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Differentiation of HELLP patients from healthy pregnant women by proteome analysis—On the way towards a clinical marker set☆

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Abstract

As the pathogenesis of the HELLP-syndrome is unknown, a complex consideration regarding the changes in the plasma as the main transport medium in the body is of great benefit because it is well available and can rapidly be investigated in the clinics. Besides that, the liver which is early affected in HELLP-syndrome produces the main part of plasma proteins. For the purpose of our study plasma protein abundances from patients with HELLP-syndrome and from control individuals were determined before and after delivery. In the differential analysis using two-dimensional gel electrophoresis, six areas with variable protein spot intensities were detected. The reference gel that we developed for HELLP plasma samples integrates the changes of plasma proteins when comparing HELLP patients to healthy women prior to and after delivery. A specific plasma protein profile for the HELLP-syndrome was generated involving protein areas that contain inter-alpha-trypsin inhibitor heavy chain H4, kininogen 1, fibrinogen gamma chain, transthyretin, haptoglobins, and serum amyloid A with statistically significant expression differences when compared to controls. The most striking difference between the majority of the gels from HELLP patients and the gels from non-HELLP samples were clearly overexpressed protein spots at about 11 kDa which were identified as serum amyloid A (SAA). This differential expression was validated and quantitatively assayed by ELISA measurements against human SAA in plasma. Our results show that significant differences in SAA expressions between healthy controls and HELLP patients were obtained, that could function as markers for the HELLP-syndrome. According to our data it is possible to draw a line of separation with no overlap between the HELLP group for which SAA plasma levels were found to be above 3.51 mg/L and the non-HELLP groups in which SAA plasma levels were below 3.51 mg/L. It now is possible to clinically elucidate if the differentially expressed proteins are suited for longitudinal studies concerning both, to function as markers or perhaps even as disease predictors that might become relevant for diagnostic tests.

Keywords: HELLP-syndrome; Proteomics; 2-DE; Two-dimensional gel electrophorsesis; Mass spectrometry; SAA ELISA

1. Introduction

*H*emolysis, *e*levated *l*iver enzymes, and *l*ow *p*latelet count syndrome (HELLP-syndrome) is a serious, life-threatening variant of severe preeclampsia in pregnant women. The incidence of the disease is reported as being 0.17–0.85% of all live births [1]. This syndrome was first described and coined as HELLP-syndrome studying 29 cases of severe preeclampsia–eclampsia complicated by thrombocytopenia, abnormal peripheral blood smear, and abnormal liver function in laboratory tests [2]. The disease manifests itself on average between the 32nd and 34th week of gestation. HELLP-syndrome will also occur postpartum in up to 30% of the cases [1]. In up to 65% of all incidents there is a severe complication on the maternal side, resulting in 3.5% of maternal death cases and of approximately 25% of perinatal mortality [3].

Abbreviations: ALT, alanine transferase; AST, aspartate transaminase; CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propane sulfonate; CHCA, a-cyano-4-hydroxy cinnamic acid; 2-DE, two-dimensional gel electrophoresis; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HELLP, hemolysis, elevated liver enzymes, and low platelet count; MALDI, matrix-assisted laser desorption/ionization; *m/z*, mass to charge ratio; PMSF, phenylmethylsulfonylflouride; SAA, serum amyloid A; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; ToF-MS, time-of-flight mass spectrometry

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Currently, neither reliable preclinical recognition nor effective prevention measures for HELLP patients are available. The diagnostic criteria used for HELLP-syndrome are variable and inconsistent [4]. The course of HELLP-syndrome is incalculable leading to the recommendation that a pregnancy beyond 32nd to 34th weeks' gestation should be immediately delivered. Before 32nd to 34th weeks' gestation, an expectant management with administration of corticosteroids is an option in a perinatal center. Patients with HELLP-syndrome that persists for more than 72 h after delivery might benefit from plasmapheresis [5]. Removal of hitherto unknown circulating mediators that are responsible for the described clinical abnormalities is considered as the principle of action of plasmapheresis therapy.

Using a global proteomics strategy, we compared plasma samples obtained from HELLP patients with those from healthy pregnant women regarding different protein expression profiles. Recently, we published data of an exploratory study using cryodetector mass spectrometry profiling of plasma samples for HELLP diagnosis [6]. Significant differences in distinct protein ion intensities between the investigated groups could be measured even without sample fractionation and enabled reliable grouping of patients and controls, respectively. The most striking difference between most of the mass spectra from HELLP patients and those from non-HELLP samples was the presence/absence of an 11.8 kDa ion signal, most likely belonging to serum amyloid A (SAA). Other significant differences in ion intensities between HELLP and control samples were observed in the peak areas of the 14.0 kDa, the 28.1 kDa, the 50.5 kDa, and the 74.5 kDa protein ion signals.

In this work we present the differential analysis of the HELLP plasma samples versus the plasma samples of healthy pregnant women using high-resolution two-dimensional gel electrophoresis. We first created a reference gel for HELLP plasma and analyzed all the detected gel areas with differentially expressed proteins which seemed specific for the HELLP-syndrome. Our results show that significant differences in protein expression patterns between healthy controls and HELLP patients were obtained that could function as marker matrices for the HELLP-syndrome. The most significant candidate for early diagnosis of HELLP-syndrome, the SAA protein abundance difference, was confirmed and quantitatively analyzed by ELISA. Our goal is to identify potential proteins in plasma that may lead to improved early detection of HELLP-syndrome.

2. Materials and methods

2.1. Patients/donors and sample collection

The study was approved by the Institutional Review Board and blood samples were taken after informed consent was given. HELLP and control samples were provided (i) from the Department of Obstetrics and Gynecology at the University of Rostock, Germany, (ii) from the Women's Hospital at the Schwerin Medical Center, Germany, and (iii) from the Women's Hospital at the Suedstadt Clinical Center Rostock, Germany. HELLP samples were obtained in the case room when HELLP-syndrome was diagnosed (Table 1). HELLP-syndrome was defined as being present, when both, clinical symptoms (Table 1) and laboratory parameters (Table 2) were unambiguous. In general, HELLP was diagnosed when (i) platelet count was less than 130,000/μL

Table 1
Patient information and clinical parameters

Patient	Age (years)	Mode of delivery	Gestation (weeks)	Manifestation	Pregnancy characteristics (wg = weeks' gestation)	Gluco-corticoides	Fetal outcome in APGAR
HELLP 1	36	Cesarean	36 + 2	HELLP	33 wg: hypertension 35 wg: right upper quadrant pain, hypertension, proteinuria, headache	+	9/9/9
HELLP 2	23	Cesarean	36 + 2	HELLP	36 wg: proteinuria, edemas	−	6/9/9
HELLP 3	23	Vaginal	35 + 1	HELLP postpartal	31 wg: hypertension, proteinuria, edemas	+	9/9/9
HELLP 4	23	Cesarean	38 + 5	HELLP	37 wg: upper quadrant pain, vomiting, edemas	−	9/10/10
HELLP 5	40	Cesarean, Stillbirth	22 + 0	HELLP	21 wg: hypertension, headache, edemas, proteinuria 21 + 7 wg: plasmapheresis	+	n.a.
HELLP 6	24	Cesarean	39 + 0	HELLP postpartal	38 wg: hypertension, right upper quadrant pain	−	9/9/10
HELLP 7	29	Cesarean	32 + 4	HELLP	32 wg: hypertension, right upper quadrant pain, headache, vomiting, proteinuria	+	5/6/10
HELLP 8	32	Cesarean	31 + 0	HELLP	30 wg: hypertension, right upper quadrant pain, vomiting	+	3/7/8
HELLP 9	24	Cesarean	35 + 5	HELLP	30 wg: edemas, 34 wg: hypertension, 35 wg: right upper quadrant pain	−	9/7/7

(+) Administered; (−) not administered; n.a.: not applicable.

Table 2
Laboratory parameters of HELLP patients

Patient	Haptoglobin[a]	Hemoglobin[b]	Hematocrit[c]	ALT[d]	AST[e]	Thrombocytes[f]
HELLP 1	0.058	5.8	0.28	171	144	39
HELLP 2	0.136	7.1	0.35	77	65	128
HELLP 3	[g]	4.9	0.24	144	178	82
HELLP 4	[g]	6.5	0.30	496	193	31
HELLP 5	0.058	7.5	0.36	65	46.8	39
HELLP 6	[g]	7.2	0.35	228	247	39
HELLP 7	[g]	5.6	0.25	1073	1117	34
HELLP 8	[g]	6.8	0.32	594	284	88
HELLP 9	[g]	6.9	0.33	90	91	164

[a] g/L; reference value: 0.3–2.0.
[b] mmol/L; reference value: 7.5–9.9.
[c] Reference value: 0.36–0.46.
[d] U/L; reference value: 0–34.
[e] U/L; reference value: 0–31.
[f] 10^9/L; reference value: 150–450.
[g] Not determined.

(normal range: 150,000–450,000/μL), (ii) AST or ALT levels were greater than 65 U/L (normal range: 0–31 U/L (AST), 0–34 U/L (ALT), and (iii) haptoglobin serum levels were below 0.136 g/L (normal range: 0.3–2.0 g/L) or hematocrit values were below 36% (normal range: 36–46%), and hemoglobin levels were below 7.5 mmol/L (normal range: 7.5–9.9 mmol/L). Two patients developed a HELLP-syndrome in the postpartum period (Table 1). Control subjects were matched with HELLP patients concerning maternal and gestational ages, body mass index, and the absence of infections, diabetes, and nicotine abuse. The control samples from pregnant women unaffected by HELLP were obtained during routine counseling in the third trimester.

For proteome analyses, venous blood samples were taken prior to delivery (t1) from seven HELLP patients (HELLP1 to HELLP7) and from six pregnant control individuals, respectively. The seven individuals that were previously affected with HELLP and the six persons from the control group were investigated again several months (mean: 6.5 months) after delivery (t2), resulting in a total of 26 individual blood samples. At time point t2 none of the investigated persons was pregnant. From each individual person at each time point blood samples (7 mL) were collected into Lithium-heparin Monovettes (Sarstedt, Numbrecht, Germany) and immediately centrifuged at 1000 rpm for 15 min at 5 °C. The supernatant (plasma) was filtered with a sterile filter (0.2 μm), and stored in aliquots of 1 mL at −80 °C until further investigations were performed. For 2-DE analysis the protein concentration was determined by the Bradford method [7] using the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany).

For ELISA measurements a total of 33 samples (9 belonging to the HELLP group (HELLP1 to HELLP9), 7 to the post-HELLP group, 10 to the healthy pregnants, 7 to the healthy mothers (post-Pregnant)) were analyzed.

2.2. 2D-PAGE analysis, densitometric image analysis, and statistical evaluation of spot volumes

2-DE and sample preparation was performed as previously described [8]. All gel runs were performed in triplicate and if possible in parallel, to minimize run to run deviations. The first dimension was carried out in an IPGphor system (Amersham Phamacia Biotech, Freiburg, Germany) on pH 4–7, 18 cm long Immobiline™ DryStrips (Amersham, Bioscienes, Uppsala, Sweden). IEF was performed in cup-loading mode by loading 900 μg of total protein for one single gel. For the second dimension a Hoefer Dalt system (Amersham Phamacia Biotech) was used and homogeneous 13.5% polyacrylamide SDS gels were generated. Second dimension electrophoresis was carried out at 100 V. Gels were stained with Coomassie Brilliant Blue G250 [9] and electronically documented as described earlier [8].

For differential gel analysis the software package Phoretix 2D Advanced Version 6.01 (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) was applied. Densitometric analysis was performed as described before [10]. Each patient or control person was represented by at least two gels. In total, 39 gels were analyzed. Nineteen gels from HELLP patients and 20 gels from the non-HELLP control groups were included in the analysis. Selected gel regions (A–F) containing protein spots of interest were compared to each other in all gels. The subsequent statistical analysis (independent double-sided *T*-test) focused on the normalized spot volume. Normalized volumes of the same spots were plotted using box and whiskers diagrams.

2.3. Sample preparation for mass spectrometric peptide mass fingerprinting

Protein spots of interest were excised from the gels with a spot picker (Flexys Proteomics picker, Genomic Solutions, Ann Arbor, MI, USA) or were excised manually as described elsewhere [8]. A low-salt digestion procedure [11,12] with the following adaptions was used. The gel plugs were washed twice with 30% acetonitrile in 25 mM ammonium bicarbonate (100 μL) and 50% acetonitrile in 10 mM ammonium bicarbonate (100 μL) solutions, respectively, shrunk by addition of 100 μL acetonitrile and dried in a speedvac evaporator. The dried gel plugs were reswollen with 5 μL of sequencing grade trypsin solution (10 ng/μL in 3 mM Tris–HCl, pH 8.5, Promega, Madi-

son, WI, USA) and incubated for 5–8 h at 37 °C. Thereafter, 5 μL of extraction solution (0.3% TFA, 50% acetonitrile, 5 mM *n*-octyl-β-D-glucopyranoside) were added and the samples were agitated at room temperature for 30–60 min before the peptide extracts were transferred by centrifugation into the 96-well collection plates.

2.4. MALDI-ToF MS and protein identification

For MALDI-ToF MS measurements sample preparations were carried out on an AnchorChip™ 600/384 target plate by applying α-cyano-4-hydroxy cinnamic acid (CHCA) as matrix [13]. The digested tryptic peptide mixtures were analyzed using a Reflex III mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with the SCOUT source, delayed extraction, and operated in positive ion reflector mode using an ion acceleration voltage of 25 kV. Measurements were externally calibrated with a commercially available peptide calibration standard (Bruker Daltonik GmbH, Leipzig, Germany). Spectra were analyzed using the BioTools software, Version 2.2 (Bruker Daltonics). Database searches were performed against SWALL (UniProt Release 4.4 that consists of Swiss-Prot Release 46.4 on 29-March-2005 and of TrEMBL Release 29.4 on 29-March-2005) using the Mascot software, Version 2.04 (Matrix Science, London, UK) setting a peptide tolerance of 80 ppm and 2 missed cleavage sites. All results were examined carefully for reliability and occurrence of multiple proteins in the same sample. Proteins were named using Swiss Prot accession numbers.

2.5. SAA ELISA test and statistical analysis

A commercially available ELISA test kit (PHASE™ RANGE Serum Amyloid A Assay, Tridelta Development Ltd., Maynooth, Ireland) was used in order to quantitatively analyze SAA expression in plasma. All measurements were performed in duplicate. First, plasma samples were diluted 1:500 with dilution buffer. Fifty microliters of a 1:100 dilution of biotinylated anti-SAA and diluent buffer and 50 μL of diluted standard and samples, respectively, were added to the well plate and incubated for 1 h at 37 °C. The absorbance was measured at 450 nm, using the Multiskan® Plus instrument (MTX Lab systems, USA). Non-parametric ANOVA test (Kruskal–Wallis test) was used for statistical analysis (SigmaStat, Version 3.0, SPSS Inc., Chicago, IL, USA). For all analyses, statistical significance was considered when $P < 0.05$ was reached.

3. Results

3.1. Selection of samples and patients

In order to search for disease-associated protein expression differences in plasma of patients suffering from HELLP-syndrome, we compared these samples with plasma proteins from healthy pregnant women in a comparable maternal and gestational age, body mass index, and non-smoking behaviour as control group. HELLP-syndrome was defined as being present when both, clinical symptoms (Table 1) were conclusive, and typical laboratory blood plasma values concerning hemolysis, elevated liver enzymes, and low platelet count were clearly beyond the normal range (Table 2). Five of nine patients suffered from right upper quadrant pain, seven of nine patients developed hypertension, three of nine described nausea or vomiting, three of nine suffered from headache and in five of nine patients a proteinuria was measured (see Table 1). Two HELLP patients revealed postpartum manifestation, consistent with literature reports [14].

To exclude individual and pregnancy-associated differences in protein expression we collected plasma from the same persons at two time points. The first time point (t1) was either during HELLP-syndrome or in the third trimester of healthy pregnancy. From both groups second samples were taken several months after delivery (t2). In total, we compared four groups to each other: HELLP (I), post-HELLP (II), Pregnant (III), and post-Pregnant (IV).

3.2. Proteome profile of plasma from HELLP patients

A reference gel (Fig. 1) for HELLP-syndrome plasma proteins was generated by assembling all detected spots of plasma proteins from both, gels of HELLP patients before and after delivery, and gels of healthy women prior to and after delivery. In all examined gels, about 550 spots each could be detected on average, consistent with previous analyses from plasma samples. A strong similarity in abundance and intensity of protein spots was found within each group and also between the groups on a first glance. Thus, an important prerequisite for comparison

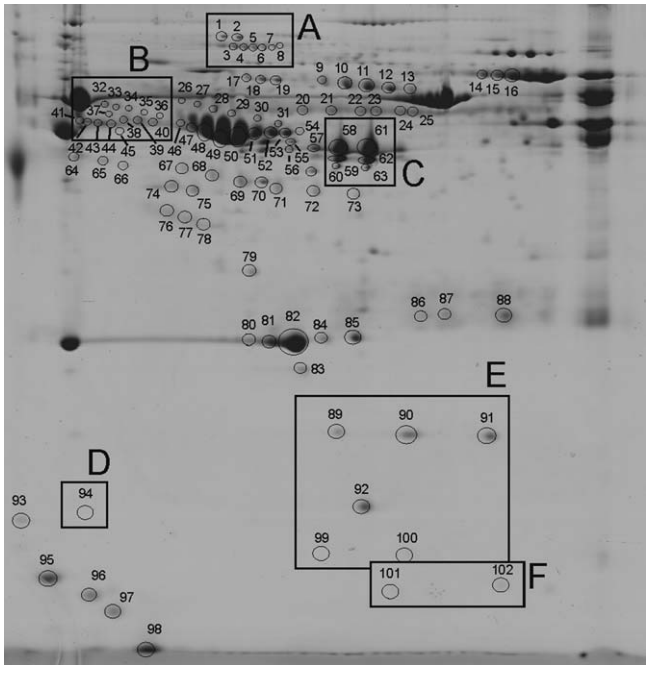

Fig. 1. Reference gel for HELLP that presents all identified protein spots and integrates all the changes of plasma protein abundances comparing HELLP patients prior to and after delivery. 2D gel (pH 4–7), stained with Colloidal Coomassie Brilliant Blue. Boxes (A)–(F) show areas with protein expression differences (cf. Fig. 2). For identity of proteins see Table 3.

Table 3
Mass spectrometrically identified proteins in plasma after 2-DE gel separation

Spot no.[a]	Protein name	Accession no.[b]
1	Alpha1-antitrypsin	P01009
2	Alpha1-antitrypsin	P01009
3	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624
4	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624
5	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624
6	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624
7	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624
8	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624
9	Hemopexin precursor (Beta-1B-glycoprotein)	P02790
10	Hemopexin precursor (Beta-1B-glycoprotein)	P02790
11	Hemopexin precursor (Beta-1B-glycoprotein)	P02790
12	Hemopexin precursor (Beta-1B-glycoprotein)	P02790
13	Hemopexin precursor (Beta-1B-glycoprotein)	P02790
14	Serotransferrin precursor (transferrin)	P02787
15	Serotransferrin precursor (transferrin)	P02787
16	Serotransferrin precursor (transferrin)	P02787
17	Alpha-1B-glycoprotein	P04217
18	Alpha-1B-glycoprotein	P04217
19	Alpha-1B-glycoprotein	P04217
20	Ig alpha-1 chain C region	P01876
21	Ig alpha-1 chain C region	P01876
22	Ig alpha-1 chain C region	P01876
23	Ig alpha-1 chain C region	P01876
24	Ig alpha-1 chain C region	P01876
25	Ig alpha-1 chain C region	P01876
26	Angiotensinogen	P01019
27	Angiotensinogen	P01019
28	Angiotensinogen	P01019
29	Angiotensinogen	P01019
30	Angiotensinogen	P01019
31	Antithrombin III	P01008
32	Kininogen 1	P01042
33	Kininogen 1	P01042
34	Kininogen 1	P01042
35	Kininogen 1	P01042
36	Kininogen 1	P01042
37	Kininogen 1	P01042
38	Kininogen 1	P01042
39	Kininogen 1	P01042
40	Kininogen 1	P01042
41	Alpha-2-hs-glycoprotein	P02765
42	Alpha-2-hs-glycoprotein	P02765
43	Alpha-2-hs-glycoprotein	P02765
44	Alpha-2-hs-glycoprotein	P02765
45	Alpha-2-hs-glycoprotein	P02765
46	Alpha1-antitrypsin	P01009
47	Alpha1-antitrypsin	P01009
48	Alpha1-antitrypsin	P01009
49	Alpha1-antitrypsin	P01009
50	Alpha1-antitrypsin	P01009
51	Alpha1-antitrypsin	P01009
52	Alpha1-antitrypsin + Vitamin D binding protein	P01009 P02774
53	Vitamin D binding protein	P02774
54	Vitamin D binding protein	P02774
55	Vitamin D binding protein	P02774
56	Vitamin D binding protein	P02774
57	Fibrinogen gamma chain	P02679
58	Fibrinogen gamma chain	P02679
59	Fibrinogen gamma chain	P02679
60	Fibrinogen gamma chain	P02679
61	Fibrinogen gamma chain	P02679
62	Fibrinogen gamma chain	P02679
63	Fibrinogen gamma chain	P02679
64	Leucine-rich alpha-2-glycoprotein	P02750
65	Leucine-rich alpha-2-glycoprotein	P02750
66	Leucine-rich alpha-2-glycoprotein	P02750
67	Haptoglobin beta chain	P00738
68	Haptoglobin beta chain	P00738
69	Apolipoprotein A-IV	P06727
70	Apolipoprotein A-IV	P06727
71	Haptoglobin beta chain	P00738
72	Haptoglobin beta chain	P00738
73	Haptoglobin beta chain	P00738
74	Zinc-alpha-2-glycoprotein	P25311
75	Zinc-alpha-2-glycoprotein	P25311
76	Apolipoprotein J clusterin	P10909
77	Apolipoprotein J clusterin	P10909
78	Apolipoprotein J clusterin	P10909
79	Alpha-1-microglobulin	P02760
80	Apolipoprotein A-I	P02647
81	Apolipoprotein A-I	P02647
82	Apolipoprotein A-I	P02647
83	Plasma retinol-binding protein	P02753
84	Apolipoprotein A-I	P02647
85	Apolipoprotein A-I	P02647
86	Serum albumin	P02768
87	Serum albumin	P02768
88	Serum albumin	P02768
89	Haptoglobin allele 2 alpha chain	P00738
90	Haptoglobin allele 2 alpha chain	P00738
91	Haptoglobin allele 2 alpha chain	P00738
92	Transthyretin precursor (prealbumin)	P02766
93	Transthyretin precursor	P02766
94	Transthyretin precursor	P02766
95	Vitronecin (fragment)	P04004
96	Vitronecin (fragment)	P04004
97	Apolipoprotein C II + apolipoprotein C III	P02652 P02656
98	Apolipoprotein A2	P02652
99	Haptoglobin allele 1 alpha chain	P00737
100	Haptoglobin allele 1 alpha chain	P00737
101	Serum Amyloid A	P02735
102	Serum Amyloid A	P02735

[a] Numbers refer to Fig. 1.
[b] SwissProt accession numbers.

of gels from plasma samples between the groups is fulfilled and enables search for disease-associated protein expression differences.

In order to obtain a general picture of the blood plasma proteins in pregnancy and in HELLP-syndrome in particular, 102 proteins were identified by mass spectrometric peptide mass fingerprinting (Table 3). Protein spots were analyzed independently from both, gels from the HELLP group (I) and gels from the healthy pregnant group (III), and showed that protein spots that were migrating to the same location contained the same protein. One of the first obvious findings was that there were differences in protein abundances that can be addressed as pregnancy-dependent. When combining the groups from pregnant women (I: HELLP + III: Pregnant) and qualitatively comparing them to the combination of non-pregnant women (II: post-HELLP + IV: post-Pregnancy), we found differences in protein expression for fibrinogen gamma (spots 58–63) and angiotensinogen (spots 26–30). Both proteins were found higher expressed during pregnancy (cf. Fig. 2).

3.3. Differential protein expression analysis between HELLP and control patients

In addition to pregnancy-related differences, there were gel areas with differentially expressed protein spots when comparing gels from HELLP patients with those from healthy pregnant women. These differences are addressed as associated with changes in the plasma caused by HELLP-syndrome (Fig. 2). Protein spots with higher abundance in the HELLP group (Table 4) were identified as kininogen 1 (spots 32–40), inter-alpha-trypsin-inhibitor heavy chain h4 (spots 3–8), and transthyretin (spots 92–94). We could also detect spots that are less expressed in all the gels from HELLP patient material (Fig. 2 and Table 4) when compared to the controls. These spots were identified as haptoglobin allele 2 α-chain (spots 89–91) and haptoglobin allele 1 α-chain (spots 99 + 100).

A striking difference in protein expression in our study were clearly overexpressed spots at about 11 kDa in the HELLP group. These protein spots were present in five out of seven HELLP patients (I) for which 2D gel analyses were performed. In all other groups, post-HELLP (II), Pregnant (III), and post-Pregnant (IV) they were absent. These spots were identified by mass spectrometric peptide mass fingerprinting as SAA (Fig. 2 and Table 4).

Table 4
Differentially expressed protein spots between HELLP patients and healthy controls

Spot no.[a]	Protein name	Accession no.[b]	Gel area[c]
3–8	Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	A
32–40	Kininogen 1	P01042	B
58–63	Fibrinogen gamma chain	P02679	C
94	Transthyretin	P02766	D
89–91	Haptoglobin allele 2 α-chain	P00737	E
99 + 100	Haptoglobin allele 1 α-chain	P00737	E
101 + 102	Serum amyloid A	P02735	F

[a] Numbers refer to Fig. 1.
[b] SwissProt accession numbers.
[c] Areas are denoted as in Figs. 1 and 2.

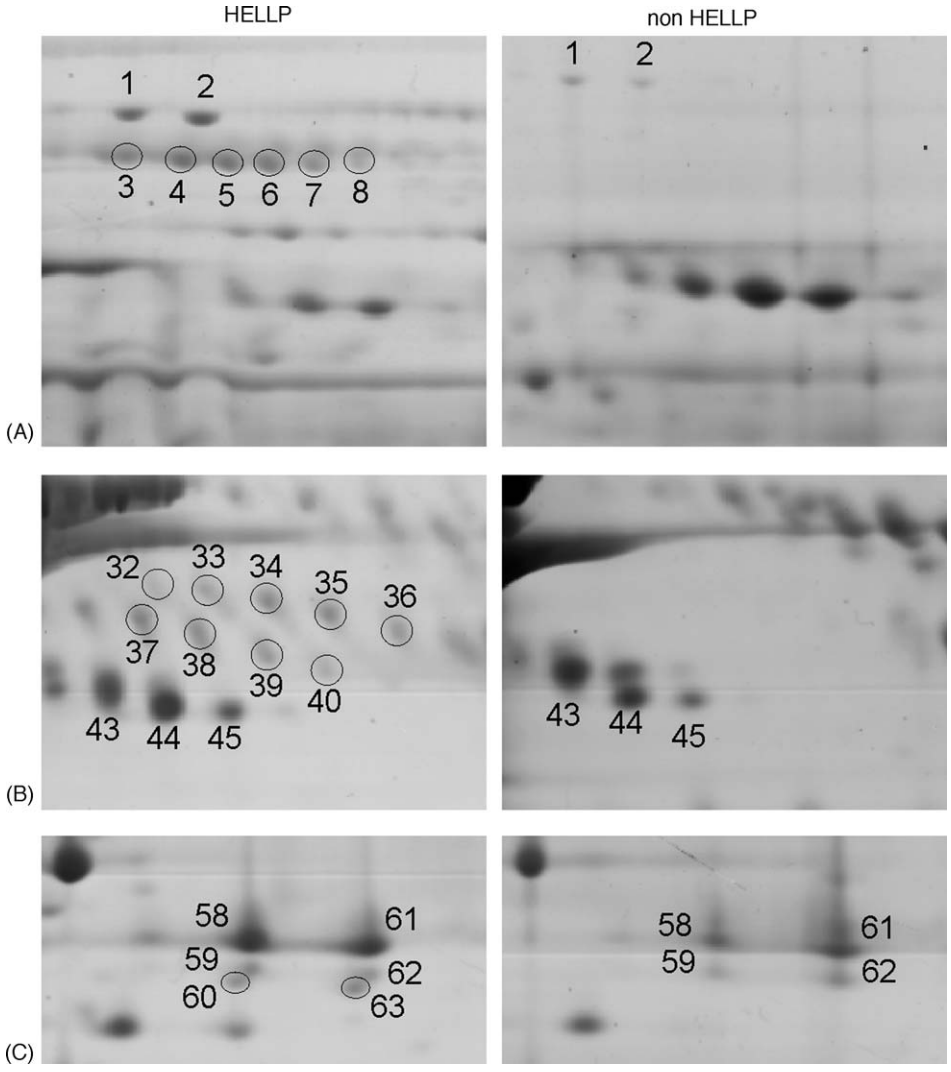

Fig. 2. Gel areas (A)–(F) present differentially expressed proteins in plasma of HELLP patients as compared to controls. Protein spots with differential abundance are circled. Numbers are identical to those in Fig. 1 and Table 3. For identity of proteins see Table 4.

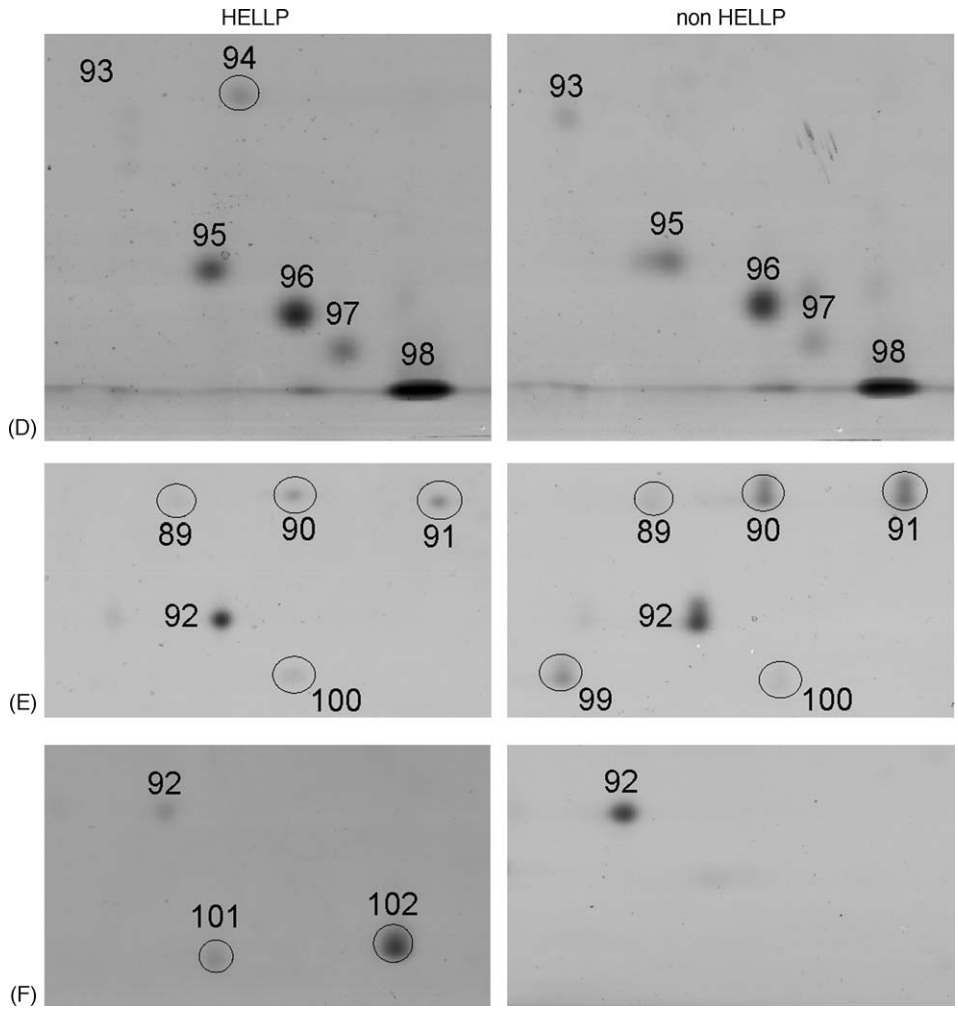

Fig. 2. (*Continued*).

Densitometric analysis of the differentially expressed proteins between HELLP and healthy pregnant controls enabled differentiation of statistically significantly different protein expression from other, less prominent protein spot differences (Fig. 3). Significant differences in protein expression during HELLP-syndrome were found in area A for inter-alpha-trypsin-inhibitor heavy chain h4 (spots 3–8; $p = 0.009$), in area B for kininogen 1 (spots 32–40; $p = 0.003$), in area C for fibrinogen gamma (spots 58–63; $p < 0.001$), in area D for transthyretin (spots 92–94; $p = 0.009$), in area E for haptoglobin (spots 89–91 and 99 + 100; $p < 0.001$), and in area F for serum amyloid A (spots 101 + 102; $p < 0.001$).

3.4. Immunoanalytical assay (SAA ELISA)

In order to investigate whether the differential expression of SAA that was suggested by the 2-DE analysis could be validated and extended to further patients, we determined plasma levels of SAA quantitatively by ELISA. This analysis allowed increasing the sample numbers. It should be mentioned, that all patient samples that were included into 2D gel analyses were also subjected to ELISA. The averaged SAA levels in plasma during HELLP-syndrome (I) is elevated as compared to the Pregnant group (III). The averaged SAA level in group I is 7.41 mg/L (3.57–10.26 mg/L), whereas the SAA level in group III is 2.28 mg/L (1.68–3.09 mg/L) (Fig. 4). Note that the highest SAA level in healthy pregnant individuals was below the lowest value of the HELLP patient group. Statistical analysis confirmed a significant difference ($p < 0.001$). The SAA levels in the non-pregnant groups were also lower as those from the HELLP group. The mean level of SAA in group II is 2.28 mg/L (1.77–3.45 mg/L) and 2.61 mg/L (2.13–3.39 mg/L) in group IV. Again statistical analysis confirmed a significantly higher SAA plasma level in the HELLP group (I) as compared to the Pregnant group (III; $p < 0.05$), to post-HELLP (II; $p < 0.05$) and to post-Pregnant (IV; $p < 0.05$) groups. According to these data it is possible to draw a line of separation with no overlap between the SAA plasma level values from the HELLP group and the SAA plasma levels from other groups. Although limited case numbers are analyzed so far, the SAA ELISA results allow a separation of HELLP versus non-HELLP patients at a cut-off value of 3.51 mg/L. Plasma samples with SAA levels above 3.51 mg/L accurately identified HELLP patients. SAA values below 3.51 mg/L

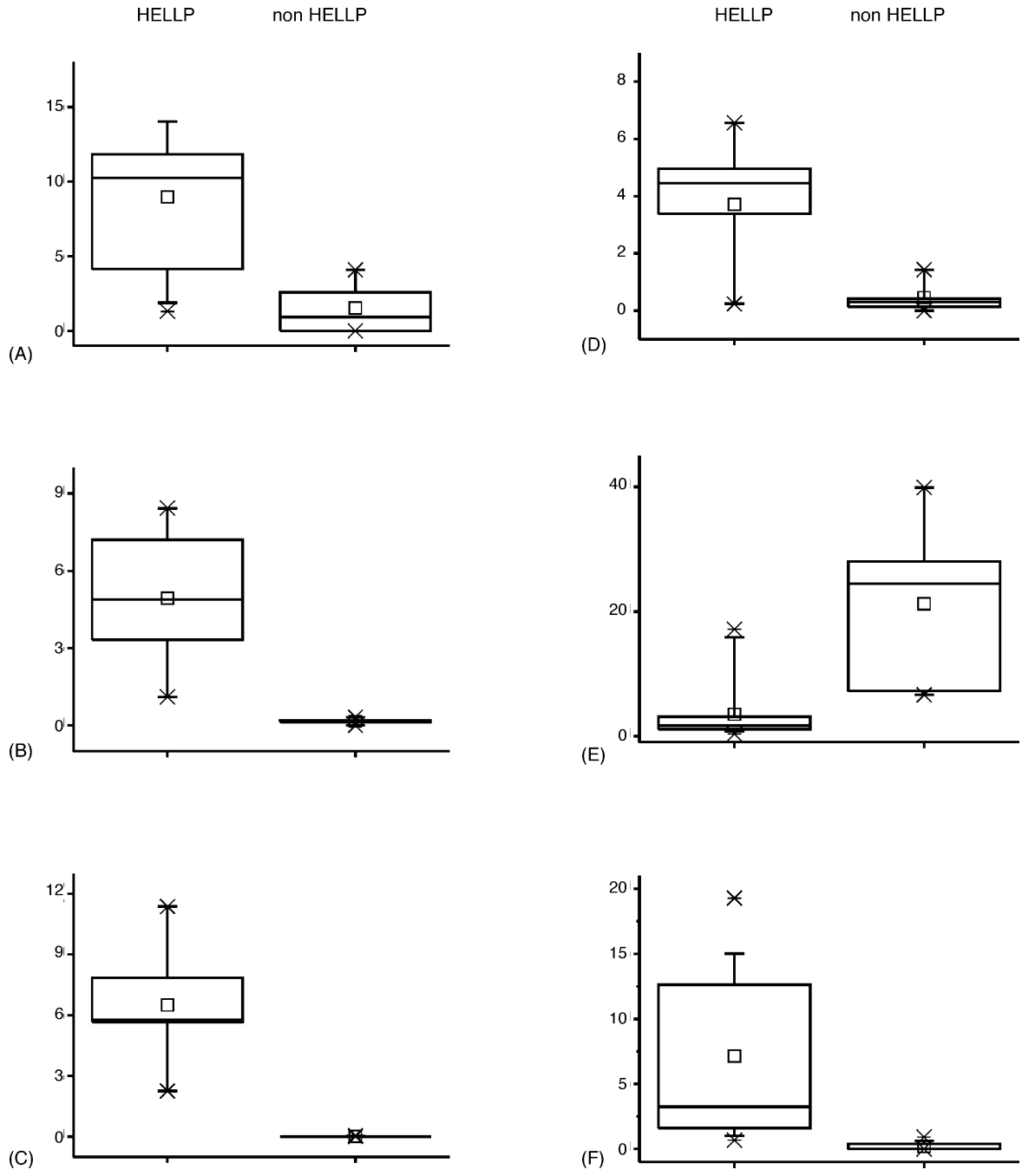

Fig. 3. Densitometric analysis of normalized spot volumes (in arbitrary units) of all spots in the respective gel areas (cf. Fig. 2). Significant expression differences are represented as "box and whiskers" plots. The HELLP group encompasses 19 gels and the non-HELLP group (healthy pregnant control group) comprises 20 gels. In the diagrams the 25th and 75th percentiles are plotted as box and the 95th and 5th percentiles as whiskers. The horizontal line within the box represents the 50th percentile. The mean value is depicted as small box. The min/max values are depicted as minus (−) signs and the 99th and 1st percentiles as crosses (×). Labeling of areas is identical to that in Fig. 2 and Table 4.

were observed in healthy pregnant women and in post-partal controls.

4. Discussion

Our proteomic approach revealed that components of acute phase proteins (SAA, fibrinogen gamma), kallikrein-kinin-system (kininogen, inter-alpha-trypsin inhibitor heavy chain 4), and transthyretin are considerably up-regulated in plasma samples of HELLP patients. The detected down-regulation of haptoglobin allele 2 α and haptoglobin allele 1 α chains may serve as positive controls for our experimental setting. Haptoglobin is an acute phase protein with a short half-life which is known to be significantly reduced in 85–97% of patients suffering from HELLP-syndrome [15]. Up to date, the determination of serum haptoglobin is considered the most sensitive method to diag-

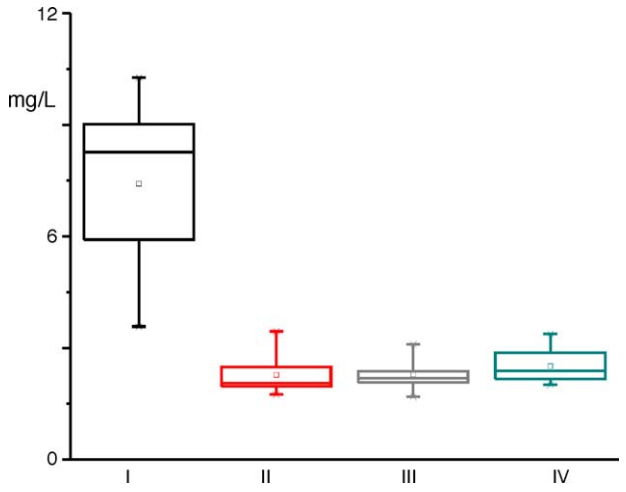

Fig. 4. Serum amyloid A ELISA data represented as box and whiskers plots. A significant ($p < 0.001$) increase of SAA levels was found in (I) the HELLP group ($n = 18$ measurements) compared to the control groups of (II) post-HELLP mothers ($n = 14$ measurements), (III) healthy pregnant women ($n = 20$ measurements), and (IV) healthy mothers/post-Pregnant controls ($n = 14$ measurements). All readings were performed in duplicate. Boxes represent the 25th–75th percentiles. The horizontal line within the boxes represents the 50th percentile. Whiskers represent the 5th and 95th percentiles. The small box represent the mean value. The min/max values are depicted as minus (−) signs and the 99th and 1st percentiles as crosses (×).

nose the hemolysis during HELLP-syndrome. Unfortunately, the decrease of haptoglobin is not detectable before the clinical symptoms manifest.

Fibrinogen gamma is also an acute phase protein which is known to increase by 100% during pregnancy and is expressed by the liver [16]. Fibrinogen is a precursor of fibrin, which is one of the main components of blood clots generated during the hemostatic response. Beyond its important role in hemostasis, fibrinogen is involved in several physiologic and pathophysiologic states, such as infection, wound healing, tumor progression, and atherosclerosis. In addition, fibrinogen has a critical role in maintaining pregnancy [17]. In its essential role in the adhesion and aggregation of platelets, fibrinogen binds to specific receptor sites on platelets. It was shown that the gamma and to a lesser extent the alpha chains carry the main sites for interaction with platelet receptor [18]. Although there are tabulated 19 separate mutations (17 of which were missense mutations of naturally occuring mutations) in the gamma chain of human fibrinogen [19], up to date there are no associations regarding fibrinogen gamma mutations and HELLP-syndrome. In general, mutations within the gamma chain of fibrinogen are not associated with serious bleeding disorders. Hence, it seems worthwhile to more closely analyze the spot pattern for fibrinogen gamma (spots 58–63) in HELLP samples for detectable sequence differences and possibly also for different post-translational modifications.

The roles of kininogens, plasma kallikrein, and transthyretin in HELLP are not clear. Transthyretin is a transport protein for both thyroxine and retinol (Vitamin A). It has been demonstrated that the transthyretin protein maintains normal levels of retinol, retinol-binding protein, and thyroid hormone in the circulating plasma [20]. Interestingly, many distinct forms of amyloidosis have been related to different point mutations in the 127 amino acid long transthyretin. Although the clinical manifestations and natural history vary, most forms have polyneuropathy as the predominant feature. Currently, more than 80 different disease-causing mutations in the transthyretin gene had been reported [21]. Only a small proportion of transthyretin mutations are apparently non-amyloidogenic. It was found that blood flow was decreased in the peripheral tissues of amyloidosis patients and suggested that this effect could be mediated in part by a decreased production of nitric oxide, also known as endothelial-derived relaxing factor [22].

The up-regulation of a 11 kDa protein (SAA) in HELLP samples was observed by cryodetector mass spectrometry [6]. Consistently, in our studies elevated SAA expession has been determined by 2-DE analyses and with a commercially available human SAA ELISA kit. Using ELISA all HELLP patients recorded elevated SAA levels as compared to the control samples in this study; even those that did not show overexpressed SAA spots in 2D gel analysis. This may be due to a lesser analytical sensitivity of 2D gel analysis as compared to ELISA. SAA abundance in plasma can elevate up to 1000-fold [23] upon inflammation. Interestingly, several reports have suggested that SAA is a marker of low grade inflammation and can predict increased risks of coronary heart disease [24,25]. In chronic inflammatory diseases, such as rheumatoid arthritis or Mediterranean fever, continuous elevation of SAA has been related to the development of AA-type amyloidosis, in which AA fibrils derived from SAA are deposited in certain vital organs and may compromise their function [26].

Recent data indicate local production of the SAA protein in histologically normal human extrahepatic tissues including placenta [27] in addition to its physiological production in the liver. It has been shown that in utero invasive first trimester trophoblast and malignant trophoblast-like choriocarcinoma cells – both representing placental derived cells of fetal origin – express acute-phase SAA transcripts [28]. Whether SAA has a protective role in fetal tissues contributing to the maintenance of cytokine balance or could adversely affect fetal growth under imbalanced cytokine conditions, remains to be resolved. To test the hypothesis of an increased placental SAA expression in HELLP patients we analyzed third trimester paraffin-embedded placental sections by immunohistochemistry. No differences were observed between HELLP and non-HELLP patients with respect to SAA protein expression (unpublished results). This is in agreement with the assumption that SAA may function as a "surrogate marker" for HELLP, representing liver involvement. It should be noted that liver-derived markers (AST and/or ALT) are indicators of liver destruction occuring in plasma by leakage. By contrast, SAA is actively produced and released into plasma. Hence, SAA principally has the potential to also function as an early marker, when detected accurately and brought into the context of HELLP.

SAA is a generic term for a family of apolipoproteins coded by different genes with a high allelic variation and a high degree of homology between species [29,30]. In humans, the SAA gene family consists of three genes (*SAA 1*, *SAA 2*, and *SAA 4*) and

a pseudogene *SAA 3*, all clustered on the short arm of chromosome 11 [31]. *SAA 1* and *SAA 2* genes, so called acute-phase genes, encode for SAA1 (the most abundant isoform) and SAA2 protein. The major contribution of the SAA protein expression differences in our study is due to SAA1, as judged from the gels. 2D gel analysis has proven successful in the discovery of SAA that hitherto was not associated with protein expression differences during HELLP. Validation of these first findings can now focus on more SAA-specific methods with higher sensitivity.

It is our intention to use SAA specific immuno-assays (ELISA, etc.) and also immuno-analytical assays for the other proteins mentioned here to investigate a broader group of patients. We thereby will validate the different expression of the proteins that were detected here and will elucidate if the differentially expressed proteins are suited for longitudinal studies concerning both, to function as clinical markers or perhaps even as predictors.

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