54764)	7	57	71	100	0.67
6.	V-set and transmembrane domain-containing protein 2A (B5MCX6)	4	50	75	100	0.54
7.	Neurocan core protein (O14594)	4	50	75	75	0.78
8.	Amyloid-like protein 1 (P51693)	11	45	55	82	0.76
9.	Neuronal cell adhesion molecule (Q92823)	27	44	59	96	0.75
10.	Pyruvate kinase PKM (P14618)	9	44	56	78	0.76
11.	Chromogranin-A (P10645)	19	42	79	94	0.67
12.	Protein kinase C-binding protein NELL2 (Q99435)	17	41	71	100	0.70
13.	Neural cell adhesion molecule (O15394)	8	38	63	87	0.82
14.	Cartilage acidic protein 1 (Q9NQ79)	16	38	56	81	0.93
15.	Seizure 6-like protein (Q9BYH1)	8	38	50	87	0.89
16.	Limbic system-associated membrane protein (Q13449)	6	33	50	100	0.79
17.	Neuroserpin (Q99574)	6	33	50	83	0.83

CO, control.
[a)] Selection criteria of statistically significant differentially abundant proteins were: >3 peptides identified, >30% with a *p*-value of <0.01, >50% with a *p*-value of <0.05, >75% of the peptides changed in the same direction.
[b)] *t*-test comparison between the groups.

Table 3. Quantification of protein AMBP and A1M*

	CO ($n = 19$)	PE ($n = 20$)	LOPE ($n = 7$)	EOPE ($n = 13$)
Concentration AMBP, median (IQR) ng/mL	10.88 (7.74–12.92)	15.24(11.42–23.76)	13.95 (9.65–19.48)	17.92 (12.21–33.17)
Log of concentration AMBP, mean (SD)	2.30 (± 0.41)	2.87 (± 0.62)[a)]	2.62 (± 0.40)	3.03 (± 0.70)[c)]
	CO ($n = 52$)	PE ($n = 47$)	LOPE ($n = 11$)	EOPE ($n = 36$)
Concentration A1M, mean (SD) ng/mL	0.27 (± 0.14)	0.35 (±0.18)[b)]	0.29 (± 0.13)	0.36 (± 0.19)[d)]

CO, control; LOPE, late-onset preeclampsia.
*Comparisons between groups with *T*-test and ANOVA with post hoc Bonferroni
[a)] $p = 0.002$ (PE vs. CO).
[b)] $p = 0.012$ (PE vs. CO).
[c)] $p = 0.001$ (EOPE vs. CO).
[d)] $p = 0.011$ (EOPE vs. CO).

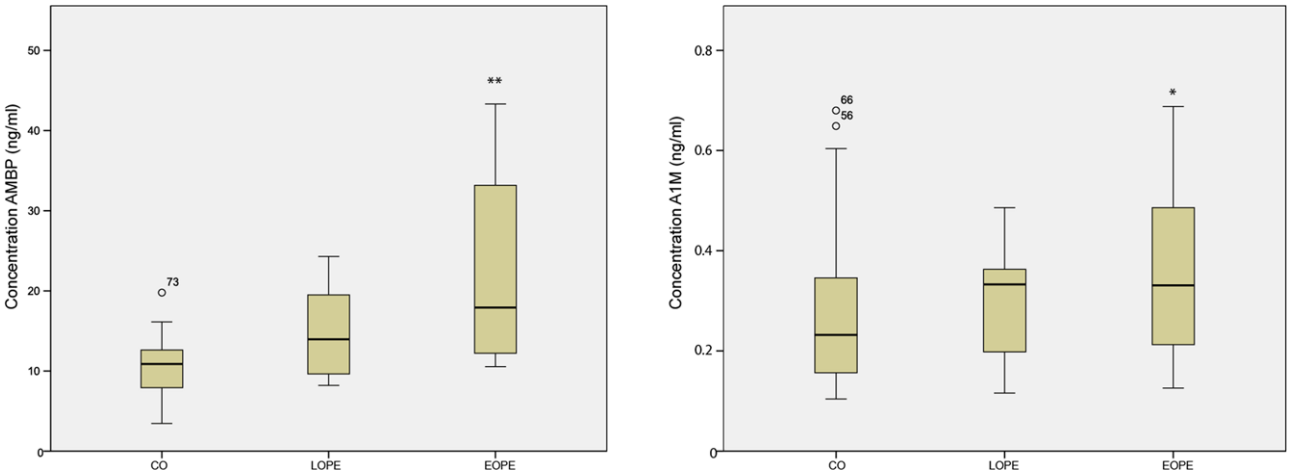

Figure 1. Boxplots of AMBP and A1M levels $^{**}p = 0.001$ (EOPE vs. CO). $^{*}p = 0.011$ (EOPE vs. CO). CO, control; LOPE, late-onset preeclampsia.

Free hemoglobin was identified in the CSF in both cases and controls. In order to exclude that the presence of hemoglobin was caused by a traumatic puncture, we repeated our experiment in exactly the same manner in four other normotensive pregnant women. We collected the first, fifth, and tenth drop of CSF. Again, free hemoglobin proved to be present in these samples, regardless of the order of the aliquots collected. When the presence of hemoglobin in the CSF samples is due to contamination, we would expect the abundance of hemoglobin the highest in the first drop before decreasing to the lowest amount in the tenth drop.

In order to find out if the free hemoglobin detected was either of maternal or fetal origin an ELISA for HbF was performed. The detection limit of this immunoassay was 0.55 ng/mL. We were not able to detect HbF in the CSF samples, because the resulting values were lower than the detection limit of the assay.

3.3 Clinical symptoms during pregnancy

In order to investigate the clinical implications of these findings we studied neurological symptoms of the study participants and their relation to AMBP and A1M levels.

In the PE group 58.8% of the participants reported complaints of headache, compared to 12.1% of the normotensive pregnant women. Twenty percent of the patients with PE reported visual complaints, which was 5.2% in the group of normotensive pregnant women (Table 1).

A positive correlation was found between visual symptoms and AMBP level in the whole group ($p < 0.01$) (Supporting Information Fig. 3).

In the subgroup of severe PE, AMBP and A1M levels tend to be lower in women who reported neurological symptoms than in women without neurological symptoms (ln AMBP: 2.9 ± 0.75 vs. 3.2 ± 0.34, A1M: 0.33 ± 0.20 ng/mL vs. 0.41 ± 0.14 ng/mL), though this difference was not statistically significant (Supporting Information Fig. 4).

3.4 Clinical symptoms postpartum

A total of 37.3% percent of PE patients (all severe PE) reported neurological symptoms from 6 wk to 1 year after delivery (Supporting Information Table 2), while none of the normotensive women reported these symptoms during their standard 6 wk checkup after delivery ($p \leq 0.0001$). A positive correlation was found between postnatally reported neurological symptoms and AMBP level ($p < 0.05$). Most reported symptoms were concentration problems and headache. In line with the neurological symptoms during pregnancy, a positive correlation was also found between postnatally reported neurological symptoms and AMBP level ($p < 0.01$) (Supporting Information Fig. 5)

4 Discussion

The current study showed a clear difference between the protein profiles of CSF from patients with PE and normotensive pregnant women. To our knowledge, this is the first study that performed high-resolution MS on CSF samples of pregnant women with and without PE. Normal pregnancy is associated with neurophysiological adaptations of the brain, including decreased inhibitory gamma aminobutyric acid type A receptor subunit expression in the cerebral cortex that lowers seizure threshold [19]. Studies have previously shown that pregnancy is a state with higher neuronal network excitability and a lower seizure threshold. This may be due to high levels of neurosteroids during pregnancy [19–22].

In addition to this, the BBB is suggested to play an important role in this process. Cipolla and co-workers investigated the function of the BBB in nonpregnant versus late-pregnant rats. They found that pregnancy caused a significant increase in BBB permeability following acute hypertension [23].

4.1 Protein AMBP and free hemoglobin

The most significantly differentially abundant protein, is protein AMBP, which was higher abundant in PE. Protein AMBP is a precursor of A1M and bikunin. A1M has been related to pregnancy and PE in several studies. Elevated serum levels of HbF and A1M in first trimester have been described to be predictive of subsequent development of PE and were also correlated with the severity of the disease [24]. In an ex-vivo placenta perfusion experiment, free hemoglobin was shown to damage the blood–placenta barrier by inducing oxidative stress. Free Hb caused structural damage to the placenta, signs of oxidative stress similar to what have been described in the PE placenta. By adding the heme-binding protein A1M to the system, the structural damage was prevented and leakage over the placenta barrier was inhibited [25, 26]. It is hypothesized that the pathogenesis of PE involves an increased expression of HbF in the placenta that due to barrier damage, leaks into the maternal circulation, causes oxidative stress, and further endothelial damage [26]. A1M is a heme-binding protein that is higher abundant and counteracts the damaging effects of free hemoglobin, shown to be elevated in PE. Free Hb might cause damage to the BBB in a similar way as described for the blood–placenta barrier. A similar process occurs in cerebral hemorrhage in preterm infants. Fragments of Hb cause cell death following intraventricular hemorrhage and upregulation of heme-scavenging proteins reduce the damage to the infants brain [27, 28]. A recent MS study showed protein AMBP also to be elevated in plasma of patients with PE, before the manifestation of clinical disease [29]. Our findings are in line with these studies, which we showed for the first time in maternal CSF. Because the brain during this disorder is at risk for damage, these proteins may be upregulated.

Interestingly, when we only look in the subgroup of severe PE, AMBP, and A1M levels tend to be lower in women who reported neurological symptoms than in women without neurological symptoms, taking into account that levels in PE were still higher than those in normotensive pregnancy, although this difference was not statistically significant. AMBP and A1M are upregulated in PE, but the upregulation and protective effect of AMBP/A1M for the CNS may fail in cases with severe PE who still report neurological symptoms. A possible explanation may be that these women are not able to oppose the toxic effects of PE properly. However, this cannot be proved with our data, since there was no functional study of the BBB. What makes it difficult is that PE has a heterogeneous phenotype. The pathophysiological process in the brain is complex and differs from patient to patient. This may also explain the variation of AMBP and A1M levels in the PE group.

Only a few previous studies investigated CSF of pregnant women by low-resolution MS [30–36]. Norwitz et al. identified nanomolar amounts of free hemoglobin in CSF of pregnant women ($n = 22$) [35]. The method of identification used in this study was SELDI-TOF mass spectroscopy. A significantly higher free hemoglobin concentration was found in the CSF in patients with severe PE than in the CSF from mild PE or controls. They stated that this toxic free hemoglobin may have a role in the cerebrovascular injuries seen in patients with severe PE. Although in line with the results described by Norwitz et al., it is a remarkable finding that free hemoglobin is present in CSF in all pregnant women, as shown in the present study. From MS data of nonpregnant healthy patients we know that CSF contains no or a very low content of free haemoglobin. None of our CSF samples contained macroscopically visible hemoglobin. The needle used in our study (25 Gauge) is not expected to cause more traumatic punctures than the needle (22 Gauge) commonly used during lumbar punctures performed by a neurologist. Furthermore, the total amount of proteins did not differ from the total amount of proteins normally found in CSF of nonpregnant subjects, which supports the idea that the presence of free hemoglobin was not likely caused by a traumatic puncture, but this event cannot be completely ruled out.

4.2 Other differentially abundant proteins in CSF of patients with PE

In this section, we will briefly describe the other differentially abundant proteins and their possible relation to PE (Table 2). Alpha-1-acid glycoprotein is an acute phase protein that increases during inflammation and protects the endothelium. It was previously identified as a predictive biomarker for PE in urine [37, 38]. Alpha-1-acid glycoprotein is constitutively expressed by human microvascular endothelial cells [39]). Interestingly, previous studies identified alpha-1-acid glycoprotein as a protein, which alters the BBB integrity in inflammatory-related diseases [40–42]. Alpha-1-antichymotrypsin is a high abundant plasma protein that is synthesized by the liver and by trophoblast cells. In previous studies, serum and urine levels of this protein were significantly higher in patients with PE. Moreover, this protein was found to be a possible predictor of PE [43, 44]. Retinol-binding protein-4 carries retinol in the blood and is higher abundant in patients with an impaired glucose regulation, obesity, diabetes, polycystic ovary syndrome, and cardiovascular disease, in fact all risk factors for PE. One previous study showed an association with EOPE [45], though another study did not identify significantly altered serum levels in PE [46]. Also, insulin-like growth factor 2 showed a positive correlation with PE in a prospective cohort study [47]. Histidine-rich glycoprotein is a protein interacting with angiogenesis and the coagulation system. It acts as a

negative acute phase reactant and its plasma levels are reduced during the last trimester of pregnancy [48,49]. The last two higher abundant proteins in the CSF were Lumican and alpha-1-B glycoprotein. These proteins have not previously been correlated to PE. The lower abundant proteins that were found in PE CSF (brain acid soluble protein 1, cadherin-13, neurosecretory protein VGF, neurocan core protein, neuronal cell adhesion molecule, seizure-6 like protein, limbic system associated membrane protein, and neuroserpin) are not previously known to have an association with PE. Most of them have a specific function in neuronal cell adhesion, neural cell growth, and interaction. It has been reported previously that decrease of neurological proteins is for instance observed in multiple sclerosis.

4.3 Strengths and limitations

A major strength of our study is that a unique set of CSF samples from (severe) preeclamptic patients and normotensive pregnant women was analyzed by using high-resolution MS. A limitation is that the cases and controls differed in parity and gestational age. However, the difference in gestational age is unavoidable when studying severe early-onset preeclamptic patients. Uncomplicated controls are rarely delivered by caesarean delivery before 37–38 gestational weeks. Another limitation is that the finding that the CSF proteome of patients with PE differed from normotensive controls may be related to the fact that during the spinal puncture most of the PE patients received intravenous administration of magnesiumsulphate (77%) and nicardipine (69%), respectively, with a neuroprotective and antihypertensive effect. Also, most women in the PE group (77%) received antepartum corticosteroids for fetal lung maturation.

A third limitation is that we were not able to compare our data with the CSF proteome of nonpregnant women. Having CSF samples of this group would be helpful in better understanding the effect on the BBB of pregnancy itself. This is a challenge for future research, since it is hard to collect these kind of samples. From an ethical point of view, CSF of healthy women without neurological diseases in the reproductive age can only be collected during spinal anaesthesia procedures for standard care. In our situation very few of these women receive spinal anaesthesia for operative procedures.

5 Concluding remarks

There is a clear difference between the protein profiles of CSF from patients with PE and normotensive pregnant controls. The finding that free hemoglobin was present in the CSF of both normotensive and hypertensive women may be explained by a different BBB permeability during pregnancy. This suggests that pregnancy itself makes the brain more vulnerable to cerebral complications, which is then aggravated in PE when protective proteins such as A1M are upregulated. However, the function of the BBB is complex and how this exactly functions during human pregnancy and PE needs to be further elucidated.

Stefan Hansson is a founder and share-holder of A1M Pharma AB, which holds patent rights on medical uses of A1M based on its heme-binding properties. The other authors have declared no conflict of interest

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