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Searching biomarker candidates in serum using multidimensional native chromatography.
II Method evaluation with Alport syndrome and severe inflammation

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ABSTRACT

Biomarker search using multidimensional native liquid fractionation of serum in microplates was evaluated. From different donors, homologous sample fractions with UV absorbance depending on state of illness were selected, and their constituents were identified and quantitated by MS. Analysis of sera of patients with Alport syndrome and severe inflammation proved the reliability of the method by confirming characteristic alterations. Moreover, 23 new marker candidates were detected for Alport syndrome, some of them being involved in matrix degradation and repair, and 33 new candidates for severe inflammation, among them α1B-glycoprotein cysteine-rich secretory protein and an apparently low molecular-weight albumin variant.

1. Introduction

The native two-dimensional (2D) liquid fractionation technique based on size exclusion (SEC, 1D) and anion exchange chromatography (AEC, 2D) described in refs. [1,2], is applied to patient sera from two disease groups. With both these groups several serum alterations may be expected suited to validate the biomarker search strategy introduced.

The Alport syndrome, a hereditary form of nephritis, is a clinically and genetically heterogeneous nephropathy. The majority of cases are transmitted as an X-linked semidominant condition due to COL4A5 mutations [3,4]. Clinically, an individually variable progressive glomerulosclerosis, hypertension, hematuria, and proteinuria may occur with consequences ranging from nephrotic syndrome to end-stage renal disease. Additionally, extra-renal manifestations may also occur, e.g., sensory-neural deafness, ocular abnormalities, and focal leiomyomatosis [5–7]. Until now reliable diagnosis has required a biopsy [8]. As in patients showing heavy proteinuria of other origin, in Alport patients the pattern of plasma proteins and their turnover may be expected to change significantly. Since collagens affected under Alport syndrome are widespread throughout the body [9] and are likely to be susceptible to proteolysis at all locations, one should expect occurrence of plasma and possibly serum constituents that may serve as valuable markers. However, no specific serum parameter has been reported for Alport syndrome up to now.

Sepsis, severe sepsis, and septic shock belong to a continuum of severe inflammation states that are defined by various vital parameters, laboratory data, hemodynamic data and data reflecting organ function. Even taken together, however, these parameters cannot diagnose sepsis reliably nor can they predict the course of disease. Instead, a combination of clinical parameters and levels of numerous constituents of plasma and other body fluids are used for diagnosis [e.g. 10]. Parameters used for

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Table 1
Characteristics of sepsis serum samples. ICU, intensive care unit; PCT, procalcitonin; APACHE, acute physiology and chronic health evaluation [26]; f, female; y, age (years).

Patient 1 f/54 y					Patient 2 f/84 y				
ICU day	Serum sample	APACHE II	PCT (μg/L)	State	ICU day	Serum sample	APACHE II	PCT (μg/L)	State
1	s3	7	2.0	Septic shock	1	s3	28	52.0	Septic shock
2	s2	9	1.1	Severe sepsis	2	s2	17	35.5	Septic shock
3	s1	12	0.6	Sepsis	5	s1	28	12.6	Sepsis
					7	s0	11	3.2	No sepsis

diagnosis are reportedly involved in networks of oxidative stress [e.g., 11], apoptosis [e.g., 12], dysregulation of innate immunity [e.g., 13], endothelial function [e.g., 14], hormone household [e.g., 15], coagulation [e.g., 13,16], complement [e.g., 17], metabolism [e.g., 14], and signal cascades [e.g., 13,18]. Furthermore, numerous marker candidates have been registered as trademarks (literature not included). Since our method and other search strategies such as 2D polyacrylamide gelelectrophoresis (PAGE) [e.g., 19], bottom-up analysis [e.g., 20], surface-enhanced laser desorption/ionization (SELDI)-based analysis [e.g., 21], and multiplexing immunoassays and array approaches [e.g., 22] may search different protein subsets for sepsis markers, our and the other methods may be used complementarily.

The protein content within each fraction of each sample is inferred from UV absorbance; when it is changed by the disease, the fraction is selected [23] for further analysis and its proteins are identified with liquid chromatography-electrospray ionization (LC-ESI) MS/MS and quantified using MS peak heights as described previously [24]. Potential biomarker candidates, i.e., constituents, the levels of which depend clearly on the disease, are detected using an efficient stepwise search strategy. Application to Alport and sepsis sera not only confirmed reported alterations due to failure of the glomerular barrier function and acute phase reaction, respectively, but also revealed some additional disease-related changes of components, showing the method to be suitable for biomarker search. Since a marker should survive the hardest handling occurring in clinical praxis, serum was chosen for biomarker search, the recommendation of the HUPO/Plasma Proteome Project for plasma in-depth analysis not withstanding [25].

2. Experimental

2.1. Materials

N-*p*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (EC 3.4.21.4, bovine pancreas) and α-cyano-4-hydroxycinnamic acid were obtained from Sigma–Aldrich Chemie (Sternheim, Germany). Sinapic acid was supplied by Fluka Chemie (Buchs, Switzerland). Vivapure Anti-HSA kit (VS-SP08HAR) was obtained from Sartorius (Göttingen, Germany). All other reagents used were of analytical grade either from Sigma, Fluka, or Merck KGaA (Darmstadt, Germany). The internal standard peptide AAGIFQFPRV-NH_2 (monoisotopic mass, 1103.62 Da) was from Jena BioScience GmbH, and the Peptide Mass Standards Kit Sequazyme was from Applied Biosystems (Darmstadt, Germany), as were Cs and Igor peptide.

2.1.1. Samples for searching new marker candidates

Twenty millilitre of blood was drawn the morning after overnight fasting. Each blood sample was drawn into S-Monovettes (Sarstedt, Germany), permitted to clot at 20 °C for exactly 2 h, and centrifuged at 2000 × *g* at 4 °C for 15 min prior to separating serum. Serum was immediately frozen at −80 °C, kept at this temperature for 1–2 days, and after transfer to our institute in liquid nitrogen until use. The sera were fractionated as described in ref. [2], briefly in Section 2.2.

2.1.2. Alport syndrome

Sera from five patients (one female (f) and four male (m), 13.9 ± 3.4 years) with histologically confirmed Alport syndrome showing proteinuria but still normal glomerular filtration rate (GFR), serum creatinine, and serum albumin concentration and age and gender matched controls (14.4 ± 5.5 years) were obtained in consensus with the Ethics Commission of the Medical Faculty, 1723-01/06.

2.1.3. Severe inflammation

From each of two patients with sepsis, several sera were obtained at different stages of severity during medical treatment, seven samples altogether, in consensus with the Ethics Commission of the Medical Faculty, 2017-05/07. The patients were not injected with blood, plasma, or plasma fractions. Samples were obtained from 5:00 to 7:00 a.m. Clinical sample characteristics are presented in Table 1.

2.1.4. Samples for analyzing alterations of albumin

Non-fractionated samples as well as their 1D- and 2D-fractions were analyzed. Albumin was quantified by enzyme immunoassay (Section 2.4) in samples of sera of septic patients (Table 1), of control sera from obviously healthy volunteers of the laboratory staff (22–64 years), and of a pool of 10 sera from healthy volunteers (6 f/4 m, 61.4 ± 7.8 years, Ethics Commission of the Medical Faculty, 1677-11/05).

2.1.5. Non-fractionated samples analyzed for alterations of α1B-glycoprotein and cysteine-rich secretory protein-3

The following samples were used for immuno-quantitation of α1B-glycoprotein (A1BG) and its agonist, cysteine-rich secretory protein-3 (CRISP-3): 96 control samples of apparently healthy blood donors of the regional transfusion service (38 f/58 m, mean age: 40.6 ± 16.1 year, range 18–79 years), sepsis sera as mentioned above, and 23 additional EDTA-plasma samples of patients classified according to the American College of Chest Physicians and the Society of Critical Care Medicine [26] (f/m, mean ± S.D. of age, range of age, APACHE II, procalcitonin: systemic inflammatory response syndrome (SIRS): 6/6, 71.9 ± 5.4 years, 63–80 years, 17.9 ± 5.8, 3.7 ± 2.3 μg/L; severe sepsis: 0/1, 58 years, 18, 1.86 μg/L; and septic shock: 3/7, 60.6 ± 9.7 years, 42–68 years, 20.3 ± 7.5, 9.6 ± 13.9 μg/L (Ethics Commission of the Medical Faculty, 0713-08/01)). Patients with SIRS due to infection were considered to fulfill the criteria [26] of severe sepsis if they showed at least one parameter of one organ dysfunction (e.g., increase of serum creatinine concentration, hypoxemia, thrombocytopenia, or metabolic acidosis), and to fulfill the criteria of septic shock if they showed arterial hypotension. Patients were excluded if they were less than 18 years old, or were pregnant, or if informed consent could not be obtained.

2.2. Separation

Pairs of 0.9 mL samples were 2D separated in parallel (cf. [2]), each pair consisting of one Alport sample and one control or pairs

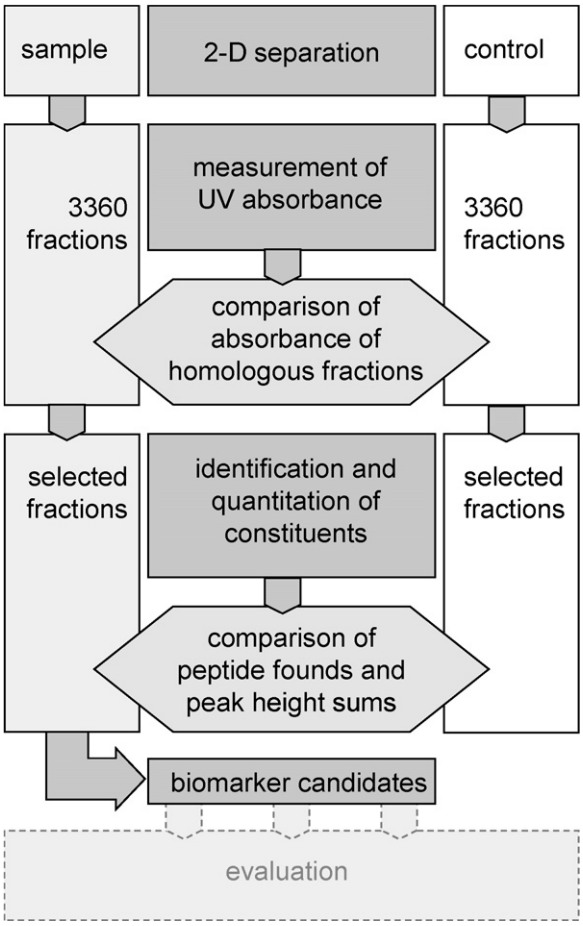


Fig. 1. Biomarker search strategy comprising multidimensional separation, comparison of total protein within homologous fractions of different origin, identification based on ESI-MS/MS sequence tags, and MS-based quantitation.

of samples belonging to adjacent states of severe inflammation. Protein concentration of 1D- and 2D-fractions was determined according to refs. [1,2]. Absorbance spectra of all fractions were determined at 205–700 nm with a Spectramax Plus^{384} microplate reader (Molecular Device, Sunnyvale, CA, USA) to exclude turbid fractions and to allow bilirubin and post-translational modifications to be searched for.

Typically, SEC of two samples and column calibration run successively within one working day, whereas a further day is needed for dialysis and parallel 2D separation of both sets of 96 1D-fractions and photometrical and fluorimetric analysis. Between separation steps, fractions and aliquots there from are stored in microplates ice-cooled and at −84 °C, respectively.

2.3. Analysis scheme for searching biomarker candidates

An overview of the search scheme is shown in Fig. 1.

2.3.1. Fraction selection

Each fractional total protein concentration was normalized by the total protein concentration of the sample to eliminate the effect of global protein concentration differences between samples.

Alport: normalized total protein concentrations of homologous 2D-fractions of patient samples and their respective controls were compared by quotient formation, $[\text{fractional protein}]_{\text{sample}}/[\text{fractional protein}]_{\text{control}}$, cf. Fig. 1. 2D-positions with quotients >2 or <0.5 were selected for further analysis if (i) at least one of the two protein concentrations exceeded 0.03 mg/mL and (ii) this 2D-position belonged to at least three neighboring fractions exhibiting uniform dependence of concentration on disease.

Severe inflammation: normalized total protein concentrations of homologous 2D-fractions of three sera obtained from patient 1 and four sera from patient 2 during decreasing severity of sepsis (cf. Table 1) were compared. The software package VisualCockpit [23] was used to locate and select homologous 2D-fractions whose protein concentration depended in a clear-cut manner on severity of disease. Briefly, concentration ratios (higher severity)/(lower severity) are calculated for neighboring stages of severity of inflammation, in the order septic shock, severe sepsis, sepsis, and SIRS. When there are only three stages (patient 1), one ratio is plotted versus the other for each fraction; in case of more stages (patient 2), the maximum ratio is plotted versus the minimum ratio. These plots permit interesting fractions to be easily selected.

2.3.2. Analysis

Selected fractions were analyzed twice: (i) proteins and peptides are identified using LC-ESI-MS/MS [2] in duplicate, (ii) for each selected fraction and each constituent stringently identified therein by sequence tags (see confidence criteria), the normalized peak height sum (S_n) of all matched tryptic peptides is determined in quadruplicate by MALDI-MS as a measure of its concentration (see below).

2.3.3. MALDI-MS

MALDI-MS of tryptic peptides [1] using α-cyano-4-hydroxycinnamic acid as matrix was performed in parallel on a target plate with pairs of Alport and control sample and with sample sets of all adjacent states of severe inflammation [24]. To this end the tryptic digest of each selected fraction was spiked with the standard peptide AAGIFQFPRV-NH_2 (see Section 2.1) with a final concentration of 0.3 μM, and was MS analyzed in quadruplicate. MS peak heights were normalized with the peak height of the standard peptide, and the normalized sum of peak heights (S_n) was calculated. S_n was normalized like global protein concentration.

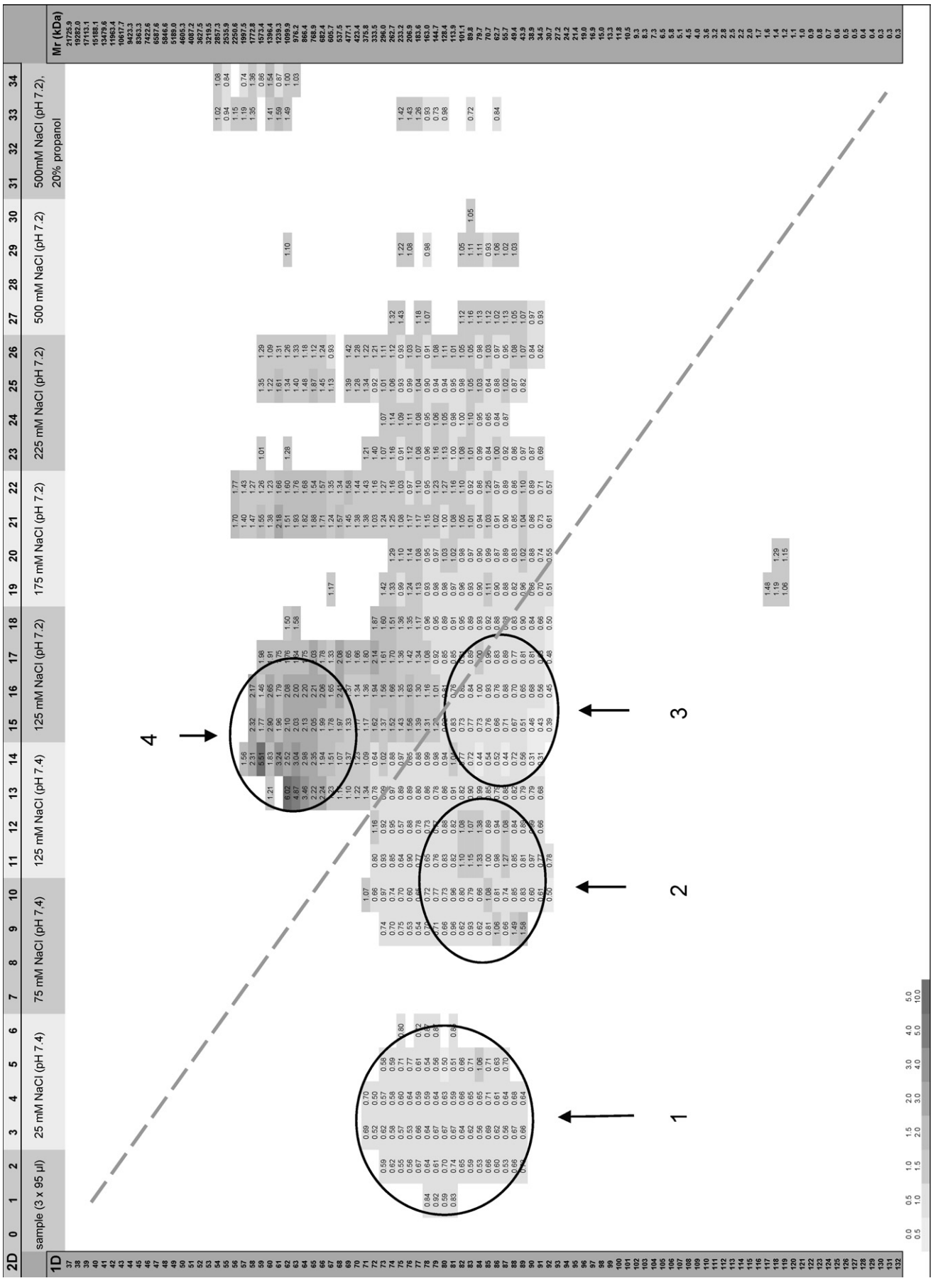
The mean value of the S_n quadruples of a protein was taken as a measure of concentration, if at least 10% of its theoretical tryptic peptides could be assigned to MS peaks according to the actual mass resolution. An in-house software package [1,24] was used. The package contains mono-isotopic peptide masses obtained by tryptic *in silico* digest of 2237 selected human protein entities that we have detected in selected fractions of patient and control serum samples up to now. MS analysis of selected fractions is the rate limiting step of candidate search. With the present equipment and software, applying both MS methods data evaluation needs several weeks per sample.

Undigested albumin was analyzed in the linear mode at 25 kV acceleration voltage, 90% grid voltage and 600 ns delay time using sinapic acid (20 mg dissolved in 1 mL of 45% acetonitrile, containing 0.1% trifluoroacetic acid) as matrix.

2.3.4. Confidence criteria designating a candidate biomarker

A protein or peptide found at a selected 2D-position (see above) is considered a candidate biomarker if the following conditions are fulfilled:

(a) The peptides were stringently identified at the selected position at least for the sample that yielded the highest total protein concentration at this position (the confidence criteria of Applied Biosystems (cf. http://www.db-


2D
1D
sample (3 x 95 µl)
25 mM NaCl (pH 7.4)
75 mM NaCl (pH 7,4)
125 mM NaCl (pH 7.4)
125 mM NaCl (pH 7.2)
175 mM NaCl (pH 7.2)
225 mM NaCl (pH 7.2)
500 mM NaCl (pH 7.2)
500mM NaCl (pH 7.2), 20% propanol
Mr (kDa)
1
2
3
4

thueringen.de/servlets/DocumentServlet?id=4054) were used for identification).

(b) Peptide founds (F_p), i.e. numbers of stringently identified tryptic peptides of the protein or peptide obtained at the selected 2D-position by ESI-MS/MS, differ at least by a factor of two between sample and control in case of Alport syndrome, or vary steadily with severity stage of disease in case of sepsis [cf. 23].

(c) At the selected 2D-position, the mean normalized height sum (S_n) of the tryptic peptides of the protein or peptide differs at least by a factor of two between fractions of sample and control in case of Alport syndrome, or S_n varies steadily with severity stage of disease in case of sepsis.

(d) The differences mentioned under b and c occur in the same direction at the selected 2D-position or a neighboring one in two randomly chosen sets of samples.

2.4. Immunoassays

Albumin immuno-reactivity was determined in 384-well microplates (Greiner Bio-One, Frickenhausen, Germany, no. 781094) according to ref. [27] with some modifications. After each incubation step, the wells were washed by dispensing and sucking 25 μL of PBS washing buffer (10 mM phosphate-buffer, pH 7.2, containing 0.140 M NaCl) containing 0.05% (v/v) Tween 20. A CyBi™-Well liquid handler with 384 channels performed five washing cycles. Twenty five microlitre of anti-human albumin antibody (DAKO Diagnostika, Hamburg, Germany) were applied per well (1 μg/mL dissolved in coating buffer: 15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0,02% (w/v) NaN_3; pH 9.6). After incubation overnight at 4 °C and washing, calibrator solutions (human albumin, Serva, 11860; concentration range 0–125 ng/mL) and samples diluted in PBS containing 1% (w/v) bovine serum albumin (BSA, Serva, 11926), and 0.1% Tween 20 so as to cover the linear range of the assay were incubated at ambient temperature for 90 min. After washing, 25 μL of goat anti-human albumin-HRP conjugate (A80-229P, Bethyl Lab., Montgomery, TX, USA) diluted 1:20,000 in PBS containing 1% BSA (Serva, 11945), 0.05% Casein (Sigma, C-3400), 5 mM Thiomersal (Roth, 6389.1), and 0.05% Tween 20 was applied and incubated at ambient temperature for 2 h. After washing, 25 μL of substrate solution (Supplementary Reagents for Enzygnost®/TMB, Behring-DADE, OUVP 17) was added, the reaction was stopped after 5–10 min and absorbance was recorded at 450 and 690 nm. An in-house program based on the pattern search algorithm [28] fitted the calibration curve to A_{450}–A_{690} calibration data and calculated albumin concentration within samples. Intra-assay precision was usually better than 10% CV. Recovery in 1D fractions of control and sepsis sera was 82.5% ($n = 2$) and 52.0 ± 27.7% ($n = 4$) and in 2D fractions, 66.9 ± 27.1% ($n = 4$) and 48.8 ± 17.1% ($n = 7$), respectively, referring to starting material. After separation had been completed, the albumin immuno-reactivity was shown to be stable in all fractions for at least 20 h at ambient temperature.

The concentration of A1BG was measured as previously described [29]. In short, samples and standards (pure A1BG ranging from 0–5 ng/ml) were diluted in carbonate buffer and incubated in 96-well flat-bottom immunoplates. After blocking with 1% BSA, rabbit anti-A1BG antiserum (Dade Behring, Marburg, Germany) was used to detect antigens followed by HRP-labeled goat anti-rabbit antibodies (Dako, Glostrup, Denmark), and visualized by color reaction with *o*-phenylenediamine and hydrogen peroxide. The concentration of CRISP-3 in samples was measured according to ref. [30], except that recently developed rabbit anti-CRISP-3 antibodies raised against native human CRISP-3 from granulocytes were used for capture and detection, and denaturation of samples was omitted.

3. Results/discussion

3.1. Alport syndrome

F_p and S_n of proteins stringently matched within 58 selected fractions were analyzed.

As expected serum 2D-fractions of Alport patients exhibit heavy alterations in protein concentration when compared with homologous fractions of age- and gender-matched controls as is exemplarily shown in Fig. 2. Decreased protein concentration is found in fractions situated below the dashed line. These fractions contain uncharged and minor charged proteins that are loosely bound to DEAE-cellulose. Albumin (dominating region 3), immunoglobulins (dominating region 1), and some apolipoproteins found below the dashed diagonal show markedly decreased levels in accordance with the reported nephrotic loss in Alport patients; a few examples are given in Table 2. Moreover, the protein concentration ratio, $c_{Alport}/c_{healthy}$, is smaller, with lower molecular-weight of the proteins in fractions situated above the dashed line (Fig. 2). A significant increase in protein concentration is found in high molecular-weight fractions containing also α-2-macroglobulin (encircled region 4 in Fig. 2). The transferrin level is increased in Alport samples (Table 2). This increase is evidently masked in the transferrin region of Fig. 2 (region 2) by altered levels of other proteins that coelute with transferrin. Gross alterations of serum protein composition that have been reported with nephrotic syndrome were used to confirm the reliability of the method, i.e., the decrease of a panel of intermediate molecular-weight proteins [31] such as immunoglobulins (Fig. 2) and albumin, and the regulatory increase of transferrin [32,33] and α-2-macroglobulin [34].

The proteins within Table 3 are shown here for the first time to be altered in patients with Alport syndrome. Some of them are increased in relation to global protein, others are found to decrease although the filtration loss has not been reported to apply to them. Among these proteins, the pregnancy zone protein, probably ubiquitin carboxyl-terminal hydrolase FAF-Y, and ADAMTS-5 could mark enhanced degradation processes; fibronectin, torsin-1B, and zinc finger protein 254 are likely to indicate ECM repair, and collagen α-1(VII) may indicate altered ECM turnover. Some cell membrane and nuclear proteins are elevated In comparison to control, probably reflecting enhanced cell turnover.

Some acute phase reactants, e.g., α-2-macroglobulin, hemopexin, and haptoglobin, are elevated, although even the high sensitivity c-reactive protein (CRP) was found with regular concentration in non-fractionated Alport samples (data not shown). This elevation may reflect the reported differential regulation and different kinetics of diverse sets of acute phase proteins [35–37] or simply accumulation due to high molecular-weight.

3.2. Severe inflammation

Since sera from different sepsis patients may differ greatly even when assigned clinically to the same stage of illness, comparing

Fig. 2. Alteration of protein distribution in serum of a patient with Alport syndrome. Serum samples of one healthy control (m/22 year) and patient 1 (cf. Table 3) were 2D fractionated in parallel using the robot [2]. Quotients of normalized protein concentration of homologous fractions ($c_{Alport}/c_{healthy}$) are indicated and coded by gray tones as well. Immunoglobulins dominate region 1, transferrin is found mainly in region 2, albumin dominates region 3, and α-2-macroglobulin is found in region 4. For details, see Section 3.

Table 2
Proteins and complexes or fragments thereof that are found to exhibit altered levels in patients in accordance with failure of the glomerular barrier function under Alport syndrome, therewith indicating validity of the platform.

Protein chain	Swiss-Prot ID	Fraction	S_n quotient	F_P quotient
Proteins with increased concentration				
α-2-Macroglobulin	P01023	59_14	3.20	1.94
Transferrin	P02787	84_11	19.48	≫1
Proteins with decreased concentration				
α-1-Antitrypsin (c)	P01009	84_14	0.28	0.36
α-1-Antitrypsin (f)		91_14	0.08	≪1
Albumin	P02768	84_14	0.18	≪1
Albumin (f)		90_14	0.08	≪1
Apolipoprotein A1 (c)	P02647	56_21	≪1	≪1
Vitamin D-binding protein	P02774	91_14	0.46	≪1

For each protein shown, the confidence criteria were fulfilled within numerous fractions. Data shown here represent typical examples. Quotients indicated in columns 4 and 5 were formed from data, Alport/healthy, obtained for the respective protein in homologous serum fractions. S_n quotient, quotient of normalized peak height sums [24]; F_P quotient, quotient of numbers of stringently identified/matched tryptic peptides (founds) as obtained with ESI-MS/MS [2]. 1D_2D-positions of fractions are indicated by two numbers, e.g., 59_14, respectively, in the column labeled "Fraction". Quotients with zero in the numerator or denominator are given as ≪1 or ≫1, respectively. (c) or (f) in column 1 indicate that the molecular-weight as deduced from SEC elution volume (1D) is markedly higher or lower than expected, indicating the appearance of a protein complex or fragment, respectively. All other proteins were found in the expected range of molecular-weight. α-2-Macroglobulin was included as an important serum component that was found elevated in many fractions, although its highest F_p quotient is slightly smaller than the limit, two, specified as a confidence criterion.

serum pools assigned to different stages, or mean and S.D. of serum sets, may lead to unreliable conclusions regarding the predictive power of their components. We avoid this unreliability by within-patient comparison, i.e., comparing differently staged sera of each single patient with each other. For patient 1 and 2, we obtained 3360 sets of three and four homologous 2D-fractions, respectively. Those with total protein content depending steadily on severity of illness were selected, a total of 69 sets. F_p and S_n of proteins stringently matched within the selected 2D-fractions were analyzed, 1150 proteins from serum fractions of patient 1 and 668 of patient 2.

Among these proteins, α-1-antitrypsin, α-1-antichymotrypsin, α-1-acid glycoprotein 1 and 2, C3, ceruloplasmin, hemopexin, haptoglobin, and α-2-macroglobulin increased with severity of disease, whereas albumin and transferrin decreased (data not shown). These alterations are reportedly related to inflammation and acute phase reaction.

Table 4 shows further protein entities that fulfill our confidence criteria for alteration and have not yet been described in this context and hence may be considered new candidate biomarkers. They are likely to reflect pathomechanisms in severe inflammation, such as (i) cell death accompanying multi-organ distress [13]

Table 3
Protein entities considered marker candidates for Alport syndrome which have not been previously reported.

Protein chain	Swiss-Prot ID	Location	Fraction	S_n quotient	F_P quotient
Proteins with increased concentration					
Fibronectin (c)	P02751	ECM, BP	65_25	18.14	≫1
Albumin (c)	P02768	BP	66_25	28.85	≫1
Hemopexin	P02790	BP	84_11	7.80	3.75
Complement factor H	P08603	BP	69_14	2.12	3.17
Haptoglobin (c)	P00738	BP	64_15	5.45	1.23
Pregnancy zone protein (c)	P20742	BP	63_15	2.43	11.5
Kinetochore-associated protein 1 (c)	P50748	C	69_14	2.01	13.50
Probable ubiquitin carboxyl-terminal hydrolase FAF-Y (c)	O00507	C	64_14	5.70	≫1
Torsin-1B (c)	O14657	ER	58_15	2.30	≫1
Properdin (c)	P27918	BP	84_11	≫1	≫1
Protein flightless-1 homolog (c)	Q13045	N	63_15	4.32	≫1
EGF-like module EMR2 (c)	Q9UHX3	CM	65_15	2.51	≫1
Myosin heavy chain 14 (c)	Q7Z406	C	68_14	≫1	≫1
Mas-related G-protein coupled receptor member F (c)	Q96AM1	CM	84_14	≫1	2.0
Zinc finger protein 254	Q86XL7	N	83_11	4.44	≫1
ADAMTS-5 (c)	Q9UNA0	ECM	64_16	19.92	≫1
Synphilin-1	Q9Y6H5	C	76_15	6.55	≫1
Proteins with decreased concentration					
α-1-acid glycoprotein 2 (c)	P19652	BP	57_21	0.09	≪1
Collagen α-1(VII) chain (f)	Q02388	ECM	87_14	0.38	≪1
N-acetylgalactosaminyltransferase II (c)	Q86X52	G	61_21	0.56	≪1
Galactose-3-*O*-sulfotransferase 3	Q96A11	G	90_14	0.24	≪1
GTPase-activating protein Spa-1 (f)	Q96FS4	C	90_14	0.46	0.18
26S proteasome regulatory subunit S1 (c)	Q99460	C	72_26	0.30	≪1

Representative data from two patients with Alport syndrome are summarized.
Serum samples from patients and age- and gender-matched healthy controls were 2D separated in parallel: patient 1, m/16 year, 63 μM serum creatinine, 35.3 mg/ml serum albumin, GFR: 187.9 ml/min/1.73 m^2, 60 erythrocytes/μl, proteinuria: per 1 mmol creatinine 479 mg total protein, 374 mg albumin, and 8 mg IgG and patient 2, f/9 year, 57 μM serum creatinine, 40.8 mg/ml serum albumin, GFR: 138 ml/min/1.73 m^2, 243 erythrocytes/μl, proteinuria: per 1 mmol creatinine 60 mg total protein, 35 mg albumin, and 3.3 mg IgG.
For details see legend to Table 2. Protein locations are indicated according to www.expasy.org. CM, cytoplasma membrane; C, cytosol; BP, blood plasma; ECM, extra cellular matrix; M, mitochondria; N, nucleus; G, Golgi apparatus. As N-acetylgalactosaminyltransferase II is an important ECM metabolizing enzyme, it was included although its S_n quotient is slightly greater than the limit, 0.5, specified as a confidence criterion. Haptoglobin was included because it is an interesting type II-acute phase protein similar to α-2-macroglobulin.

Table 4
Protein entities considered marker candidates for severe inflammation, which have not been previously reported.

Protein chain	Swiss-Prot ID	Pat.	Cell location	Tissue specificity	Fraction	S_n quotient	F_P quotient
Proteins with increased concentration							
Puromycin-sensitive aminopeptidase (f)	P55786	1	C	–	86_18	8.2	≫1
Transcription initiation factor TFIID subunit 4 (f)	O00268	1	N	–	88_22	31.1	≫1
α-taxilin	P40222	2	C	–	86_17	6.0	5.0
Cytoplasmic acetyl-CoA hydrolase 1 (monomer)	Q8WYK0	2	C	Liver	86_21	1.7	≫1
Tryptophan 5-monooxygenase 2	Q8IWU9	2	n.f.	Brain	86_17	2.6	≫1
Microtubule-associated protein 1B (f)	P46821	2	C	Neurons	86_17	2.0	≫1
Transmembrane protease, serine 13	Q9BYE2	2	CM	–	86_21	2.2	1.7
ADP-ribosylation factor GTPase-activating protein 3	Q9NP61	2	C	–	86_17	1.7	≫1
ATP- dependent helicase SMARCA2 (f)	P51531	2	N	–	86_17	1.7	≫1
Centaurin-delta 1 (f)	Q8WZ64	2	C	–	86_17	2.7	≫1
TNFR- related death receptor-6 (f)	O75509	2	CM		86_17	2.7	≫1
RAS protein activator-like 1 (c)	Q9UJF2	2	C	–	73_10	1.8	≫1
GMP-PDE beta (f)	P35913	2	CM	Retina	86_17	4.1	≫1
Ran-binding protein 17 (c)	Q9H2T7	2	C, N	–	74_2	2.0	≫1
Protein C6orf165	Q8IYR0	2	n.f.	n.f.	86_17	2.1	≫1
Synapse-associated protein 90 (f)	P78352	2	CM	Brain	91_22	2.3	2.0
Cadherin, neural type, 2	P55289	2	CM	Brain	86_17	4.9	1.3
26S proteasome non-ATPase regulatory subunit 1 (f)	Q99460	2	C	–	91_22	3.0	≫1
Coatomer beta subunit (f)	P53618	2	C	–	86_17	3.3	3.0
Proteins with decreased concentration							
α-1B-glycoprotein	P04217	1	BP	Liver, secretory	83_14	≪1	≪1
Leucine-rich α-2-glycoprotein	P02750	1	BP	Liver*, secretory	88_14	0.014	≪1
ADAMTS-12 (f)	P58397	1	ECM	–	69_13	0.4	≪1
Protein flightless-1 homolog (c)	Q13045	1	N, C	–	70_13	0.012	≪1
Isocitrate dehydrogenase [NAD] subunit α	P50213	1	M	–	89_14	0.018	≪1
SCAN domain-containing protein 2 (c)	Q9GZW5	1	N	–	67_13	≪1	≪1
SCAN domain-containing protein 2	Q9GZW5	1	N	–	87_21	0.4	≪1
Multidrug resistance-associated protein 4 (f)	O15439	1	CM	–	86_22	0.5	≪1
Vitronectin (c)	P04004	1	BP, ECM	–	77_17	0.3	≪1
Infertility-related sperm protein Spag-1 (f)	Q07617	1	C	Testis	86_23	0.2	≪1
Albumin (f)	P02768	1			88_18	0.3	≪1
Albumin (f)		2	BP	Liver, secretory	91_16	0.2	≪1
Kininogen-1	P01042	2	BP	Liver, secretory	86_17	0.5	≪1
Exportin-1 (f)	O14980	2	C, N	–	84_2	0.4	≪1
Calbindin (c)	P05937	2	C	–	74_2	0.4	≪1
Proteins with increased and decreased fractional concentrations							
Vitamin D-binding protein (c2)	P02774	1	BP	Liver, secretory	85_14	890	≫1
Vitamin D-binding protein (c1)				–	83_14	≪1	≪1
Elongation factor 1-beta (c2)	P24534	1	C	–	77_18	0.3	≪1
Elongation factor 1-beta (c1)		2		–	74_2	11.0	≫1
Aggrecan core protein (f1)	P16112	1	ECM	Cartilage	80_22	2.4	≫1
Aggrecan core protein (f2)		1		–	90_14	0.03	≪1
Mitochondrial intermediate peptidase (c1)	Q99797	1	M	–	69_13	3.1	≫1
Mitochondrial intermediate peptidase (c2)		1		–	59_32	0.4	≪1

Confidence criteria were fulfilled for each patient for pairs of adjacent states of illness.
Data shown here belong to the strongest alterations found. Quotients, higher/lower severity, indicated in columns 7 and 8 were formed from data belonging to adjacent degrees of severity which were obtained for the respective protein in homologous serum fractions. Notations see Table 2.
Locations were taken from www.expasy.org: CM, cytoplasma membrane; C, cytosol; BP, blood plasma; ECM, extra cellular matrix; M, mitochondria; N, nucleus; *, probably also secreted by neutrophil granulocytes [55]; n.f., location not found; Pat., patient.

(cf. Table 4, nuclear, cytosolic, mitochondrial and plasma membrane proteins), (ii) degranulation (leucine-rich α-2-glycoprotein), and (iii) matrix degradation (aggrecan core protein, vitronectin, ADAMTS-12). Cytosolic Puromycin-sensitive aminopeptidase is not only an indicator of cell death by necrosis, but it is also related to apoptosis induction [38] and antigen processing [e.g. 39]. Table 4 contains mitochondrial marker candidates for sepsis, i.e., isocitrate dehydrogenase [NAD] and intermediate peptidase, whereas Struck et al. [40] have reported another mitochondrial constituent, mitochondrial carbamoyl phosphate synthase instead.

Vitamin D-binding protein and vitronectin are multifunctional proteins that show great promise as biomarkers. In addition to transporting vitamin D, vitamin D-binding protein scavenges actin, activates macrophages, captures endotoxin, and regulates angiogenesis and immune responses [41,42]. Two complexes containing vitamin D-binding protein were observed to vary in different way in concentration (Table 4). The serum level of free vitamin D-binding protein has been reported to decrease under non-survival multi-organ failure. [e.g. 43]; complexed entities of the protein, however, may vary in different way because they may partake in different reactions. The masses of both altered protein forms (complex 1: 67 kDa, complex 2: 86 kDa) found do not match those of complexes formed of monomeric or polymeric actin plus free vitamin D-binding protein nor the free vitamin D-binding protein itself. Vitronectin has been described as a positive acute phase reactant and member of the hemopexin super-family. Vitronectin is involved in regulating pericellular proteolysis, hemostasis, complement dependent immune response and T-cell response, angiogenesis, cell adhesion and motility, and activating PAI-1 [e.g. 37,44–46]. Thus it is likely to be involved in the systemic dysregulation of coagulation, endothelial function and immune defense in sepsis. Besides confirming the reported increase of the monomer (data not shown), however, a high

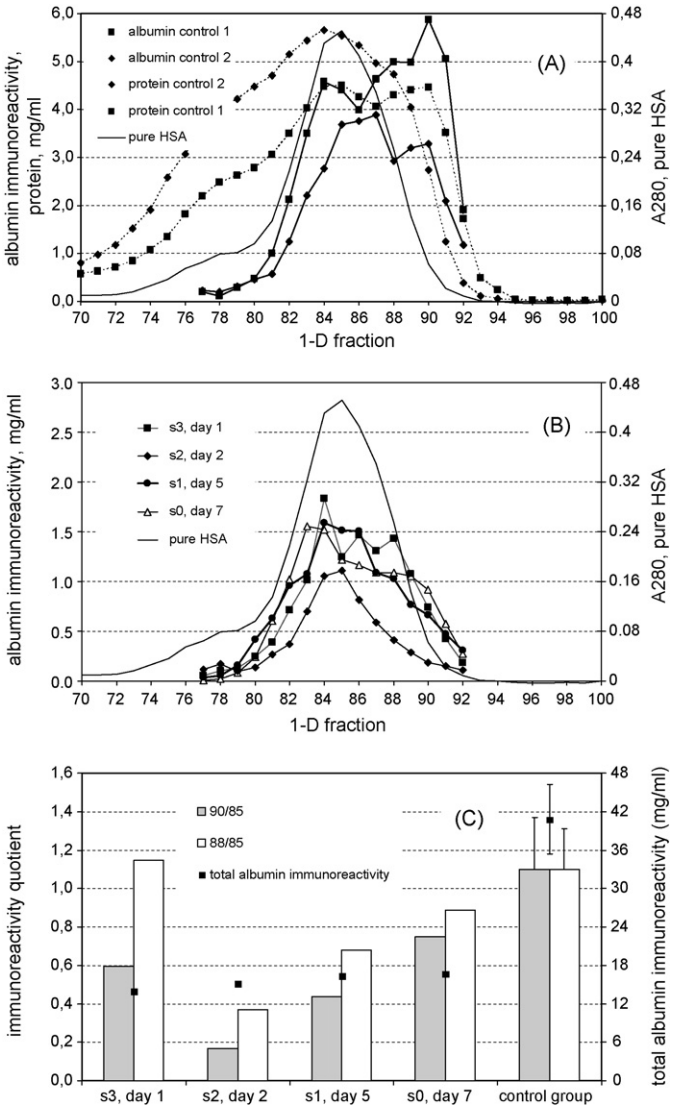


Fig. 3. Distribution of albumin immuno-reactivity within 1D-fractions of normal and septic sera. (A) SEC chromatograms of pure HSA and sera from two healthy controls; control 1, m/45 year; control 2; f/30 year; (B) SEC chromatograms of pure HSA and four sera from patient 2; (C) albumin immuno-reactivity in serum fractions and non-fractionated sera from patient 2 and controls. The control group consists of 4 samples, i.e., control 1, control 2, control 3 (m/64 year), and pooled serum of 10 healthy volunteers (6 f/4 m, 61.4 ± 7.8 years); septic sera s3-s0 are from patient 2, f/70 year, cf. Table 1. Albumin concentrations in non-fractionated and 1D-separated sera were determined by ELISA in quadruplicate (see Section 2.4). For quotients of albumin concentrations in different 1D-fractions, mean values of quadruplicates were taken. The fraction numbers refer to nominator and denominator, they are indicated in the inset. Albumin concentrations are given as mean values of quadruples (1D-fractions of patient 2) and mean value ± standard deviation of the mean values indicated by antennas (control group).

molecular-weight variant of the protein as seen from the SEC fraction number was found here to decline under severe inflammation (Table 4).

The distribution of albumin immuno-reactivity was determined by immunoassay in fractions of sera originating from two patients at various days of illness and six controls including one pool of 10 healthy persons. Surprisingly, 1D separation of all control sera revealed two albumin species differing in apparent mass. Examples are shown in Fig. 3. One albumin species (1D-fractions 84–86) exhibits 67.5 ± 2.2 (n = 6) kDa as determined for 1D-fraction 85 by column calibration, thus fitting the albumin monomer. This species coelutes with pure fatty-acid-free human albumin; its MALDI-MS peaks cover a mass range from 65800 to 67800 Da with a main peak mass of 66453 ± 113 Da (n = 4). This species is also found in sepsis sera with a main peak mass of 66480 ± 166 Da (n = 4; data not shown). The other albumin species elutes in 1D-fractions 88–91, exhibiting an apparent molecular-weight range from 47 to 33 kDa according to column calibration. Surprisingly, fraction 90 shows one dominant protein only, with a normal monomeric albumin mass as determined by MALDI-MS and SDS-PAGE. Moreover, normal monomeric albumin mass was also found by MALDI-MS and SDS-PAGE for albumin from both fractions 85 (normal apparent mass) and 90 ("low" apparent mass) after purification on anti-human albumin gel (Vivapure Anti-HSA-kit). Additionally, the coverages of albumin peptide mass fingerprints were equal in both these purified fractions. These data suggest the presence of a modified albumin species within fraction 90 rather than the presence of a low-molecular-weight fragment. This species obviously interacts more strongly with the SEC column matrix and could possibly be nicked and/or extended in shape. Work is currently in progress to elucidate its molecular nature. Nevertheless, the particular apparent low-molecular-weight species of albumin is lost in sepsis sera to a higher extent than is total albumin (Fig. 3). Most proteomics procedures remove high abundant proteins from serum prior to analyzing the sample. Among these proteins, however, albumin was found here to deserve closer attention. Albumin heterogeneity has been reported for naturally occurring high molecular-weight aggregates [e.g. 47] and minor molecular-weight alteration [e.g. 48,49]. Due to ligand binding one may expect not only molecular-weight variants but also conformation heterogeneity [50,51].

Homologous 2D-fractions from samples of increasing severity exhibited decreasing A1BG (Table 4). Its physiological agonist CRISP-3 was identified by MS in 2D-fractions, but S_n could not be quantified by our equipment. The immuno-reactivity of both A1BG and CRISP-3 was determined in non-fractionated samples (Fig. 4). Both protein levels indeed were much lower at all inflammatory states. Because of the limited number of samples at hand, however, different degrees of severity could not be discriminated and a more thorough evaluation is needed. A1BG acts as a binding protein for CRISP-3, a neurotoxin-like substance secreted from neutrophilic granulocytes [29,52], and may thereby prevent CRISP-3 from affecting systemic processes during innate host defense. Possibly, formation of complexes interferes with the immuno-quantitation of both proteins as shown for leucine-rich α-2-glycoprotein 1 and the apoptotic marker cytochrome c [53]. Contrarily, in non-survivors A1BG has been shown to increase in serum [19]. After our above reported analyses were completed, [54] published results found with a proteomic approach different from ours; they confirmed the decrease of A1BG and leucine-rich α-2-glycoprotein in sepsis; however, some alterations they found were opposite to those reported here, e.g., with increasing severity, transferrin increased instead of decreased and vice versa for kininiogen 1, hemopexin, haptoglobin, and ceruloplasmin.

The results presented were obtained after primary selection of serum fractions with total protein content (i) above 0.1 mg/mL as required for subsequent analysis and (ii) depending in a clear-cut manner on stage of disease, thus indicating presence of possible markers. Although condition (i) will limit the number of fractions considered and condition (ii) may render fractions with minor or opposite variations of component levels overlooked, the analysis revealed that a remarkable number of components hitherto not considered may be biomarker candidates, among them are also proteins with minor concentration. However, the time needed for analysis would not permit hundreds of subjects as

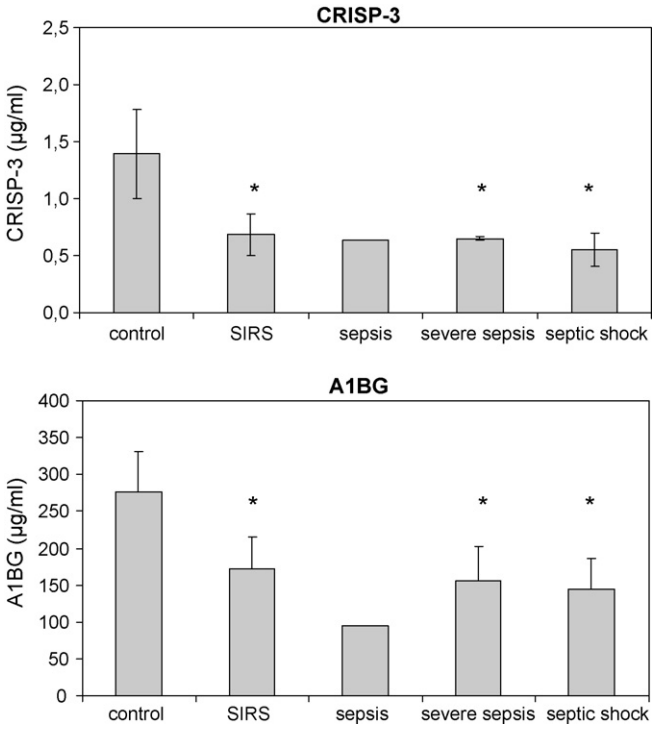


Fig. 4. Immuno-reactivity of CRISP-3 and A1BG under severe inflammation. Concentrations are given as mean ± standard deviation indicated by antennas. Concentrations that are significantly lower than the control ($p < 0.01$, Studentïs t-test using SPSS for Windows 14.0) are marked by an asterisk. The following numbers of non-fractionated samples were tested: control, 96; SIRS, 12; sepsis, 1; severe sepsis, 2; and septic shock, 11.

necessary for clinical validation. Nevertheless, with the search strategy proposed even with a small number of probands of both model diseases, a considerable set of known serum alterations could be rediscovered. Additionally, alteration of two candidate biomarkers selected by MS quantitation could be confirmed by immune assays indicating the platform suitable for biomarker discovery. Thus, the new biomarker candidates presented here have to be evaluated carefully with respect to reproducibility, accuracy, sensitivity, and their diagnostic and/or prognostic value applying independent methods to a number of non-fractionated samples sufficient to obtain statistically significant results.

4. Conclusion

Orthogonal combination of classical non-denaturing chromatographic procedures for analyzing fractions of normal and patient sera by MS and immuno-reactivity proved to yield new biomarker candidates for renal failure and severe inflammation. Although only fractions with total protein above 0.1 mg/mL were selected for analysis, also some minor serum components could be shown to vary according to the stage of illness, i.e., to be marker candidates. The separation and quantitation methods introduced have to be combined with high-throughput techniques for re-enrichment of fractions that were diluted by each separation step, and MS equipments with higher sensitivity, throughput and mass resolution in order to enhance biomarker yield.

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