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PAPER

A proteomic approach to identify plasma proteins in patients with abdominal aortic aneurysm

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Our aim was to identify the key proteins involved in the pathogenesis of AAAs. To explore the possible pathogenetic mechanisms involved in AAA, we analyzed by proteomics modifications in plasma proteome of patients with AAA. Therefore, the present study analyzed the soluble plasma proteins using two dimensional electrophoresis (2-DE) and mass spectrometry (MS). We identified 33 protein spots, 31 of which show an up-regulation in AAA patients whilst the expression level of 2 protein spots is reduced. We confirm a number of biomarkers associated with AAA that have been previously identified by various authors. We identified a significant increase of a class of proteins such as fibrinogen, α1-antitrypsin and haptoglobin in plasma from AAA patients. The presence of these proteins in human AAA plasma may be related to the inflammatory processes active in these subjects. We have seen a negative correlation between the vitamin D-binding protein (DBP) and hemoglobin subunit β and AAA presence. DBP levels have been found to increase in AAA wall tissues by others and this discrepancy with our results could be due to the different analysis source. We wanted to analyze the factors measurable in plasma-associated rather than in tissue-associated markers because the application of circulating biomarkers in diagnostic laboratories would be relatively simple. DBP is very important for vascular remodelling and it may have an important role in the protection of vascular walls. In plasma tissue this protein reduces platelet aggregation and extends coagulation time. No one protein identified in this study has the biologic plausibility to be used singularly as a biomarker of aneurysmal disease due to inadequate specificity. The effect of using multiple biomarkers combined with clinical factors requires investigation in carefully designed population-based studies and these studies need to select the criteria of choice to define healthy controls very carefully. Clearer identification of various markers is needed, possibly using other proteomic techniques to screen for new candidates such as gel-free proteomic technology that enables us to handle larger groups of subject compared to gel-based proteomic technology.

Introduction

Abdominal aortic aneurysm (AAA) affects 4%–8% of men and 1.5% of women over age 60 years and usually remains asymptomatic until rupture.[1] AAA rupture is a medical emergency with a mortality rate as high as 80%–90%, 40–50% of deaths occurring before reaching the hospital. Because elective surgical repair has mortality rates less than 5%, great interest exists for population screening which is currently based on ultrasonography (US), a highly accurate, and noninvasive screening tool. However, although US screening of men aged 65 or more can indeed reduce mortality from ruptured AAA,[2] a US based national screening programme may also result in identifying a large number of patients unfit for surgery but requiring a continued observation. Therefore, the need for additional strategies to refine cost effectiveness of screening programmes is currently advocated.[3]

A number of biomarkers associated with AAA presence such as plasma fibrinogen, D-dimer, and IL-6 have now been identified in cross-sectional case–control studies. Most studies have not assessed the validity of these markers as diagnostic tests for AAA because sensitivity and specificity appear to be inadequate when hypothesizing the use of one single biomarker for diagnosis.[4] The effect of using multiple biomarkers combined with clinical factors requires investigation in large, carefully designed population-based studies. Proteomics has been extensively employed to investigate cancer and other

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diseases, but there are currently no reports concerning the proteomic study of plasma from patients affected by abdominal aortic aneurysm. Very recently Urbonavicius *et al*. were the first to identify proteins associated with the size of the AAA by proteomic analysis of the vessel walls.[5] They report positive correlations between GAPDH, annexin A4, a fragment of transforming growth factor-β-induced protein ig-h3 (TGFBIp)/β ig-h3 and the expansion of AAA. They also found a negative correlation between apolipoprotein H, fibrinogen β chain, ApoA-I and albumin and AAA size. Some protein spots were of very low abundance or were ambiguously identified.

To explore the possible pathogenetic mechanisms involved in AAA, we analyzed by proteomics modifications in plasma proteome of patients undergoing aortic aneurysm repair. Our aim was to identify the key proteins involved in the pathogenesis of AAAs. Therefore, the present study analyzed the soluble plasma proteins using two dimensional electrophoresis (2-DE) and mass spectrometry (MS). We identified 33 protein spots, 31 of which show an up-regulation in AAA patients whilst the expression level of 2 protein spots is reduced.

Materials and methods

Subjects investigated

Plasma from 8 patients scheduled for abdominal aortic aneurysm (AAA) repair through elective aortic reconstructive surgery was investigated. The control group included 6 age-matched volunteers. Exclusion criteria were diabetes, evidence of peripheral vascular disease, recent history of myocardial infarction, chronic intestinal inflammatory disease, widespread cancer, left ventricular ejection fraction inferior to 40%, symptomatic congestive heart failure, significant segmental wall motion abnormalities prior to dobutamine stress echocardiography, new wall motion abnormalities detected during dobutamine stress echocardiography and inability to understand informed consent. Experimental protocols were approved by the local Ethical Committee and written consent was obtained from each enrolled subject.

Experimental protocol

Venous blood samples (2 ml) were collected in K_2EDTA-containing tubes on the day of their admission to General Hospital. Blood was centrifuged at 4 °C, 1000 *g* for 10 min, and the isolated plasma samples were stored at −80 °C until use.

Protein concentration of each sample was determined by the standard Bradford method (Bio-Rad).

Two-dimensional electrophoresis

An aliquot of 6.25 µl of plasma was mixed with 10 µl of a solution containing 10% (w/v) sodium dodecyl sulfate (SDS) and 2.3% (w/v) dithioerythritol (DTE). The sample was heated to 95 °C for 5 min and then diluted to 500 µl with a solution containing 8 M urea, 4% (w/v) 3-cholamidopropyl dimethylammonium-1-propane sulfonate (CHAPS), 40 mM Tris, 65 mM DTE.[6] Isoelectric focusing (first dimension) was carried out on nonlinear wide-range immobilized pH gradients (IPGs; pH 3.0–10 NL; 18 cm-long IPG strips; GE Healthcare, Uppsala, Sweden) and achieved using an EttanTM IPGphor™ system (GE Healthcare, Uppsala, Sweden). IPG strips were rehydrated with 60 µg of the plasma sample for analytical run, and with 400 µg of plasma for preparative-run, diluted in 350 µl of rehydration buffer containing 8 M urea, 4% (w/v) CHAPS, 65 mM DTE, and 0.5% IPG buffer with the same pH range as the Immobiline DryStrips and a trace of bromophenol blue, for 1 h at 0 V and for 8 h at 30 V, at 20 °C. The strips were focused according to the following electrical conditions at 16 °C: 200 V for 1 h, from 300 to 3500 V in 30 min, 3500 V for 3 h, from 3500 to 8000 V in 30 min, and 8000 V until a total of 80 000 V per h was reached. After isoelectric focusing separation, the strips were equilibrated with 10 ml of a solution containing 50 mM Tris-HCl pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 2% (w/v) DTE for 12 min and with 10 ml of a solution containing 50 mM Tris-HCl pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 2.5% (w/v) iodoacetamide and a trace of bromophenol blue for 12 min. The second dimension was carried out on 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) at 10 °C and 40 mA per gel constant current until the dye front reached the bottom of the gel. Analytical gels were stained with ammoniacal silver nitrate.[7] The exposure time for silver staining was optimized to avoid overexposure of some gels in comparison to others. The mass spectrometry preparative gels were stained with colloidal Coomassie blue G-250 (Sigma-Aldrich).

Image analysis and statistics

Two-DE images were scanned by using the Epson expression 1680 PRO scanner. For each investigated subject, three technical replicates were performed and only spots present in all the replicates were taken into consideration for subsequent analysis.

A cut-off limit was considered to limit interference due to individual physiological changes, in particular each spot intensity volume was processed by background subtraction and then normalized between gels as a proportion of total protein intensity from entire gel using ImageMaster 2D Platinum 6.0 software (GE Healthcare). Relative spot volume (%V) ($V_{\text{single spot}}/V_{\text{total spots}}$, where V is the integration of the optical density over the spot area) was used for quantitative analysis in order to decrease experimental errors. The mean values of the %V were compared among the two groups (AAA patients and age-matched volunteers) by Student's *t*-test analysis of variance (ANOVA) and by non-parametric Wilcoxon rank-sum test (for continuous variable), using the Graphpad Prism4 software. The spots selection criteria for MS were: (1) fold-change to select differentially expressed protein spots. Regarding the error range of spot detection which is determined by evaluating the reproducibility of 2-DE gels, we set the threshold at $\geq$ 2-fold change for considering a protein spot differentially expressed (a variation of the mean %V of above 2-fold among the two groups); (2) p-value $\leq$ 0.05 to select differentially expressed protein spots that displayed a statistically significant variation of the mean %V among the two groups. p-value was calculated by Student's *t*-test and by non-parametric Wilcoxon rank-sum test (for continuous variable), using the Graphpad Prism4 software. Only the differentially expressed protein spots with a threshold of $\geq$ 2-fold change and with a p-value $\leq$ 0.05 were selected for mass spectrometry analysis.

Protein identification by MALDI-TOF mass spectrometry

Protein identification was carried out by peptide mass fingerprinting on an Ettan matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) Pro mass spectrometer (Amersham Biosciences), as previously described.[8,9] Electrophoretic spots, visualized by the colloidal Coomassie staining protocol, were manually excised, destained, and acetonitrile dehydrated. Successively, they were rehydrated in trypsin solution and in-gel protein digestion was performed by an overnight incubation at 37 °C. From each excised spot, 0.75 μl of recovered digested peptides were prepared for MALDI-TOF MS by spotting them onto the MALDI target, allowing them to dry and then mixing them with 0.75 μl of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid). After application of the matrix to the dried sample and drying, tryptic peptide masses were acquired. Protein identification by Peptide Mass Fingerprints search was performed using MASCOT version 2.2 as the search engine (Matrix Science, London, UK, http://www.matrixscience.com) through the Swiss-Prot/UniprotKB database, release 57.11, 24 November 2009.

Protein identification was achieved on the basis of corresponding experimental and theoretical peptide-fingerprinting patterns. Search parameters were: peptide mass tolerance of 100 ppm; a single trypsin missed cleavage; alkylation of cysteine by carbamidomethylation as fixed modification, and oxidation of methionine as variable modification. The query was first opened to the entire database in order to highlight any artifactual identification and after that restricted to human proteins. The criteria used to accept identifications included the extent of sequence coverage, the number of matched peptides, and the probabilistic score obtained by using Mascot software, as reported in Table 3. In detail, the identifications were accepted as positive when at least 4 matching peptides and at least 10% peptide coverage of theoretical sequences matched, setting the probabilistic score at $p < 0.05$. Regarding the probabilistic score, the threshold Mascot MS protein score greater than 56 was considered significant (p-value < 0.05). (The Mascot score is $-10\log(P)$, where P is the probability that the observed match is a random event.) Tryptic digests that did not produce MALDI-TOF unambiguous identifications were subsequently acidified with 2 μl of a 1% trifluoroacetic acid solution, and then subjected to electrospray ionization (ESI) ion trap MS/MS peptide sequencing using an LCQ DECA ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Acidified samples were enriched using ZIP-TIPTM pipette tips for sample preparation (Millipore, Billerica, MA, USA), previously equilibrated in 50% acetonitrile solution and abundantly washed in 0.1% trifluoroacetic acid. Tryptic peptide elution from the ZIP-TIPTM matrix was achieved with a 70% methanol and 0.5% formic acid solution; and 3 μl of concentrated sample solution was then loaded into the nanospray needle. MS/MS database searching was performed by Mascot MS/MS ion search software (http://www.matrixscience.com) in the Swiss-Prot/UniprotKB database (release 57.11, 24 November 2009). The following criteria were applied: MS accuracy ±1.2 Da, MS/MS mass accuracy ±0.6 Da, peptide precursor charge 2+, monoisotopic experimental mass values, trypsin digestion with one allowed missed cleavage, fixed carbamidomethylation of cysteine and variable oxidation of methionine. The probabilistic score for the LC-MS/MS analysis was obtained using Mascot software. The Mascot individual ions score greater than 35 was considered significant (p-value < 0.05). (Ions score is $-10\log(P)$, where P is the probability that the observed match is a random event.)

Results

Subjects

Table 1 summarizes the clinical characteristics of the eight AAA patients and the six control subjects enrolled for the present study. The patients were on waiting list for elective aortic reconstructive surgery. The overall mean age of the AAA group and the age-matched control group is 65.6 ± 6.9. However the age of AAA patients was slightly higher than that of the control group. Values of smoking, blood pressure and heart rate were higher among patients with AAA. No other cardiovascular risk factors differed between the groups. No patients received cardiovascular drugs or other support of cardiac function during the observation period. The anthropometric parameters did not differ between the two types of subjects.

Plasma protein profiles of AAA and control subjects

Protein profiles of plasma samples from eight patients with abdominal aortic aneurysm (AAA) and from six healthy controls were analyzed by 2-DE. For each investigated subject, plasma proteins were separated by 2-DE gel and the resulting silver-stained gels were analyzed using ImageMaster 2D Platinum version 6.0 (GE Healthcare). To obtain statistically significant results, each plasma protein sample was run in triplicate. Relative spot volume (%V) ($V_{\text{single spot}}/V_{\text{total spots}}$, where V is the integration of the optical density over the spot area) was used for quantitative analysis in order to decrease experimental errors. The circulation half-life of low molecular weight plasma proteins is directly related to their binding affinity to high abundant carrier proteins.[10,11] This observation has been supported by proteomic based studies that have detected a significant amount of low molecular weight proteins in specific carrier protein fractions (serum albumin).[12] To avoid an alteration of proteomic results we decided not to remove highly abundant proteins such as albumin and IgG.

Table 1 Clinical characteristics of the investigated groups

	Abdominal aortic aneurysm group (n = 8)	Age-matched control group (n = 6)
Male/female	6/2	6
Age, years	68 ± 6	62 ± 7
Weight, kg	72 ± 8	68 ± 11
Height, cm	170 ± 5	176 ± 6
Systolic blood pressure, mmHg	128 ± 9	100 ± 12
Diastolic blood pressure, mmHg	78 ± 10	70 ± 5
Heart rate, beats per min	71 ± 10	68 ± 8
Smokers (n)	6	2

This would cause the removal of a broad range of other low mass and low abundant physiologically important regulatory and/or transient proteins.[13] Fig. 1 shows a representative two dimensional gel image of AAA plasma (panel A) and of control plasma (panel B). Approximately 900 spots in each silver nitrate-stained gel, ranging from 10 to 200 kDa with a pH between 3 and 10, can be visualized by ImageMaster software. When the 2-DE gels of plasma proteins from the patients were compared with those from healthy controls, many chains of spots that may represent different degrees of protein post-translational modifications (PTMs) and/or different isoforms of the same protein were found to be significantly over-expressed. When gels corresponding to the plasma proteins from the healthy subjects were used as reference in 2-DE gel analysis, the densities of 33 protein spots were found to be changed in the gels corresponding to plasma proteins from AAA patients.

Identification of differentially expressed proteins

The comparative analysis revealed that in AAA patients 33 protein spots were differentially expressed by approximately $\geq$ 2-fold change and with a p-value ≤ 0.05. The locations of these 33 differentially expressed protein spots are marked with arrows and numbers in the representative gels shown in Fig. 1. Of these spots, 31 were up-regulated and 2 down-regulated in comparison to the healthy subjects. A list of the up- and down-regulated proteins as well as their biological functions according to their Gene Ontology (GO) biological process is summarized in Tables 2 and 3. Table 2 reports all the identified proteins, their relative amounts expressed as the mean $\pm$ standard deviation of the relative spot volume (%V) ($V_{\text{single spot}}/V_{\text{total spots}}$, where V is the integration of the optical density over the spot area) and statistical analysis (fold change $\geq$ 2 and p-value < 0.05). A group of 4 protein spots (7, 15, 18 and 23) show a significant increase using the ANOVA test (see Table 3), in contrast they show a non-significant increase (p-values: 0.0526, 0.0704, 0.0593 and 0.0810 respectively) using the non-parametric Wilcoxon test. Despite this discrepancy these 33 differentially expressed protein spots were excised from preparative Coomassie-stained 2-DE gels, in-gel digested with trypsin, and subsequently analyzed by MS. All identified proteins are described in Table 3. As shown in the table the sequence coverage (number of the identified residues/total number of amino acid residues in the protein sequence) of the proteins identified, by peptide mass fingerprinting, ranges from 12% to 72%, depending on the size and amount of each protein. The identification of protein spot 21 with low protein quantity was further confirmed by electrospray ionization (ESI)–ion trap MS/MS peptide sequencing, the relative results are reported in Table 3. It is known that plasma proteins are affected by post-translational modifications. Among the 33 protein spots we found several isoforms of nine proteins (α1-antitrypsin, complement factor B, serotransferrin, fibrinogen α chain, fibrinogen γ chain, hemopexin, haptoglobin α chain, haptoglobin β chain, ApoJ) that differed in the molecular weight and/or in the pI (values reported in Table 3). These modifications could be due to protein degradation or to post-translational modifications such as glycosylation, phosphorylation, oxidation, disulfide bond formation. Using Mascot research we explored the presence of such PTMs. We obtained results with a significant Mascot MS score for all proteins using oxidation of methionine as variable modification in the research parameter. For example α1-antitrypsin (spot 2) was more oxidised than spot 1 (5 residues instead of 1); fibrinogen α chain, spots 10, 11, 12 differ also in oxidation rate; apolipoprotein J, spot 27 is oxidised unlike spot 28. Moreover as reported by Chalkley R.J. *et al.*[14] there are some flaws in the current search engines software used for PTMs: they mostly apply an intensity threshold to the peak list to limit 'noise peaks' and this can eliminate peaks that contain basal

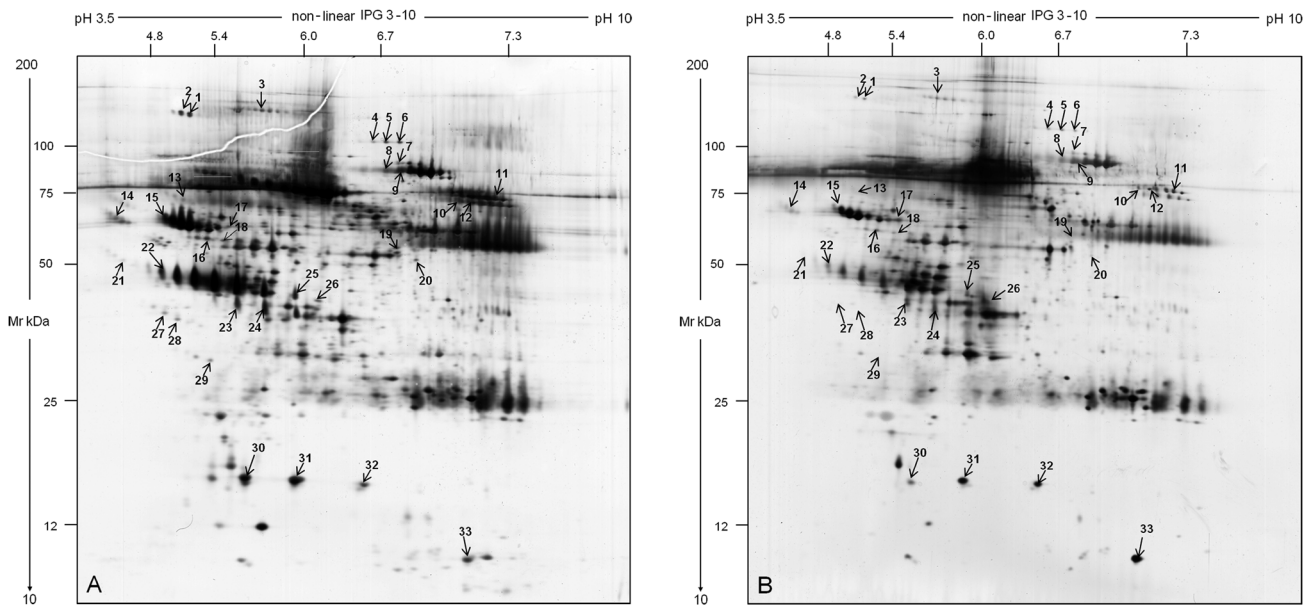

Fig. 1 Representative silver-stained gels of plasma proteins. Arrows and numbers indicate protein spots identified by MS. (A) Representative 2-DE gel of AAA plasma. (B) Representative 2-DE gel of control subject plasma.

Table 2 Quantitative data and statistical analyses of protein spots whose intensity levels significantly differed among plasma of patients with AAA and age-matched control group

Spot	Average spot volume% in AAA patients (arbitrary units)[a]		Average spot volume% in age-matched control group (arbitrary units)[a]		Volume ratio (AAA/control group)	ANOVA (p value)[b]	Wilcoxon (p value)[c]
	Mean	SD	Mean	SD			
1	0.1311	±0.0526	0.0619	±0.0294	2	0.0138	0.0127
2	0.1159	±0.0510	0.0542	±0.0277	2	0.0205	0.02
3	0.0716	±0.0375	0.0259	±0.0087	2.8	0.0131	0.0007
4	0.0718	±0.0404	0.0234	±0.0079	3	0.0141	0.02
5	0.1448	±0.0896	0.0577	±0.0299	2.5	0.0427	0.028
6	0.0908	±0.0461	0.0342	±0.0239	2.6	0.0186	0.0008
7	0.1253	±0.0668	0.0418	±0.0191	3	0.0122	0.0526[d]
8	0.2467	±0.0988	0.1025	±0.0301	2.4	0.0050	0.02
9	0.4741	±0.1389	0.2124	±0.0744	2.2	0.0013	0.0007
10	0.5053	±0.2235	0.2487	±0.1021	2	0.0234	0.033
11	0.1138	±0.0499	0.0374	±0.0198	3	0.0042	0.0036
12	0.0920	±0.0300	0.0439	±0.0298	2	0.0115	0.02
13	0.0380	±0.0165	0.0136	±0.0054	2.8	0.0047	0.0055
14	0.3056	±0.1918	0.1103	±0.0490	2.8	0.0328	0.0127
15	0.7965	±0.5520	0.1507	±0.0842	5	0.0157	0.0704[d]
16	0.1141	±0.0616	0.0348	±0.0176	3.2	0.0104	0.0117
17	0.1242	±0.0754	0.4092	±0.2643	−3.3	0.0126	0.0278
18	0.1633	±0.0910	0.0724	±0.0290	2.2	0.0375	0.0593[d]
19	0.1129	±0.0413	0.0520	±0.0447	2.2	0.0217	0.0167
20	0.0560	±0.0245	0.0266	±0.0090	2	0.0167	0.02
21	0.0253	±0.0105	0.0089	±0.0042	2.8	0.0036	0.0047
22	0.8825	±0.4315	0.3820	±0.1890	2.3	0.0217	0.0168
23	0.4240	±0.1253	0.2100	±0.1872	2	0.0245	0.0810[d]
24	0.5385	±0.2086	0.1932	±0.0865	2.8	0.0026	0.0066
25	0.3655	±0.1470	0.1115	±0.0393	3.3	0.0015	0.0081
26	0.0892	±0.0369	0.0427	±0.0061	2	0.0104	0.0024
27	0.0559	±0.0191	0.0257	±0.0132	2.2	0.0063	0.02
28	0.2375	±0.1306	0.0538	±0.0151	4.4	0.0053	0.007
29	0.0515	±0.0213	0.0204	±0.0135	2.5	0.0088	0.0117
30	1.2734	±0.7087	0.0927	±0.0426	13	0.0017	0.0007
31	1.5263	±0.6779	0.5537	±0.2059	2.7	0.0056	0.0027
32	0.4484	±0.1623	0.2233	±0.0924	2	0.0105	0.0233
33	0.4299	±0.2330	1.1583	±0.4739	−2.7	0.0025	0.0013

[a] The volume percentage (%V) is calculated as $V_{\text{single spot}}/V_{\text{total spots}}$ (V is the integration of the optical density over the spot area) using ImageMaster 2D platinum 6.0 software (GE Healthcare). [b] p-value calculated group by analysis of variance (ANOVA). [c] p-value calculated group by the non-parametric test (Wilcoxon rank-sum) using the GraphPad Prism4 software. $p \leq 0.05$ was considered statistically significant. [d] See Results.

information regarding the localization process, moreover they do not clearly indicate site assignment reliability *i.e.* they ignore the ambiguity issue. In most cases, the solution is the manual verification and this introduces a wide range of variability in the results. Studies show that inflammation with infiltrates of macrophages and lymphocytes is an important feature of AAA.[15,16] Indeed, several of the proteins whose expression increased in AAA patients were proteins involved in the acute phase. Moreover, in patients with AAA, elevated levels of various plasma markers of inflammation have been reported. Among these proteins we identified extracellular matrix proteins: three different isoforms of fibrinogen α chain (spots 10, 11, 12) and two isoforms of fibrinogen γ chain (spots 16 and 18).

Fibrinogen is an essential glycoprotein since it is a precursor to fibrin. It is formed by three different chains α, β, and γ and increases the aggregation of platelets, endothelial cells, and leukocytes. This aggregation is responsible for leukocyte and platelet activation leading to cell release of mediators. Plasma fibrinogen concentrations have been reported by Al-Barjas to correlate positively with AAA.[17] As previously reported[13] in AAA patients high levels of fibrinogen are associated with fibrin degradation products such as D-dimer which has multiple pro-inflammatory effects. Fibrin degradation may mediate the observed increase of acute phase reactants in AAA pathogenesis.[18] We identified three isoforms of Complement Factor B (spots 4, 5, 6), this protein is part of the alternative pathway of the complement system which plays a central role in innate immunity and participates in autoimmunity, debris removal and response to tissue injury as it can operate without antibody participation. Although the literature on the role of the complement system in human AAA is scant, studies on mice with AAA disease present evidence that the classic and alternative pathways of the complement system are activated in AAA tissues. The authors formulated the hypothesis that the complement system participates in the development of AAA by providing the necessary chemotactic signal that attracts neutrophils to the aortic wall.[19]

Complement Factor B circulates in the blood as a single chain polypeptide producing the catalytic subunit Bb, a serine protease that associates with the C3b-fragment to form the alternative C3 convertase pathway. To date, few reports have described the deposition of C3 and antibodies in human AAA tissues.[20] Our data strengthen the hypothesis of a relationship between human AAA and the complement system. Among the

Table 3 Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) and MS/MS analyses of proteins whose levels significantly differed among patients with AAA and age-matched control group

Spot	Protein name	Swiss-Prot entry[a]	Biological function[b]	MALDI-TOF MS			Predicted pI/Mr[f] (kDa)	Exp. pI/Mr[g] (kDa)
				Score[c]	Seq. cov.[d] (%)	No. of matched masses[e]		
1	α1-Antitrypsin	P01009	Protease inhibitor	145	32	11	5.37/46.88	5.05/131.36
2	α1-Antitrypsin	P01009	Protease inhibitor	231	48	18	5.37/46.88	5.00/132.63
3	α2-Macroglobulin (fragment)	P01023	Protease inhibitor	128	12	13	6.00/164.61	5.54/134.35
4	Complement factor B	P00751	Complement fraction	130	20	10	6.67/86.85	6.41/111.05
5	Complement factor B	P00751	Complement fraction	108	18	9	6.67/86.85	6.50/111.87
6	Complement factor B	P00751	Complement fraction	170	31	15	6.67/86.85	6.60/111.87
7	Serotransferrin	P02787	Ion transport	232	32	21	6.81/79.28	6.59/99.01
8	Serotransferrin	P02787	Iron ion transport	144	30	17	6.81/79.28	6.50/95.26
9	Serotransferrin	P02787	Iron ion transport	315	43	27	6.81/79.28	6.68/95.26
10	Fibrinogen α chain	P02671	Coagulation factor	186	21	21	5.70/95.66	7.07/81.64
11	Fibrinogen α chain	P02671	Coagulation factor	174	23	20	5.70/95.66	7.33/80.72
12	Fibrinogen α chain	P02671	Coagulation factor	160	20	20	5.70/95.66	7.15/81.64
13	Kininogen-1 (fragment)	P01042	Coagulation factor	101	18	10	6.34/73.00	5.01/80.46
14	α2-HS-glycoprotein	P02765	Protease inhibitor	117	28	9	5.43/40.10	4.62/72.71
15	α1-Antitrypsin	P01009	Protease inhibitor	218	48	18	5.37/46.88	4.90/72.36
16	Fibrinogen γ chain	P02679	Coagulation factor	243	60	18	5.37/52.11	5.15/64.44
17	Vitamin D-binding protein	P02774	Transport protein	204	46	16	5.40/54.53	5.28/68.95
18	Fibrinogen γ chain	P02679	Coagulation factor	132	34	11	5.37/52.11	5.25/63.82
19	Hemopexin	P02790	Transport protein	168	32	13	6.55/52.39	4.98/51.94
20	Hemopexin	P02790	Transport protein	132	29	12	6.55/52.39	6.72/56.79
21	Leucine-rich α2-glycoprotein	P02750	Hemostasis	DLLLPQPDLR[h]			6.45/38.38	4.66/56.60
22	Haptoglobin (β chain)	P00738	Hemoglobin binding protein	127	24	11	6.13/45.86	4.89/52.79
23	Haptoglobin (β chain)	P00738	Hemoglobin binding protein	121	25	11	6.13/45.86	5.35/43.88
24	Haptoglobin (β chain)	P00738	Hemoglobin binding protein	107	25	11	6.13/45.86	5.57/42.90
25	Haptoglobin (β chain)	P00738	Hemoglobin binding protein	111	27	10	6.13/45.86	5.81/46.21
26	Haptoglobin (β chain)	P00738	Hemoglobin binding protein	105	21	10	6.13/45.86	5.99/44.81
27	Apolipoprotein J	P10909	Transport protein	144	27	9	5.89/53.03	4.90/41.75
28	Apolipoprotein J	P10909	Transport protein	101	21	7	5.89/53.03	4.98/40.30
29	Apolipoprotein A-I	P02647	Transport protein	208	50	15	5.56/30.76	5.17/31.84
30	Haptoglobin (α chain) (fragment)	P00738	Hemoglobin binding protein	74	15	5	6.13/45.86	5.41/16.08
31	Haptoglobin (α chain)	P00738	Hemoglobin binding protein	53	17	5	6.13/45.86	5.81/15.93
32	Haptoglobin (α chain)	P00738	Hemoglobin binding protein	68	17	8	6.13/45.86	6.34/15.51
33	Hemoglobin subunit β	P68871	Transport protein	157	72	8	6.75/16.10	7.12/9.83

[a] SwissProt/UniprotKB accession number. [b] Biological function of the identified proteins according to their Gene Ontology (GO) biological process. [c] MASCOT MS score (Matrix Science, London, UK; http://www.matrixscience.com). MS matching score greater than 56 was required for a significant MS hit (p-value < 0.05). [d] Sequence coverage = (number of the identified residues/total number of amino acid residues in the protein sequence) × 100%. [e] Number of peptide masses matching the top hit from Ms-Fit PMF. [f] Predicted pI and Mr according to protein sequence. [g] Experimental pI and Mr based on the calculation using software ImageMaster 2D Platinum 6.0 (GE Healthcare). [h] Sequence tag identified by ESI-Ion trap MS/MS peptide-sequencing with Mascot MS/MS score: 53. The Mascot individual ions score greater than 35 was considered significant (p-value < 0.05).

acute phase proteins over-expressed in AAA patients we found five isoforms of haptoglobin β chain (spots 22, 23, 24, 25 and 26) and three isoforms of haptoglobin α chain (spots 30, 31 and 32). This protein is one of the major plasma glycoproteins and its levels often increase during inflammation, tissue damage and diseases such as cancer and arthritis.[21] It is composed of α and β polypeptide chains that are covalently associated by disulfide bonds. Many authors suggested that α and β polypeptides are synthesized *via* a common precursor polypeptide.[22] Haptoglobin is a positive acute phase protein that binds free hemoglobin and removes it from blood circulation to prevent kidney injury and iron loss following hemolysis. Also, by binding free hemoglobin, haptoglobin acts as a potent antioxidant and an immunosuppressor of lymphocyte function.[23] Indeed, in correlation with this, we found a reduced level of hemoglobin subunit β (spot 33) in plasma of AAA patients. This reduced level of hemoglobin in plasma from AAA patients could be related to the increased level of haptoglobin which is responsible for the hemoglobin removal from the plasma of these subjects.

We identify other acute phase proteins such as α2-HS-glycoprotein (spot 14), α1-antitrypsin (spots 1, 2 and 15), α2-macroglobulin (spot 3 protein fragment) and leucine-rich glycoprotein (spot 21) as up-regulated in AAA patients, thus suggesting a generalized pro-inflammatory state in these subjects. The protein α1-antitrypsin is an inhibitor of serine

proteases. Its primary target is the protein elastase, but it also has a moderate affinity for plasmin and thrombin. It irreversibly inhibits trypsin, chymotrypsin and plasminogen activator. Even though the physiological function of leucine rich glycoprotein is still unknown, changes in the concentrations of this protein were identified by Bini *et al.* using 2-DE in human serum during bacterial infection and in serum of patients with meningitis.[24] Among the acute phase proteins we identify hemopexin (spots 19 and 20), a glycoprotein released into plasma where it binds heme with high affinity. The better characterized function of hemopexin is heme scavenging at the systemic level by transporting heme to the liver. This is important both in physiologic heme management for heme–iron recycling and in pathologic conditions associated with intravascular hemolysis to prevent the pro-oxidant and pro-inflammatory effects of heme. Hemopexin is not only a systemic scavenger for heme but also acts as a multifunctional agent in important processes such as iron homeostasis, antioxidant protection, pathway signalling to promote cell survival and gene expression.[25] We found another binding transport protein which can bind iron: three isoforms of serotransferrin (spots 7, 8 and 9). This protein is responsible for the transport of iron from sites of absorption and heme degradation to those of storage and utilization. Serotransferrin may also have a role in stimulating cell proliferation and acute phase response.[26]

We found two up-regulated apolipoproteins in AAA patients: two isoforms of ApoJ (clusterin: spots 27 and 28) and ApoA-1 (spot 29). Recently van Dijk and colleagues have shown a protective effect of exogenous ApoJ *in vitro* on ischaemically altered cardiomyocytes. They hypothesized that ApoJ is involved in reducing infarction damage.[27] Other authors have reported that ApoJ mRNA was expressed in smooth muscle cells after vascular injury, suggesting that ApoJ contributes to restenosis post-angioplasty.[28] Even though AAA and atherosclerosis have many risk factors in common, studies investigating the association between lipids/lipoproteins and AAA have provided different results.[29]

Only a limited number of studies have explored apolipoprotein concentration in relation to AAA. In a population-based study of AAA the plasma levels of apoA-1 were found to be decreased in AAA patients in comparison to those in healthy subjects; this decrease was independently correlated with the reduced levels of other apolipoproteins.[30] ApoA-1 is the primary protein constituent of high-density lipoproteins (HDL). Low circulating levels of HDL is a risk factor for cardiovascular diseases and is associated with arterial endothelium dysfunction. In this study, both proteins ApoA-1 and ApoJ are positively associated with AAA. An increasing number of studies demonstrate that lipoprotein concentrations in subjects with AAA are higher than that in healthy control subjects yet correspond to that found in patients with atherothrombosis.

The relation between lipoproteins and AAA progression is currently unclear and a clearer identification of such correlation is needed, possibly using proteomic techniques.

The few protein spots whose levels are reduced in the AAA patients in comparison to the healthy controls were vitamin D-binding protein (spot 17) and hemoglobin subunit β (spot 33).

The vitamin D-binding protein (DBP) is the major plasma carrier protein of vitamin D and its metabolites. This protein is of potential importance for vascular remodelling and may contribute to an observed protective role of vitamin-D sterols in vascular diseases development. Urbonavicius and collaborators[5] were the first to find that, by proteomic approach analyzing vascular walls, this protein is positively correlated with the size of AAA. However, regarding the up-regulation of DBP expression levels, the authors reported that a collagen fragment was identified in the DBP spot. For this reason they could not accurately establish if its expression levels increased in AAA patients. In our experimental conditions we encountered a completely different behaviour of the DBP expression level in AAA patients. As reported in Table 2 the protein (spot 17) shows an evident decrease in expression in comparison to the control (volume ratio AAA/control group = −3.3). Further studies are required to confirm the potential and clinical role of DBP in this human disease and to further investigate whether and how this protein participates in the pathogenesis of the disease.

Discussion

Using a proteomic approach we identified a significant increase of a class of proteins such as fibrinogen, α1-antitrypsin and haptoglobin in plasma from AAA patients. The presence of these proteins in human AAA plasma may be related to the inflammatory processes active in these subjects. We have seen a negative correlation between the vitamin D-binding protein and hemoglobin subunit β and AAA presence. DBP levels have been found to increase in AAA wall tissues by Urbonavicius *et al.* and this discrepancy with our results could be due to the different analysis source. We wanted to analyze the factors measurable in plasma-associated rather than in tissue-associated markers because the application of circulating biomarkers in diagnostic laboratories would be relatively simple. DBP is very important for vascular remodelling and it may have an important role in the protection of vascular walls. In plasma tissue this protein reduces platelet aggregation and extends coagulation time.

Furthermore, only proteins whose quantity exceed approximately 3 ng and with a molecular mass between 10 and 200 kDa are detected by the 2-DE technique. Proteins in very low abundance are extremely difficult to identify using this gel-based proteomic technique. The present work is merely an example of the use of 2-DE and MS to identify several proteins at a given time in plasma of patients with AAA. Thus, further studies are needed to firmly establish which specific proteins or fragments participate in this disease's development.

In conclusion, in this study we confirm a number of biomarkers associated with AAA that have been previously identified by various authors. No one protein identified in this study has the biologic plausibility to be used singularly as a biomarker for aneurysmal disease due to inadequate specificity. The effect of using multiple biomarkers combined with clinical factors requires investigation in carefully designed population-based studies and these studies need to select the criteria of choice to define healthy controls very carefully. Clearer identification of various markers is needed, possibly

using other proteomic techniques such as gel-free proteomic methodologies to screen for new candidates.

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