Proteomic identification of differentially expressed proteins in aortic wall of patients with ruptured and nonruptured abdominal aortic aneurysms

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Objective: **To compare the basic proteomic composition of abdominal aortic aneurysm (AAA) wall tissue in patients with nonruptured and ruptured aneurysms.**

Methods: **A proteomic approach with two-dimensional gel electrophoresis (2D-PAGE) and mass spectrometry (MS) was used to identify differentially expressed proteins in AAA tissue from nine patients with nonruptured and eight patients with ruptured AAA. Computerized image analysis was used to detect protein spots. Differentially expressed protein spots were in-gel digested and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Western blot analysis was used to confirm differential expression.**

Results: **Seven differentially expressed proteins were detected among 745 protein spots, selecting spots whose average relative volumes differed more than twofold between the nonruptured and the ruptured group. Four protein spots were up-regulated in the ruptured group, and three were down-regulated. Five of the spots were identified. Among the upregulated spots, No. 605 was identified as peroxiredoxin-2. The up-regulation was confirmed by Western blotting. No. 381 was identified as an actin fragment. Two spots, Nos. 719 and 499, could not be identified. Among the down-regulated protein spots, No. 130 contained two peptides; one reliably determined peptide, FEDGVLDPDYPR, is found in vitronectin. Another peptide, QIDNPDYK, was borderline significant and found in calreticulin. The down-regulation of vitronectin was confirmed by Western blotting. Spot Nos. 193 and 199 both contained peptides from albumin with actin also present in No. 199.**

Conclusion: **The identified proteins suggest that the aortic wall of ruptured aneurysms responds to a stressful condition and that proteolytic degradation of the cytoskeleton and connective tissue may be part of the response. (J Vasc Surg 2009;49:455-63.)**

Clinical Relevance: **The use of biomarkers in medicine lies in their ability to detect disease and to support diagnostic and therapeutic decisions. Proteins are the main functional outcomes of genes, so proteomics will lead biology and medicine beyond genomics. The identification, quantification, classification, and functional assignment of proteins will be essential to the full understanding of the molecular events of AAA progression. New research and novel understanding of the molecular basis of the disease reveal an abundance of exciting new biomarkers that present a promise for use in the everyday clinical practice. Consequently, the blood level of an ideal biomarker should have: high sensitivity (increased pathologically in the presence of the disease), high specificity (not increased in the absence of the disease), add information about the risk or prognosis, change in accordance with the clinical evolution, reflecting the current status of disease, be reproducible (as determined by the low coefficient of variation), and be easy and less expensive to determine. The identification of such biomarkers also causes identification of potential pathogenetic pathways, and may thus open possibilities for pharmacological inhibition of growth and provide a tool for monitoring this inhibition.**

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Ruptured abdominal aortic aneurysm (AAA) is a serious clinical problem that requires an emergency repair associated with a high mortality rate.[1] Growth and rupture of AAA is characterized by inflammation of the arterial wall, increased expression of matrix-degrading metalloproteinases (MMPs) and their inhibitors, degenerative changes in elastin, and collagen and apoptosis of the smooth muscle cells of the media.[2] An effort to prevent rupture by early detection of AAA may reduce very high mortality rates associated with rupture by providing the opportunity for elective repair. Regrettably, it is often difficult to predict an adverse outcome.

Proteolytic degradation of the elastin and collagen fibers as well as different proteases and their inhibitors have

been found in the wall of the enlarging AAA.[3-5] However, the processes that characterize AAA rupture are poorly understood. If reliable predictors of rupture can be determined, they hold the potential to provide for the indication of surgery and may reveal modifiable risk factors for AAA progression and rupture.

In recent years, genomics has increased the understanding of many diseases. Although genetic mutations and/or altered gene expression may underlie a disease, the biochemical basis for diseases is caused by distortions in the proteins. We have used a combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for protein separation and visualisation followed by mass spectrometry (MS) protein identification.[6] We report the differential expression of seven proteins in the ruptured group vs the nonruptured group. The differential expression of the proteins in the disease is discussed in relation to the functions of the proteins.[7]

MATERIALS AND METHODS

Patients and tissue sampling

AAA wall samples were used from 17 patients who underwent infrarenal AAA repair between 1998 and 2000 at the Department of Vascular Surgery, Viborg Hospital. Eight had ruptured AAA.

Full AAA thickness tissue were collected from the anterior wall of the aneurysm at the maximal dilated place, cleaned from blood, fat, and foreign tissues. Areas of definite, hard calcification were discarded. When tissue was taken from the patient, in a few days it was frozen at −20°C. For longer term storage, the tissue was transferred to a −80°C freezer. The maximal anterior-posterior diameter of AAA was measured by computed tomography (CT) scanning within a month before surgery. In ruptured cases, this was done immediately before surgery.

All patients, or close relatives as deputies in cases of incapacity of the patient, were informed and gave consent to this procedure. This study was approved by the Scientific Ethical Committee and reported to the data protection authorities.

Two-dimensional gel electrophoresis

Approximately 1 g of complete AAA wall tissue was frozen in liquid nitrogen, crushed with a hammer between plastic sheets, dissolved in lysis buffer (pH 3-10 NL), homogenized with an Ultra-Turax homogenizer and centrifuged at 14,000 rpm for 10 minutes. Rehydration buffer was added to the supernatant and first dimension was run on pre-cast immobilized pH gradient strips (18 cm; pH 3-10 NL) with the Multiphor II system (GE Healthcare, Chalfont St. Giles, UK). The second dimension was performed on home made polyacrylamide gels (12% T, 3% C) that were fixed in 50% (v/v) methanol, 12% (v/v) acetic acid, or 0.0185% (v/v) formaldehyde for at least 1 hour or overnight. They were then washed three times for 20 minutes in 35% (v/v) ethanol, pretreated for 1 minute in pretreatment solution (0.02% (w/v) $Na_2S_2O_3$, $5H_2O$) and rinsed two times for 3 minutes in water. Staining of gels was performed for 20 minutes in 0.2% (w/v) $AgNO_3$, 0.028% (v/v) formaldehyde after which they were rinsed two times for 20 seconds in water. Development was carried out in development solution (6% [w/v] Na_2CO_3, 0.0185% [v/v] formaldehyde, 0.0004% [w/v] $Na_2S_2O_3$, and $5H_2O$) for approximately 3 minutes and was stopped in stop solution (50% [v/v] methanol, 12% [v/v] acetic acid). Finally, the gels were dried between cellophane sheets and sealed in plastic bags.

Analysis of two-dimensional gels

Analysis of gels was performed essentially as previously described.[8] In short, the dry and transparent silver stained gels were scanned in the transmissive mode on a GS-710 imaging densitometer from Bio-Rad (Hercules, Calif) using the Quantity One software package. Gel images were exported as 16-bit gray-scale TIFF files that were imported into the Melanie II (Swiss Institute of Bioinformatics, Geneva, Switzerland) 2-D PAGE analysis software package. After background subtraction, the protein spots were automatically defined and quantified with the feature detection algorithm as described in the manual. Spot intensities were expressed as relative volumes in percentages (%VOL) by integrating the optical density of each pixel in the spot area (VOL) and dividing with the sum of volumes of all spots detected in the gel. About 10 to 12 of the spots were used as landmarks. One of the gels used in an analysis comparison was selected as a reference gel to which each other gel used in the analysis was aligned and matched to as described in the manual. In the reference gel, each spot (feature) is assigned with a number. The quality of the match made by the computer was critically evaluated in each case and necessary editions and corrections were done manually.

Comparison of groups

Comparisons were performed between two groups of gels (group A-nonruptured AAA and group B-ruptured AAA) first by selecting spots with an average relative volume in one group differing by more than twofold with the average relative volume in the other group. Significant differences among the selected spots were calculated using a two-sample *t* test ($P < .05$). These data were exported to Excel for further analysis. Only data that could be considered as normal distributed as checked by a Shapiro-Wilcox analysis were used. Comparisons were made between pairs of groups with eight to nine gels in each group. Only well focused spots were considered.

Identification of proteins by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Gels containing protein spots selected for identification were re-swelled in water. The cellophane sheets were peeled off so the protein spots could be excised from the gels. Proteins were subjected to in-gel tryptic digestion and identification, essentially as previously described.[8] In short, the digested sample was dried and the peptides resuspended in 12 μl buffer A (water/acetonitrile/formic acid,

Table I. Patient characteristics

	Nonruptured n = 9	*Ruptured n = 8*	*Total n = 17*	*P*
Gender: males/females	6/3	5/3	11/6	NS
Age (y) Median (range)	71.6 (68-75)	75.4 (68-83)	73.5 (68-83)	NS
Aneurysm diameter (mm) Median (range)	56.2 (35-80)	57 (49-90)	56.6 (35-90)	NS
Smoking status (smokers/nonsmokers)	5/4	4/4	9/8	NS
NSAID (Y/N)	4/5	2/6	6/11	0.62
Statins (Y/N)	0/9	0/8	0/17	0.99
Outcome (A/D)	9/0	5/3	14/3	0.08

97.7/2/0.3, V/V/V). Peptides were separated using an inert nano LC system composed of a FAMOS micro auto sampler, a Switchos micro column switching module, and an Ultimate micro pump from LC Packings (San Francisco, Calif). After preconcentration of 5 μl of the sample on the precolumn the peptides were eluted from the analytical column with a gradient made by mixing decreasing volumes of buffer A with increasing volumes of buffer B (water/acetonitrile/formic acid, 9.7/90/0.3, V/V/V). The peptides were eluted into the nano electrospray ion source of the quadruple time-of-flight Q-TOF Ultima mass spectrometer (Micromass, Manchester, UK). MS survey scans were acquired using MassLynx 4 SP4 (Waters, Milford, Mass) at a rate of 1 per second from m/z 400 to 2000. The instrument was operated in a data-dependent MS to MS/MS switching mode where doubly, triply and quadruply charged peptide ions detected in MS survey scans triggered a switch to MS/MS for obtaining peptide fragmentation spectra with an interval of m/z values 50 to 2000. The obtained raw data were processed using ProteinLynx GlobalServer 2.1 (Waters, Milford, Mass). The processed data were used to search the SwissProt database with the on-line version of the Mascot MS/MS ion search facility (Matrix Science, Ltd., London, UK, http://www.matrixscience.com).[9] Searching was performed with doubly and triply charged ions with two missed cleavages; a peptide tolerance of 50 ppm, one modification, Carbamidomethyl-C and an MS/MS tolerance of 0.05 Da. Contaminating proteins including keratins, trypsin, and bovine serum albumin were disregarded. At least one "bold red" peptide was required in the search. The peptides are reported for proteins with scores giving a less than 5% probability that the observed match was a random event.

Western blotting

For one-dimensional Western blotting protein concentrations of samples were determined using a noninterfering assay (NI Protein Assay, Geno Technology Inc., St Louis, Mo). Identical amounts of protein from each sample (5 or 10 μg total protein) were added to each lane of a 10% to 20% and 4% to 20% Tris-Glycine gel (Invitrogen, Carlsbad, Calif). After electrophoresis, the proteins were transferred to nitrocellulose membranes. For immunodetection of the proteins the nitrocellulose sheets were incubated overnight at 4°C in phosphate-buffered saline (PBS) (2.7 mM KCl, 1.8 mM KH_2PO_4, 10.1 mM Na_2HPO_4, 140 mM NaCl, pH 7.3) containing 0.05% Tween-20 and 5% skimmed milk. The membranes were washed five times in PBS with 0.05% Tween-20. Rabbit polyclonal antibody to peroxiredoxin-2 (Abcam, Cambridge, UK) was diluted to 1:2000, rabbit polyclonal antibody to vitronectin sc-28929 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) was diluted 1:1000, and rabbit polyclonal antibody to calreticulin (Abcam, Cambridge, UK) was diluted 1:1000. The blots were incubated with antibodies for 1 hour at room temperature. After five washes in PBS with Tween-20, the blots were incubated for another hour with 1:1000 diluted peroxidase-conjugated swine antirabbit IgG (Dako, Glostrup, Denmark). Finally, the membranes were washed five times in PBS with Tween-20 and developed with the enhanced chemiluminescence technique (Amersham Biosciences Inc.). Blots were quantified using Imaging Densitometer GS710 and Quantity One software (Bio-Rad, Switzerland).

RESULTS

There were 11 male and 6 female patients. The mean age was 73.5 ± 6.8 years and the age range was 66 to 83 years. The characteristics of the patients are given in Table I. There is no significant difference between the groups of nonruptured vs ruptured with respect to gender, age, aneurysm diameter, smoking status, or ingestion of nonsteroidal anti-inflammatory drugs.

Comparison of protein expression profiles

Fig 1 shows representative gels of aortic tissue from nonruptured aneurysms (*A*) and aortic tissue from acute ruptured aneurysms (*B*). In all analyses, the nonruptured aortic tissue was used as reference. About 745 paired protein spots were visualized. Seven protein spots were found to be more than twofold differentially expressed at the significance level of $P < .05$. Four proteins were up-regulated and three were down-regulated (Fig 2). They are all indicated with arrows in Fig 1.

Protein identification

All seven proteins were excised from the rehydrated gels and subjected to in-gel tryptic digestion for subsequent identification by LC-MS/MS. Among seven spots that were excised for identification, five of the spots contained peptides for putative identification, two up-regulated spots

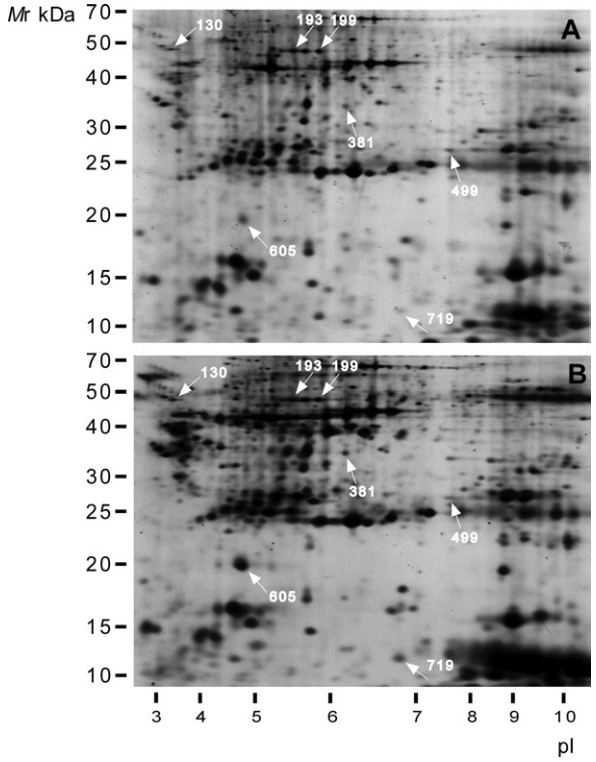

Fig 1. Two-dimensional gel images of non-ruptured abdominal aortic aneurysms (**A**) and ruptured abdominal aortic aneurysms (**B**). Proteins that were significantly differentially expressed more than two-fold are indicated with arrows (upward pointing, up-regulated; downward pointing, down-regulated). Five of the proteins were identified using LC-MS/MS as indicated in Table I where also the degree of up- and down-regulation is given.

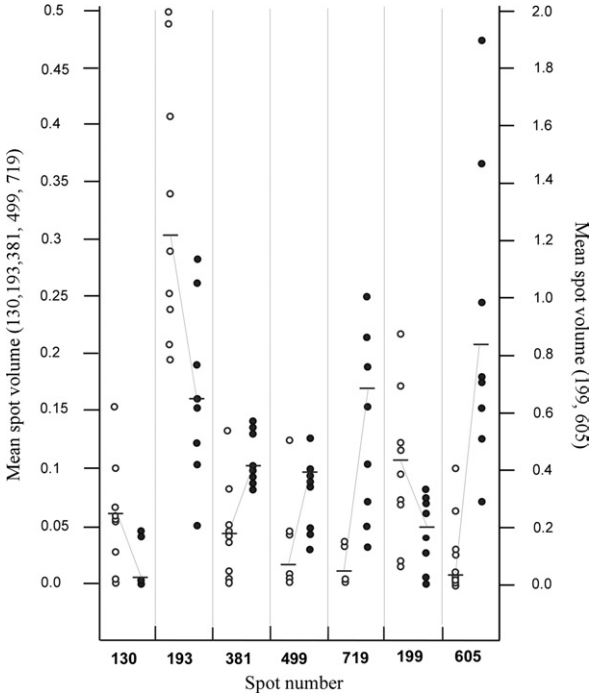

Fig 2. Protein levels as spot volume percentages of seven significantly and more than two-fold differentially expressed proteins from non-ruptured (○) and ruptured (●) abdominal aneurysms. The mean values in each group are indicated and connected.

and three down-regulated spots. Table II shows the peptides that were obtained and used to identify the proteins and the degree of differential expression. Examples of the spectral data that were used to identify the proteins are given in Figs 3 and 4.

Spot 719. Spot 719 was 19.5-fold up-regulated in the ruptured group. Although several spots were excised for identification, we obtained no certain identification probably because of very low amounts present in the spot.

Spot 605. Spot 605 was up-regulated 8.1-fold in the ruptured group as determined by 2D-PAGE. The spectral data used for the identification is shown in Fig 3. The three peptides are present in peroxiredoxin-2 with a score at 125 well above the significance limit indicating that the identification was very reliable. The Western blot analysis of peroxiredoxin-2 (Fig 5) revealed expression of an approximately 20 kDa protein band, which confirmed the 2D-PAGE analysis. Western blot analysis indicated that the peroxiredoxin-2 level was significantly 2.2-fold up-regulated in aortic tissue from ruptured aneurysms compared with aortic tissue from nonruptured aneurysms, operated electively (Fig 5).

Spot 499. Spot 499 was up-regulated in the ruptured group by 4.9-fold. The spot was not identified, although excised several times, probably due to low amounts of protein present.

Spot 381. Spot 381 was identified as a fragment of either beta or gamma actin. The fragment was 2.7-fold up-regulated in the ruptured group.

Spot 130. Spot 130 was 5.4-fold down-regulated in ruptured AAA walls. The database search revealed two peptides that fitted the spectral data as shown in Fig 4. One peptide, FEDGVLDPDYPR, quite reliably fitted (score 54) the spectrum as seen in Fig 4, *A*. This peptide is found in the multifunctional glycoprotein vitronectin and is also present in the 40-kDa aortic aneurysm associated protein-40 (AAAP-40) that possesses similarity to vitronectin, fibrinogen as well as several calcium binding proteins. The latter protein AAAP-40, however, possesses a lower molecular mass 40 kDa than observed from the 2D gels, ie, around 50 kDa. Another peptide, QIDNPDYK, also fitted the spectral data (score 36) shown in Fig 4, *B*. However, the score obtained in the search was only 36, which is just borderline significant as seen in Table II. Thus, this identification is less reliable than in case of vitronectin. An additional search using another search program (Gobal server) also scores vitronectin (score 12.5; probability 100%) significantly higher than calreticulin (score 10.4; probability 12.49%). Moreover, Western blot analysis of vitronectin expression revealed the presence of mainly two protein bands with

Table II. Proteins that are differentially expressed in ruptured abdominal aortic aneurysms vs nonruptured abdominal aneurysms

Protein No.	*Peptides analyzed*	*MS identification*	*Accession (SwissProt) (pI; Mr)*	*Mascot score*[a]	*Level in ruptured group vs level in nonruptured group*
719	NI[b]				19.5
605	LSEDYGVLK (110-118) TDEGIAYR (119-126) QITVNDLPVGR (139-149)	Peroxiredoxin-2	PRDX2_HUMAN (5.67; 21.747)	125	8.09
499	NI[b]				4.85
381	GYSFTTTAER (197-206)	Actin, cytoplasmic 1 (Beta-actin)	ACTB_HUMAN or ACTG_HUMAN (5.29; 41.7-41.8)	53	2.67
199	LVNEVTEFAK (66-75) YLYEIAR (162-168) YICENQDSISSK (287-298) VPQVSTPTLVEVSR (439-452)	Albumin	ALBU_HUMAN	103	0.5
	AGFAGDDAPR (20-29) EITALAPSTMK (317-327)	Actin, gamma-enteric smooth muscle (smooth muscle gamma actin)	ACTA_HUMAN, ACTB_HUMAN, ACTC_HUMAN, ACTG_HUMAN, ACTH_HUMAN or ACTS_HUMAN (5.31; 41.7-41.8)	55	
193	LVNEVTEFAK (66-75)	Albumin	ALBU_HUMAN (6.25; 69.3)	60	0.5
130	FEDGVLDPDYPR (230-241) QIDNPDYK (279-286)[c]	Vitronectin Calreticulin	VTNC_HUMAN (5.7; 54.3) CALR_HUMAN (4.3; 48.1)	54[c] 36[c]	0.18

[a]A Mascot score above 36 indicates a significant identity when searching in the total database ($P < .05$).
[b]Not identified
[c]The score for vitronectin is well above the significance level whereas calreticulin is of borderline significance. This result was also obtained when searching was performed with a different search program (Global server) giving a significantly higher score for vitronectin (score 12.5; probability 100%) and an insignificant score for calreticulin (score 10.4; probability 12.49%).

molecular masses at about 70 and 50 kDa. It is likely the 50 kDa band corresponds to spot 130 as detected by 2D-PAGE (Fig 1). The two bands were quantitated together revealing a 2.9-fold significant down-regulation of vitronectin in aortic tissue from ruptured aneurysms (Fig 5). Western blot of calreticulin was unreliable, since it gave several weak bands indicating that the amount of the protein in the tissue was too low to give a clear and specific reaction with the antibody used.

Spot 193. Spot 193 was identified as albumin being twofold down-regulated in the ruptured AAA group.

Spot 199. Spot 199, which was twofold down-regulated in the ruptured group, was identified with several peptides as albumin. The spot also contained some actin peptides where several isoforms are possible based on the two peptides identified, aortic smooth muscle actin (ACTA_HUMAN), beta actin (ACTB_HUMAN), gamma actin (ACTG_HUMAN), cardiac alpha actin (ACTC_HUMAN), enteric smooth muscle gamma actin (ACTH_HUMAN), and skeletal muscle alpha actin (ACTS_HUMAN).

DISCUSSION

Four mechanisms seem to be relevant for the pathogenesis of aneurysm formation: proteolytic degradation of aortic wall connective tissue, inflammation and immune responses, biomechanical wall stress, and molecular genetics.[10] However, the specific pathogenetic mechanisms underlying the progression of nonruptured into ruptured abdominal aneurysms are still largely unknown. In this article, we present the first proteomic study comparing nonruptured with ruptured abdominal aneurysms using 2D-PAGE and MS identification. We detected differentially expressed proteins in tissue from ruptured aneurysms compared with nonruptured AAA tissue. Among these, we identified two up-regulated proteins and three down-regulated in the wall of ruptured AAA and suggest an association between these proteins and rupture of the aneurysm in the human abdominal aorta. Three of the identified proteins were also analysed by Western blot analysis. Two of the antibodies gave reliable results and confirmed the differential expression observed by 2D-PAGE.

Spot 605 identified as peroxiredoxin-2. In the present study, peroxiredoxin-2 was up-regulated 8.1-fold in the ruptured AAA group compared with nonruptured AAA as determined by 2D-PAGE. Western blot analysis confirmed the significant up-regulation of peroxiredoxin-2 in ruptured aortic tissue. Peroxiredoxins are a family of antioxidative proteins that share a common reactive cysteine residue in the N-terminal region and eliminate hydrogen peroxide (H_2O_2).[11] Intracellular peroxiredoxins have been implicated in cellular antioxidant defence and the regulation of many cellular processes including cell proliferation, differentiation, and apoptosis.[12-14] Overexpression of genes for peroxiredoxin-2 in human endothelial cells

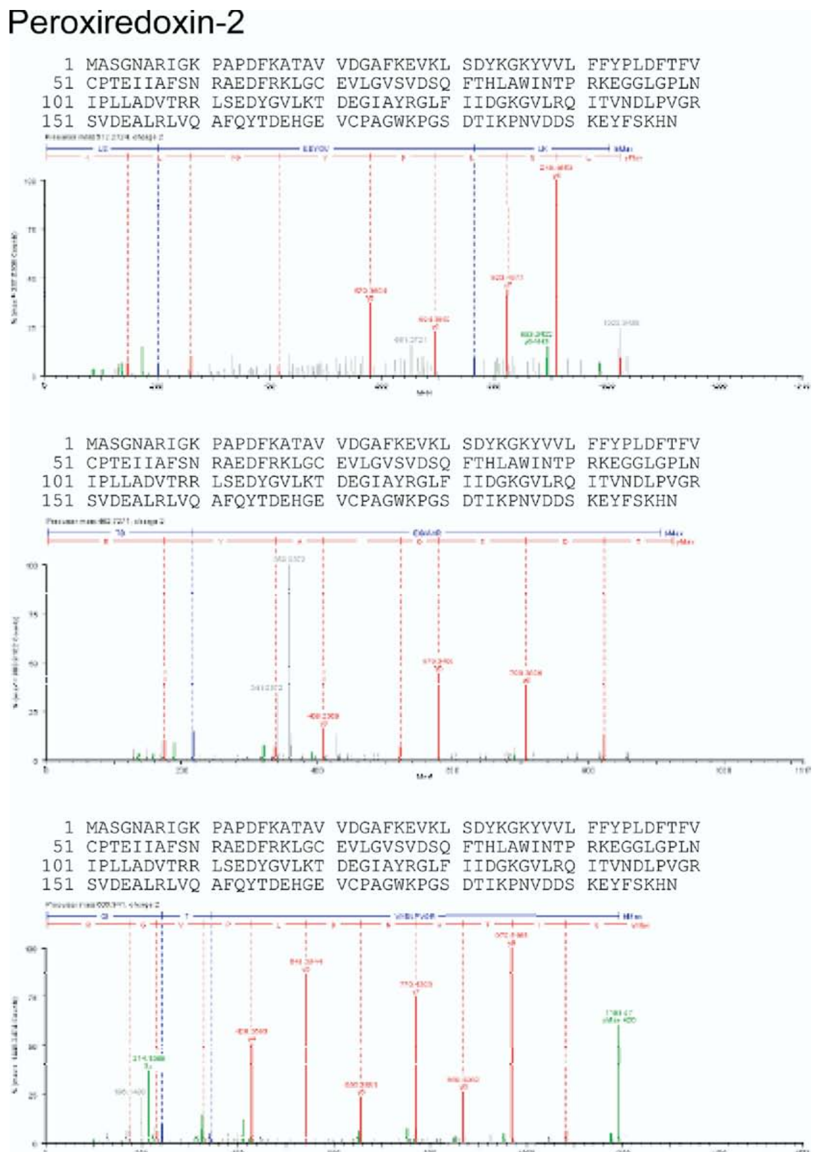

Fig 3. Identification of spot No. 605 by LC-MS/MS. Three tandem mass spectra used to reliably identify spot 605 as peroxiredoxin-2. The identified peptides are indicated with bold in the sequence.

leads to cellular resistance to elevated levels of H_2O_2.[15] The increase in peroxiredoxin-2 concentration may be secondary to specific mechanisms involved in the response to injury associated with ruptured AAA. However, the role of reactive oxygen species (ROS) and antioxidants in aneurysm disease is an area of great interest as well. Among ROS, the accession of superoxide anion (O_2^-) contributes to the functional and structural changes of the vessel wall. Superoxide (O_2^-) levels in aneurysmal tissue in human beings is 2.5-fold higher than adjacent, nonaneurysmal aortic tissue and 10-fold higher than control aorta.[16] This pro-oxidant environment has important implications for matrix degradation and activates MMPs suggesting a possible role of oxidative stress in the pathogenesis of abdominal aortic aneurysms.[17,18] Previous experiments indicate that ROS and oxidative stress are locally enhanced in AAA and may play a pivotal role in the pathologic progression of AAA.[19,20] Collectively, these studies provide support for the hypothesis that oxidative stress is an important component in formation and enlargement of AAA through activation of MMPs and induction of apoptosis within the aortic wall.

Spot 193 and 199 were identified as albumin. Albumin was found to be twofold down-regulated in the ruptured AAA group based on spot 193. This could be due to a response to the rupture of the aneurysm. Vascular inflammation and proteolytic degradation are implicated in the pathogenesis of AAA. The down-regulation could re-

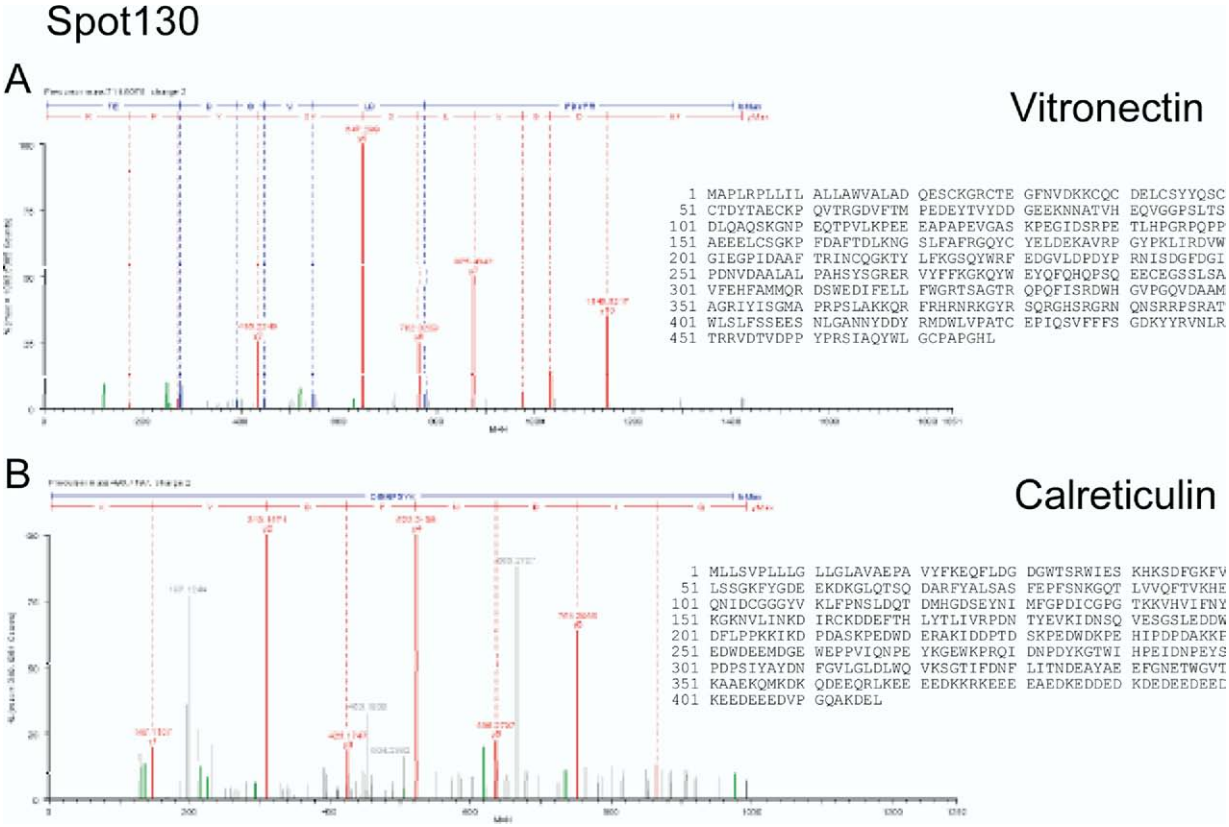

Fig 4. Identification of spot No. 130 by LC-MS/MS. From spot No. 130 two tandem mass spectra were obtained. **A,** Shows a high quality spectrum that is compatible with a peptide found in vitronectin (shown with bold). **B,** A different peptide from spot No. 130 showed a borderline significance with a peptide in calreticulin (shown with bold).

sult from dilution due to an inflammatory based edema of the wall or as a result of an increased proteolytic degradation of albumin in the wall.

Spot 199 and 381 are identified as actin. Actin was found in spot 199 together with albumin. In spot 381, an actin fragment was found to be 2.7-fold up-regulated in ruptured AAA. Actin exists ubiquitously as a structural constituent of the cytoskeleton and plays a role in motor activity. Up-regulation of an actin fragment may be due to an increased proteolytic degradation of actin in the ruptured aneurysm.

Spot 130 is likely identified as vitronectin. Vitronectin is likely to account for the major changes of spot 130. Although spot 130 contained spectra from two different proteins the major content, and thereby, the major change in protein is likely to be vitronectin based on the quality of the data used for identification. This interpretation was further substantiated by Western blot analysis that also revealed significant down-regulation of two vitronectin bands in the ruptured abdominal aorta group. Vitronectin is an adhesive multifunctional glycoprotein circulating in the plasma, and in the extracellular matrix, including the vascular wall, and play key roles in the migration, attachment, and differentiation of cells.[21-23] This protein also has several other functions in the complement, coagulation, and fibrinolytic system. Limited proteolysis by plasmin converts vitronectin into defined fragments, which are detectable at sites of inflammation and angiogenesis.[24] Plasmin is important in the degradation of matrix glycoproteins such as fibronectin, vitronectin, and laminin, all of which have been reported to bind plasminogen activator inhibitor-1 (PAI-1). [25,26] Decreased PAI-1 activity leads to increased activity of urokinase-like-plasminogen activator (u-PA) and tissue-type-plasminogen activator (t-PA) to promote cellular migration and matrix remodeling. The data also suggest that MMPs such as MMP-9, MMP-2, and MMP-12 may be responsible for the pathological degradation and/or normal turnover of vitronectin.[27,28] Therefore, the degradation of the matrix, which occurs during tissue remodelling involves both plasminogen activators and activators of metalloproteases,[29,30] and vitronectin provides unique regulatory links between cell adhesion and proteolytic enzyme cascades. In a rat model, blocking of t-PA activity by PAI-1 overexpression prevents formation of aneurysms and arterial rupture by inhibiting MMP activation.[31]

Taken together, the present results suggest that the increased concentration of peroxiredoxin-2, the putative

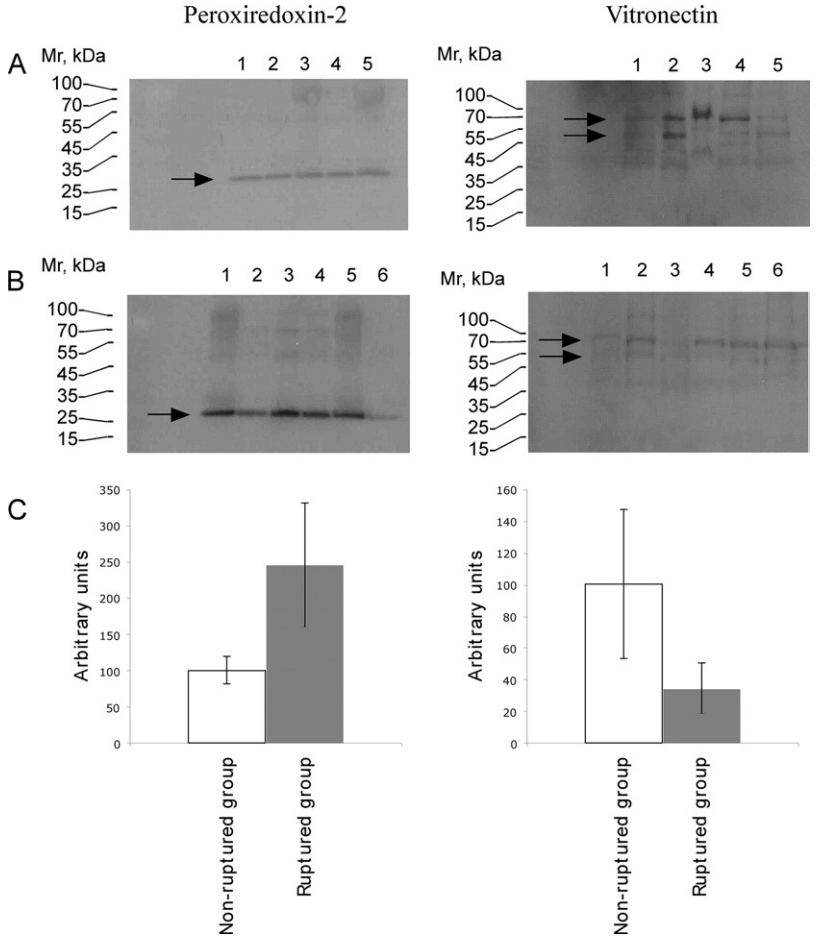

Fig 5. Western blotting analysis using anti-peroxiredoxin-2 and anti-vitronectin on 5 patient samples (1-5) from non-ruptured AAA group (**A**) and 6 patient samples (1-6) from ruptured AAA group (**B**). The bands indicated with arrows are significantly differentially regulated in the ruptured group using t test ($P < .05$). Peroxiredoxin-2 is up-regulated and two vitronectin bands quantitated together (indicated with arrows) are down-regulated in the ruptured group (**C**).

decreased concentration of vitronectin and albumin found in the wall of ruptured AAA patients as well as the up-regulation of an actin fragment may be related to a change in proteolytic activity and inflammatory response linked with AAA progression and rupture. AAA size is also a strong predictor of AAA rupture. In our material, there was no significant difference in size between the groups being averagely 56.2 mm in the nonruptured group and 57 mm in the ruptured group (Table I). In order to standardize the material, each sample was taken at the anterior wall of the AAA at the maximal dilated circumference. However, some protein changes may be dependent on the proximity to the actual site of rupture. We cannot rule out that, with this procedure, we have overlooked other proteins that also could be important predictors for rupture.

Overall, it is important to notice that a number of limitations apply to the present study. A relatively small number of patients were analyzed. Furthermore, a limited set of proteins are detected by the 2D-PAGE technique, ie, only proteins above a level of about 3 nanogram between pI approximately 3 and 10 and molecular mass between about 10 and 200 kDa. A number of proteins found to be differentially expressed in ruptured vs nonruptured AAA tissue were not identified in the present study, a 4.9-fold and a 19.5-fold up-regulated protein. The proteins are probably of very low abundance and, therefore, difficult to identify with the presently available technique.

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AUTHOR CONTRIBUTIONS

Conception and design: SU, JL, HV, BH
Analysis and interpretation: SU, JL, HV, GU, EH, BH
Data collection: JL
Writing the article: SU, JL, BH
Critical revision of the article: SU, JL, HV, GU, EH, BH
Final approval of the article: SU, JL, HV, GU, EH, BH

Statistical analysis: SU, GU, JL, BH
Obtained funding: JL, EH, BH
Overall responsibility: JL, HV, EH, BH

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