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**A Combined Proteomic and Transcriptomic Approach Shows Diverging Molecular Mechanisms in Thoracic Aortic Aneurysm Development in Patients With Tricuspid- And Bicuspid Aortic Valve**

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**Keywords:** Proteomics, 2D-DIGE, LC-MS/MS, PCA, OPLS-DA

**Abbreviations:**

TAA	thoracic aortic aneurysm
TAV	tricuspid aortic valve
BAV	bicuspid aortic valve
PCA	principal component analysis
PC	principal component
OPLS-DA	orthogonal projections to latent structures discriminant analysis
MVA	multivariate data analysis
RMA	subsequent robust multichip average normalization
ECM	extra cellular matrix
SMC	smooth muscle cells
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
DTT	dithiothreitol
FA	formic acid
ACN	acetonitrile
2D-DIGE	2 dimensional fluorescence difference gel electrophoresis
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
IEF	isoelectric focusing
PMT	photo multiplier tube
MALDI-TOF MS	matrix assisted laser desorption/ionization time of flight mass spectrometry
PTMs	peptide mass fingerprints
SAP/APC	serum amyloid P component
AD	Alzheimer's disease
SSA	senile systemic amyloidosis
ACTA2	actin, aortic smooth muscle

ACTN1	alpha actinin 1
ACTN4	actinin, alpha 4
ALB	albumin
ANXA 1	annexin A1
ANXA4	annexin A4
ANXA5	annexin A5
APCS	amyloid P component, serum
CLIC4	chloride intracellular channel 4
ENO1	enolase 1, (alpha)
FBLIM1	filamin binding LIM protein 1
FGB	fibrinogen beta chain, isoform CRA_d
FGG	fibrinogen gamma chain
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSN	gelsolin
HBA1	hemoglobin subunit alpha 1
HBB	hemoglobin, beta
HBD	hemoglobin subunit delta
HSP90AB1	heat shock protein 90kDa alpha (cytosolic)
HSPA1L	heat shock 70kDa protein 1-like
HSPB1	heat shock protein 27
KIAA1429	KIAA1429 protein
LDHA	lactate dehydrogenase A
LGALS1	lectin, galactoside-binding, soluble, 1
MFAP4	microfibrillar-associated protein 4
MYL6	myosin, light chain 6
MYL9	myosin regulatory light polypeptide 9

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MYL12B	myosin regulatory light chain 12B
OGN	osteoglycin
PARK7	Parkinson disease (autosomal recessive, early onset) 7
PDLIM3	PDZ and LIM domain 3
TAGLN	transgelin
TAGLN2	transgelin 2
TF	transferrin
TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)
TINAGL1	tubulointerstitial nephritis antigen-like 1
TPM2	tropomyosin 2 (beta)
TTR	transthyretin
TUBB	tubulin, beta
VIM	vimentin
YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3 protein beta/alpha)
YWHAQ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3 protein theta)
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3 protein zeta/delta)
TGFβ	transforming growth factor β
FERMT2	fermitin family member 2, also known as kindlin2 and Mig2
LIM	Lin-11, Isl-1 and Mec-3

## Summary

Thoracic aortic aneurysm is a pathological local dilatation of the aorta, potentially leading to aortic rupture or dissection. The disease is a common complication of patients with bicuspid aortic valve, a congenital disorder present in 1-2% of the population. Using 2 dimensional fluorescence difference gel electrophoresis proteomics followed by mRNA expression, and alternative splicing analysis of the identified proteins, differences in dilated and non-dilated aorta tissues between 44 patients with bicuspid and tricuspid valves was examined. The pattern of protein expression was successfully validated with LC-MS/MS. A multivariate analysis of protein expression data revealed diverging protein expression fingerprints in patients with tricuspid compared to the patients with bicuspid aortic valves. From 302 protein spots included in the analysis, 69 and 38 spots were differentially expressed between dilated and non-dilated aorta specifically in patients with tricuspid and bicuspid aortic valve, respectively. 92 protein spots were differentially expressed between dilated and non-dilated aorta in both phenotypes. Similarly, mRNA expression together with alternative splicing analysis of the identified proteins also showed diverging fingerprints in the two patient groups. Differential splicing was abundant but the expression levels of differentially spliced mRNA transcripts were low compared with the wild type transcript and there was no correlation between splicing and the number of spots. Therefore, the different spots are likely to represent post-translational modifications. The identification of differentially expressed proteins suggests that dilatation in patients with a tricuspid aortic valve involves inflammatory processes whereas aortic aneurysm in patients with BAV may be the consequence of impaired repair capacity. The results imply that aortic aneurysm formation in patients with bicuspid and tricuspid aortic valves involve different biological pathways leading to the same phenotype.

## Introduction

Thoracic aortic aneurysm (TAA) is a pathological widening of the aorta, resulting from degeneration of extra cellular matrix (ECM) resulting in loss of smooth muscle cells in the tunica media. TAA is a prevalent complication in individuals with a bicuspid aortic valve (BAV), a common congenital disorder present in 1-2% of the population (1). The origin of BAV is believed to be a consequence of abnormal aortic cusp formation during valvulogenesis. The presence of BAV predisposes for progressive dilatation of the ascending aorta and thereby contributes to an increased risk of aortic aneurysm that can eventually lead to fatal rupture or dissection of the aorta. The pathophysiological basis for the commonly occurring aortopathy in patients with BAV is not known. However, previous studies on molecular mechanisms in TAA have shown that patients with tricuspid aortic valve (TAV) have diverging mRNA expression profiles compared to patients with BAV (2, 3) as well as diverging alternative splicing fingerprints in the transforming growth factor-beta signaling pathway (4). These results suggest that different molecular mechanisms are involved in TAA formation in patients with TAV and BAV.

In the present study we sought to analyze protein and gene expression profiles in dilatation of ascending aorta between TAV and BAV in order to identify novel candidate proteins associated with dilatation in the two phenotypes. Our approach to proteomic analysis involved 2 dimensional fluorescence difference gel electrophoresis (2D-DIGE), followed by gel-free isotope tag based LC-MS/MS that was used as independent validation for the 2D-DIGE analysis. Multivariate data analysis (MVA) was applied to spot (2D-DIGE) and protein (LC-MS/MS) expression levels in patients with dilated and non-dilated aorta. In addition, we also analyzed the mRNA and exon expression of the identified proteins in order to study to what extent the changes in mRNA expression and/or splicing reflect the differences observed between TAV and BAV at protein level. The results imply that the

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two phenotypes, BAV and TAV, indeed have different molecular fingerprints leading to divergent molecular mechanisms of dilatation at both proteomic and transcriptomic levels.

## **Experimental Procedures**

### *Clinical samples*

Aortic biopsies were obtained through the Advanced Study of Aortic Pathology (ASAP) (5). Written informed consent from all participants had been obtained according to the declaration of Helsinki and with approval by the ethics committee of the Karolinska Institute (application number 2006/784-31/1). The study included BAV and TAV patients undergoing aortic valve surgery with a reconstitution of a dilated aorta and patients that only underwent aortic valve surgery (non-dilated controls) at the Karolinska University Hospital, Stockholm, Sweden. Aortic biopsies were taken from the anterior (convex) part of the aorta *i.e.* the site of aortotomy a few cm above the aortic valve. All patients were accepted for surgical treatment of aortic valve disease (aortic stenosis, ICD code: I35.1, or aortic regurgitation, ICD code: I35.0) and/or dilatation of the ascending aorta (ICD code: I71.2) (5). All aortic biopsies were obtained with the patient on normal thermic cardiopulmonary bypass after aortic crossclamping and myocardial arrest with the patient in general anesthesia. Induction of anesthesia was obtained by fentanyl, propofol, tracrיום and midazolam whereupon sevoflurane was used until initiation of bypass during which propofol and fentanyl were used. None of the patients had significant coronary artery disease according to coronary angiography. Marfan syndrome patients were excluded from the study. Definition of dilatation was based on an aorta dilatation of >45 mm (dilated) or <40 mm (non-dilated). Thoracic aorta dilatation measurements were taken at the point of the aorta showing maximal dilatation. The measurements were performed during anesthesia using transesophageal echocardiography. Tissue biopsies were frozen on dry ice directly after excision at the surgery. The samples were thereafter stored at -80°C until the day of protein extraction. The samples were anonymized directly after the surgery prior to the experimental procedures and analysis.

*Protein extraction and sample labeling for analytical and preparative gels*

The intima-media tissue layers from 22 dilated (6 TAV and 16 BAV) and 22 non-dilated (15 TAV and 7 BAV) aorta tissues were dissolved in a cell lysis buffer (2D protein extraction buffer V from GE Healthcare including 30 mM Tris buffer pH 8.0) including a protease inhibitor cocktail, Complete Mini Roche EDTA free (in order to prevent protease degradation of proteins). There were in total 14 females and 30 males. 25 patients had hypertension and 7 patients were diagnosed with diabetes. The tissue samples were, thereafter, mechanically homogenized using TissueLyzer system (Qiagen) according to manufacturer's instructions. The samples were subsequently sonicated in a water bath sonicator at 4°C (30 seconds sonication at high intensity followed by 30 seconds resting in 8 minutes), pelleted in a microcentrifuge at 12000g in 10 min at 4°C. The protein contents in the supernatant were measured with the Bradford protein assay (BioRad). The samples were aliquoted and stored at -80°C. The volume of protein lysate equivalent to 5 µg total protein was used for labeling with CyDye DIGE Fluor Labeling Kit for Scarce Samples (GE Healthcare) according to manufacturer's instructions. The internal standard was composed of all the samples pooled together and labeled with Cy3 while each sample individually was labeled with Cy5. 500 µg of the pooled internal standard samples were used for preparative gels and labeled with Cy3 and subsequently subjected to Silver staining (Serva) in order to be able to pick interesting spots. Two separate preparative gels were prepared, the same spots picked from both gels and identified individually thereby securing the spot-protein ID relationship.

*Two-dimensional differential gel electrophoresis*

The internal standard Cy3 samples and the individual Cy5 labeled samples were pooled and dissolved in DeStreak rehydration solution (GE Healthcare) with 0.5% 3-10 non-linear pH range (NL) IPG buffer (GE Healthcare) to a final volume of 450 µl. The mixture was thereafter deposited on the 1st dimension isoelectric focusing (IEF) Immobiline DryStrip pH 3-10 NL, 24 cm (GE Healthcare). The strips were covered with mineral oil and left for passive rehydration for 24 h on a protean IEF system

(BioRad). After rehydration, IEF was performed at 20°C at 300 V step and hold for 3 h, 1000 V gradient for 6 hours, 8000 V gradient for 3 h; it was finally completed at 8000 V step and hold for 3 h. The experimental plan was designed to allow for each sample to be thawed only once prior to first dimension IEF. 12 samples at time were subjected to IEF. The IEF strips were thereafter frozen at -80°C prior to second dimension. The samples were run on the second dimension within the recommended limit of time from CyDye manufacturer (GE Healthcare) of one month. The IPG strips were subjected to second dimension horizontal electrophoresis HPE™ Flat Top Tower system (Serva) according to the manufacturer's instructions. Briefly, the procedure was as follows below. The equilibration buffer, anode- and cathode buffers, cooling fluid, large second dimension gels with plastic backing and buffer wicks were provided by the manufacturer (Serva). The strips were equilibrated prior to the second dimension in dithiothreitol (DTT) in equilibration buffer. The second dimension flatbed non-fluorescent gels were put onto the ceramic cooling plate with cooling fluid between the gel and the plate (the system has room for running four gels in parallel). The equilibrated first dimension strip was put into the already existing slot in the second dimension gel (with gel side down for gel-gel contact). The second sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) dimension with four gels was run over night according to the program below. Step 1: 100 V, 28 mA, 4W, 30 min; Step 2: 200 V, 52 mA, 12 W, 30 min; Step 3: 300 V, 80 mA, 20 W, 10 min; first dimension strips were removed after Step 3, thereafter Step 4 was launched: 220 V, 20 mA, 8 W, overnight; Step 5: 1500 V, 160 mA, 120 W, 3 h. Care was taken as to include both dilated and non-dilated samples in one run including 12 first dimension and four second dimension samples.

#### *Gel scanning and analysis*

Both the analytical and preparative gels were scanned on a Typhoon FLA 9000 laser scanner (GE Healthcare). The excitation wavelength for Cy3 is 532 nm and for Cy5 633 nm, the emission wavelengths are 580 and 670 nm, respectively. Care was taken that no spot on any of the analytical

gels was saturated; therefore the photo multiplier tube (PMT) of the scanner was adjusted for each gel separately to be just below the level of saturation. Progenesis SameSpots (NonLinear Dynamics) gel image analysis software was used to align the images and export the normalized spot volumes for subsequent statistical data analysis.

#### *Protein identification*

109 protein spots were excised from the two preparative gels, destained and subjected to in-gel digestion with trypsin (modified, sequence grade porcine, Promega) (6). The tryptic peptides were concentrated and desalted on a micro C18 ZipTip (MilliPore) and eluted with 65% acetonitrile, containing the  $\alpha$ -cyano-4-hydroxycinnamic acid matrix, and applied directly onto the metal target followed by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Ultraflex III TOF/TOF, Bruker Daltonics). Peptide mass fingerprints (PTMs) were generated following the manufacturer's instructions and presented with the proprietary software FlexAnalysis v3.3. Peptide spectra were internally calibrated using autolytic peptides from trypsin ( $m/z$  842.51, 1045.56, 2211.11, and 3337.76). Searches in the NCBI nr sequence data base (the current available at time of search) using the ProFound tool (<http://prowl.rockefeller.edu/prowl-cgi/profound.exe>) were made in order to identify the proteins. One missed cleavage, and partial oxidation of methionine were allowed during the search. All searches were performed with a tolerance of  $\pm 0.03$  Da. Criteria for acceptance of identified proteins was based on score value derived by the search engine ProFound together with the number of missed cleavages and physical migration on the 2D gel compared to theoretical values.

#### *RNA extraction and Array hybridization*

mRNA expression analysis and mRNA extraction and preparation has been presented previously (2). Briefly, the intima-medial layer of the vascular specimens were isolated by adventicectomy, incubated with RNAlater (Ambion) and homogenized using FastPrep method (Qbiogene, Irvine) with

Lysing Matrix D tubes (MP Biomedicals, Germany). The RNA samples were hybridized and scanned at the Karolinska Institute microarray core facility. Affymetrix GeneChip® Human Exon 1.0 ST arrays and protocols were used.

### *Statistical data analysis*

A series of different multivariate statistical tools have been used to analyze protein expression, meta probe set (gene) as well as core probe set (exon) expression. These tools include principal component analysis (PCA) (7), and orthogonal projections to latent structures discriminant analysis (OPLS-DA) (8, 9). The multivariate models presented were all mean centered and unit variance scaled prior to the analysis. The model complexity was estimated according to cross-validation (10). The software used for multivariate models was Simca p+12.0.1x64 (Umetrics), unless otherwise stated. Import of Affymetrix GeneChip Exon ST 1.0 CEL files, subsequent robust multichip average (RMA) (11) normalization followed by SI calculations were made using OneChannelGUI package for R (12).

Multidimensional data obtained by screening several patient samples with regard to protein/gene/exon level expression can be considered as a set of patient vectors, where the expression of all proteins/genes/exons in a particular row defines the position of the vector in the multi-variable space, thereby describing the particular patient sample with regard to its protein/gene/exon expression. Each patient sample will thereby have a unique expression profile that displays a summary of all the proteins/genes/exons present in the data set, *i.e.* an expression fingerprint for that particular patient (4). PCA explains the variance-covariance structure of a set of variables by using linear combinations of those (7). The linear combination represent a new coordinate system that is obtained by rotating the original variable space. The new axes acquired will represent the directions with maximum variability where PC1 indicates the direction of the highest variability followed by consecutive PCs with diminishing variability orthogonal to the previous PC or PC plane.

OPLS-DA is a multivariate analysis method of choice when a quantitative relationship between a data matrix  $\mathbf{X}$ , in our case expression levels of different proteins/genes/exons, and a vector,  $Y$ , containing qualitative values (class belonging) need to be analyzed together (8). OPLS-DA separates the systematic variation in  $\mathbf{X}$  into two parts, one that is linearly related to  $Y$  and one that is unrelated (orthogonal) to  $Y$ . The systematic variation observed in  $\mathbf{X}$  that is not correlated to the response set  $Y$  is through this mathematical operation removed. The opportunity arises thereby to study only the variation that is correlated to the classification of interest. Two new units obtained from PCA and OPLS-DA, scores and loadings can be further examined. The scores give information about the patients projected onto a lower dimensional plane. The loadings, on the other hand, reveal how the PC model plane is inserted into the variable space and thereby can be used to interpret the meaning of the scores. Quality parameters for all multivariate analysis models (derived from cross-validation) performed in this study are shown in Supplemental Table 1.

#### *Protein level statistical data analysis*

Protein expression data (302 normalized spot volumes from 2D-gel) were subjected to both PCA and OPLS-DA analyses with regard to TAV (15 non-dilated and 6 dilated) and BAV (7 non-dilated and 16 dilated) patients separately with dilated and non-dilated qualitative  $Y$  variable. Patients were color coded in both TAV (8 females) and BAV (6 females) PCA according to sex in order to find out whether there are sex specific groupings in the data set. The results showed that no such groupings could be observed (data not shown). The significance of differentially expressed proteins was analyzed by means of loadings in OPLS-DA models for TAV and BAV including jack-knife confidence levels (9, 10) of the loadings. The significance was calculated as  $ABS(\text{loading}) - ABS(\text{jack-knife confidence interval})$ ; if positive indicates significant protein. The TAV model loadings were plotted against BAV model loadings in a combined model scatter plot in order to identify TAV and BAV specific differential protein expression events. The combined model scatter plot is informative since it reveals several

important characteristics about the data. To start with, the level of contribution of each protein spot to the model can be interpreted, the further away a protein spot is situated from the origin, the higher is the impact on the model. Furthermore, shared and discriminative proteins spots between the two models can be identified. A novel protein, not shown to be involved in thoracic aortic aneurysm previously, LDH, found to be statistically significant according to jack-knife confidence levels, was chosen for validation with Western blot. In an additional analysis PCA and OPLS-DA were applied to 22 non-dilated patient samples with TAV and BAV in contrast to the other analyses that are based on non-dilated versus dilated aorta tissue samples in TAV and BAV respectively.

#### *mRNA expression level statistical data analysis*

In the present study, PCA (7) was applied to meta probe set (gene) expression data for 43 unique genes that were identified by protein expression analysis and 81 patients including 51 dilated (15 TAV, 36 BAV) and 30 non-dilated (14 TAV, 16 BAV) previously published thoracic aorta intima/media samples (4). Both PCA and OPLS-DA were applied to this data set. GAPDH, HBA1, and MYL12B were extracted from Full evidence level. All other genes were extracted from Core evidence level and Unique hybridization target. Prior to the multivariate analyses, the mRNA expression data (meta probe set) was RMA normalized, scaled to unit variance (UV) and mean centered as described above. Dilated and non-dilated samples were color coded in the scores plot and the first three PCs (contributing the most to the variation in the multidimensional data space). The analyses were performed on TAV and BAV aorta tissue subsets separately, with dilated and non-dilated samples as two discriminant Y variable groups. The significance of differentially expressed genes was analyzed using loadings in OPLS-DA models for TAV and BAV including jack-knife confidence levels (9, 10) of the loadings as described above.

#### *Exon level statistical data analysis*

The exon and mRNA level data were preprocessed with Robust Multichip Average (RMA) (11), followed by splice index (splice index =  $\log_2(\text{probe set intensity}/\text{transcript expression level})$ ) calculation on core level, UV scaling and mean centering on 393 probe sets that belong to the 43 genes analyzed above. Thus, the exon level data was normalized with respect to whole transcript (gene) expression. Both PCA and OPLS-DA were applied to this data set. Genes with only one probe set (HSP90AB1, TAGLN2, and TUBB) together with the three genes (GAPDH, HBA1, and MYL12B) that were extracted from Full evidence level at gene level were excluded from exon analysis. The significance of differentially expressed exons was analyzed by means of loadings in OPLS-DA models for TAV and BAV including jack-knife confidence levels (9, 10) of the loadings, as described above.

#### *LC-MS/MS validation*

Four volumes of ice cold acetone was added to protein extracts (in the lysis buffer used above for protein extraction) from 21 selected patients (5 TAV non-dilated, 5 TAV dilated, 5 BAV non-dilated, and 6 BAV dilated, all of which also were included in the 2D-DIGE analysis), and each mixture was inverted three times and incubated at  $-20\text{ }^{\circ}\text{C}$  for 1 h. A 10 min centrifugation at  $12\ 000\ g$  ( $4\text{ }^{\circ}\text{C}$ ) followed, after which the supernatant was discarded and the pellet was washed with 0.5 ml of acetone. After re-dissolving the pellet in 1 % SDS, each sample was further diluted in two steps to 0.2% SDS, and protein concentration determined with the Dc protein assay (Bio-Rad). According to the concentrations estimated, equal total protein amounts from each sample were taken and TEAB (triethylammonium bicarbonate buffer, pH 8.5) was added to 50 mM (and SDS diluted to 0.1%). Reduction of cysteines was done by addition of  $2\ \mu\text{l}$  of 50 mM dithiothreitol and heating to  $55\text{ }^{\circ}\text{C}$  for 30 min. Carbamidomethylation followed by addition of  $4\ \mu\text{l}$  of 100 mM iodoacetamide. Trypsin was added 1:50 (ratio trypsin:substrate), and digestion proceeded overnight at  $37\text{ }^{\circ}\text{C}$ . From the resulting peptide mixtures, equal amounts were taken from each of the 21 samples to produce an internal standard. Three sets of 8 samples (each set contained 7 patient samples, with both non-dilated and dilated tissue types represented, together with 1 internal standard) were labeled with iTRAQ8plex

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following the manufacturer's instructions. After pooling, all iTRAQ8-labeled peptide sets were cleaned by strong cation exchange solid phase extraction (SCX-SPE, strata-X-C, Phenomenex). Each of the three pools was then re-dissolved in LCMS solvent A (97% water, 3% acetonitrile (ACN), 0.1% formic acid (FA)) to obtain 1 µg/µl solutions.

The LC auto sampler (HPLC 1200 system, Agilent Technologies) injected 1 µl into a C18 guard desalting column (ZORBAX 300SB-C18, 5µm bead size, 5x0.3mm, from Agilent). We then used a 15 cm long C18 picofrit column (100 µm internal diameter, 5 µm bead size, Nikkyo Technos Co., Tokyo, Japan) installed on to the nano electrospray ionization (NSI) source. Solvent B was composed of 5% water, 95% ACN, and 0.1% FA. At a constant flow of 0.4 µl/min, the curved gradient went from 2% solvent B up to 40% solvent B in 4h. Online LC-MS was performed using a hybrid LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Precursors were isolated with a 2 m/z window. We enabled "preview mode" for FTMS master scans, which proceeded at 30000 resolution (profile mode). Data-dependent MS/MS (centroid mode) followed in two stages: firstly, the top 5 ions from the master scan were selected for collision induced dissociation (CID, at 35% energy) with detection in the ion trap (ITMS); and after, the same 5 ions underwent higher energy collision dissociation (HCD, at 37.5% energy) with detection in the orbitrap (FTMS). The entire duty cycle lasted ~3.5 s. Dynamic exclusion was used with 90 s duration. Each iTRAQ8plex pool was run in analytical duplicates.

All MS/MS spectra were searched by Sequest under the software platform Proteome Discoverer 1.3.0.339 (Thermo) against the human SwissProt protein sequence database (downloaded from uniprot.org on 20120202) using a target-decoy strategy. Precursor mass tolerance of 10 ppm and product mass tolerances of 0.02 Da for HCD-FTMS and 0.8 Da for CID-ITMS were used. Additional settings were: trypsin with 1 missed cleavage; carbamidomethylation on cysteine and iTRAQ-8plex on lysine and N-terminal as fixed modifications; and oxidation of methionine and phosphorylation on

serine, threonine or tyrosine as variable modifications. Quantitation of iTRAQ-8plex reporter ions was done using an integration window tolerance of 20 ppm. Reported results have 1% false discovery rate at peptide level. The number of entries searched in the data base were according to the following: Swissprot human subset downloaded from uniprot.org on 20120202 was searched with 35818 protein entries containing 20909407 amino acid residues.

286 proteins were identified as common in the three pools from LC-MS/MS. All the identified proteins were subjected to OPLS-DA analysis where dilated samples were analyzed versus non-dilated samples in TAV and BAV separately (according to the statistical data analysis for 2D-DIGE data). Furthermore, 35 2D-DIGE identified proteins that were also found to be present in LC-MS/MS data were analyzed separately with dilated versus non-dilated samples in TAV and BAV separately and the results were compared in a combined model scatter plot where TAV model loadings were plotted against BAV model loadings.

#### *Western blot*

Fluorescent western blot technique, ECL Plex (GE Healthcare) with Cy5 coupled secondary (goat anti-rabbit) antibody was used for validation of differentially expressed LDH and Transferrin proteins. Transferrin was used as internal standard. Both of the primary antibodies were of rabbit origin, diluted 1:200 from Santa Cruz. The secondary antibody was diluted 1:2500. Proteins were separated using SDS-PAGE electrophoresis on 1mm thick pre-made gradient (4-12%) gel (Invitrogen). The same amount of protein (17.5µg) was loaded for all the samples (4 TAV non-dilated and 4 TAV dilated). The membranes were scanned with a Typhoon scanner (GE Healthcare), using the red (633) laser and 670 BP 30 filter setting for Cy5 detection.

#### *Immunohistochemistry*

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Immunohistochemistry was performed on paraffin embedded sections taken from aorta of dilated and non-dilated patients, stained with anti-human TTR (SC-13098), VIM (SC-66002), LDH (SC-33781), TGM2 (SC-20621), all purchased from Santa Cruz, CA.

## Results

### *Protein separation and detection by 2D DIGE analysis*

Our approach to proteomic analysis of patients with TAA involved 2D-DIGE experiments of 44 patients with either TAV (15 with non-dilated aorta and 6 with dilated ascending aorta) or BAV (7 with non-dilated and 16 with dilated ascending aorta). Protein spots were detected using the SameSpots software in protein extracts from the intima/media region of the aorta (Figure 1). Inter-gel matching was performed through the inclusion of the internal standard on each gel. Apparent protein spots (302 spots) were subsequently included in further analysis based on visual inspection of each individual spot (Figure 1). The subsequent analysis was performed on sample/standard normalized spot volumes, thereby improving the accuracy of protein quantitation between samples from different gels.

### *Multivariate data analysis of protein expression data showed differential protein expression patterns in TAV and BAV*

The normalized spot volumes were subjected to multivariate data analyses, PCA and OPLS-DA. Both PCA and OPLS-DA were performed on TAV and BAV patients separately (Figure 2, Supplemental Table 1). The non-supervised PCA score plot in PC1-PC3 plane showed that non-dilated (black) and dilated (red) samples separate well in both TAV and BAV patients. This clustering was further highlighted when a supervised OPLS-DA model with class-belonging (dilated or non-dilated) was performed (Figure 2, Supplemental Table 1). It is clear that the dilated and non-dilated tissue samples form two different clusters along the predictive component, tp1, describing correlation with the Y vector, in OPLS-DA scatter plot in both TAV and BAV models. This indicates that both patients with TAV and BAV show different protein expression profiles in non-dilated and dilated tissue samples.

In order to find out what protein spots that are responsible for the clustering seen in the score plots,

the loadings were analyzed (Figure 3). The contribution of each protein spot to the separation seen among the patients (Figure 2) can be visualized as loadings of the two separate OPLS-DA models, patients with TAV and patients with BAV (Figure 3). Jack-Knife confidence levels derived from cross-validation of the two models were calculated and visualized together with the loadings (Figure 3 A-C). The significance was calculated as  $ABS(\text{loading}) - ABS(\text{jack-knife confidence interval})$ ; if positive indicates significant protein spot (Supplemental Table 2, Figure 3). This calculation resulted in 69 differentially expressed protein spots between dilated and non-dilated aorta specific for TAV (Figure 3B) and 38 differentially expressed protein spots specific for BAV (Figure 3C). As shown in Figure 3D, 92 protein spots were commonly differentially expressed in both TAV and BAV patients (Figure 3A). The contribution of loadings to the TAV and BAV models is also visualized in a combined model scatter plot where loadings for the TAV and BAV models are plotted against each other (Figure 3E). In this respect, proteins that are situated in the beginning and in the end of the diagonal (red dots in Figure 3E) show shared protein expression patterns between TAV and BAV, proteins that had higher expression in dilated samples are situated in the upper right hand corner while proteins that display higher expression in non-dilated samples can be found in the lower right hand corner on the opposite side of the diagonal in the combined model scatter plot (Figure 3E). In similar matter, TAV specific proteins can be found along the X-axis; if higher in dilated tissue samples on the right of origin and if higher in non-dilated samples on the left side of the origin (blue dots in Figure 3E). The corresponding BAV specific proteins can be seen on the Y-axis; if higher in dilated tissue samples in the upper part of the plot (positive values) and if higher in non-dilated tissue samples in the lower part of the plot (negative values along the Y-axis) (green dots in Figure 3E).

The multivariate statistics indicate that a larger proportion of protein spots are differentially expressed in patients with TAV compared to patients with BAV. In addition, an analysis based on only non-dilated patients was performed (Supplemental Table 1, Supplemental Figure 1). The analysis was based on TAV vs BAV OPLS-DA as well as a non-supervised PCA where TAV and BAV patients have

been color-coded after the analysis was performed. It is clear from the model quality parameters (Supplemental Table 1) that the model is not satisfactory, neither for PCA nor for OPLS-DA. This showed that the two clusters of TAV and BAV cannot be separated in non-dilated patients, based on the protein expression of the 302 protein spots included in the analysis. There were thereby very few protein spots that showed significance in the model (Supplemental Figure 1C).

### *Protein identification*

Protein spots chosen for identification by MALDI-TOF are marked with numbers in Figure 1 and the combined model scatter plot in Figure 3. In total, 109 protein spots were chosen for identification (Supplemental Table 3) including spots differentially expressed between dilated and non-dilated aorta only in TAV, BAV or both TAV and BAV. Furthermore, protein spots that did not show any differential expression were also included (situated close to origin in Figure 3E). The protein identification of the vast majority of identified spots showed satisfactory sequence coverage and expectation values (Supplemental Table 3). The identification of all 109 spots is shown in Supplemental Table 3. The 109 spots identified correspond to 43 individual proteins (keratin excluded). Protein ID of all identified proteins are included in Table 1. One representative of each cluster of spots with same identification is shown. The selection criteria of the representative protein spot included significance in the majority of the spots, *e.g.* if all the spots or the majority of spots in a certain cluster of proteins with same identification (Supplemental Table 3) were significantly differentially expressed between dilated and non-dilated aorta samples in TAV and/or BAV, one of the spots from that particular cluster was included in the new table (Table 1) and color coded according to the fold change and significance. In the case where all spots or the majority of spots in a certain cluster did not display any significance, that cluster is represented by an empty field (*e.g.* transferrin, TF in Table 1, Supplemental Table 3).

LC-MS/MS was chosen as a separate method of protein quantification on a subset of patients included in the 2D-DIGE analysis in order to validate the results from 2D-DIGE analysis. In the LC-MS/MS experiments, 21 patients (5 TAV non-dilated, 5 TAV dilated, 5 BAV non-dilated, and 6 BAV dilated) were included, all of which also can be found in the 2D-DIGE analysis. Same multivariate analysis techniques as for 2D-DIGE experiments were also applied on LC-MS/MS data, PCA and OPLS-DA (Supplemental Figure 2 and 3, Supplemental Table 1). Out of 43 individual proteins identified in the 2D-DIGE analysis, 35 were also found and extracted from the LC-MS/MS analysis (Table 2, Supplemental Table 4). The significance was derived from loadings in OPLS-DA as ABS (loading) – ABS (jack-knife confidence interval); if positive indicates significant protein (Supplemental Table 5, Supplemental Figure 2). The significance was calculated as non-dilated versus dilated tissue samples in TAV and BAV models separately (Supplemental Figure 2). This calculation resulted in 12 differentially expressed proteins between dilated and non-dilated aorta in TAV and 16 differentially expressed proteins in BAV (Table 2). In comparison with 2D-DIGE experiments, 57% and 71% of significant differentially expressed proteins were validated with LC-MS/MS analysis in TAV and BAV respectively (Table 2). Furthermore, 286 proteins identified as common in all three LC-MS/MS pools, were subjected to OPLS-DA analysis according to above and summarized together with the 35 proteins identified by 2D-DIGE in a combined model scatter plot for TAV and BAV models. It can be seen in Supplemental Figure 3A that 2D-DIGE identified proteins (colored as red, green and blue) are found as highly contributing proteins in TAV and BAV models even in a model where all LC-MS/MS proteins were included. When comparing to the corresponding combined model scatter plot of LC-MS/MS data where only 2D-DIGE identified proteins were included (Supplemental Figure 3B), it is clear that the two analyses are, by and large, following the same pattern, *e.g.* MYL6 (P60660), and PARK7 (Q99497) are both situated in the upper right hand corner in both Supplemental Figure 3A and B, indicating that both have significantly higher expression level in dilated samples in both TAV and BAV patients. Similarly, HBB (P68871), and HBA1 (P69905) are both situated in the lower left

hand side corner in both Supplemental Figure 3A and B, indicating that both have significantly higher expression levels in non-dilated samples in both TAV and BAV patients. On the other hand, LDHA (P00338) and MYL9 (P24844) have significant higher expression levels in dilated samples only in TAV and BAV respectively in both Supplemental Figures 3A and B. In summary, these results suggest that proteins identified as significant in 2D-DIGE analysis also are found in an overall analysis of all identified proteins with LC-MS/MS as highly contributing proteins in both TAV and BAV models.

Lactate dehydrogenase (LDHA) is one of the proteins that showed significant protein differential expression between dilated and non-dilated aorta samples in both 2D-DIGE and LC-MS/MS experiments (Table 1, and 2) and was, therefore, chosen for differential protein expression validation with Western blot. TF was chosen as internal standard for Western blot validation due to the fact that it did not show any significant differential expression between dilated and non-dilated aorta. Four TAV patients with non-dilated aorta and four TAV patients with dilated aorta tissues were used for Western blot experiments (Figure 4). The results showed that LDH, when normalized to TF expression, was significantly up regulated in dilatation compared to non-dilated aorta ( $p = 0.015$ ) (Figure 4B). The differential expression of LDH in Western blot experiment is thus in concordance with the corresponding differential expression seen in the 2D gel and LC-MS/MS analyses.

Immunohistochemical staining of sections from the four patient groups was performed using antibodies directed against differentially expressed proteins selected from Table 1. We chose anti human antibodies against LDH, TGM2, and VIM (all up-regulated in dilated TAV), anti-TTR (down-regulated in dilated BAV). Overall, the four differentially expressed proteins chosen for IHC staining were localized in endothelial and smooth muscle cells in the vessel walls, giving further support to the 2D gel results.

A literature search for the function of proteins differentially expressed between dilated and non-dilated aorta in TAV and BAV is presented in Table 3. Scrutinizing the function of proteins that are specifically regulated in each group suggests that dilatation in BAV and TAV develops via different mechanisms. In particular, proteins related to inflammation were associated with dilatation in patients with TAV whereas in BAV, proteins involved in repair processes were detected. Protein function in TAV and BAV is further covered in the discussion section.

*Aortic mRNA expression of the identified proteins differs between patients with TAV and BAV in dilated and non-dilated aorta*

mRNA expression corresponding to the 43 individual proteins was subjected to multivariate data analysis, in order to discover which genes that are differentially expressed between dilated and non-dilated aorta in patients with TAV and BAV (Supplemental Figure 5, Supplemental Table 6, Table 4). The analysis, based on data from 29 TAV and 52 BAV patients (4), resulted in 16 and 14 differentially expressed genes according to multivariate statistics (significant according to jack-knife confidence levels and loadings) in TAV and BAV, respectively. Dilated and non-dilated patients formed separate clusters in PCA in both TAV and BAV analyses as seen in three dimensional score plot in Supplemental Figure 5A and D. This was further highlighted by a good model (Supplemental Table 1) and a good separation in OPLS-DA (Supplemental Figure 5B and E). Diverging expression fingerprints leading to dilatation in TAV and BAV were evident at the transcript level, with 7 and 5 genes differentially expressed only in TAV and BAV respectively (Table 4). However, although mRNA expression was able to separate patients with dilated and non-dilated aorta, mRNA expression correlated poorly to the protein expression (Table 1 and 5).

*Multivariate data analysis of exon expression data*

Gene level normalized exon expression (splice index) corresponding to the 43 individual proteins (393 probe sets), were subjected to multivariate data analysis in order to analyze differential splicing of the identified proteins (Supplemental Figure 6, Table 5, Supplemental Table 7 ). PCA showed a separation of dilated and non-dilated clusters in both TAV and BAV (Supplemental Figure 6 A and B). The separation between dilated and non-dilated patients in both TAV and BAV, however, was evident foremost by the good OPLS-DA model (Supplemental Table 1) and subsequent separation seen in the OPLS-DA score plot (Supplemental Figure 6 B and E). The number of significantly differentially expressed exons between dilated and non-dilated aorta samples (normalized with respect to the whole transcript level, *i.e.* splice index) according to jack-knife confidence levels and loading values from OPLS-DA were 112 and 97 in TAV and BAV respectively (Supplemental Table 7, Supplemental Figure 6 C and F). The contribution of loadings to the TAV and BAV models was visualized in a combined model scatter plot (Supplemental Figure 6G) where loadings for the TAV and BAV models are plotted against each other accompanied by a Venn diagram (Supplemental Figure 6H). The combined model scatter plot clearly illustrated that 43 significant gene level normalized exons were common between TAV and BAV, 69 were TAV specific while 54 were BAV specific. From the multivariate analysis it was thereby evident that TAV and BAV patients show diverging exon expression fingerprints in the identified proteins between dilated and non-dilated aorta tissues, implying that different protein isoforms may be present in TAV compared to BAV.

## Discussion

In the present study, TAA associated protein expression in patients with BAV and TAV was identified using 2D-DIGE together with multivariate statistics. Moreover, the analysis of protein spots was combined with microarray mRNA and exon expression analysis. Our results collectively demonstrated that TAA formation in patients with BAV has clearly diverging expression fingerprints compared to the patients with TAV, at all three levels of gene, exon and protein analysis. This study provides further support to our previously performed mRNA expression analyses including all expressed genes in the human genome (2).

2D-DIGE proteomic results were validated using an independent, peptide based proteomic method, LC-MS/MS and 57% and 71% of the identified proteins were validated in TAV and BAV respectively. It is important to notice that 2D-DIGE analysis results in a number of protein spots that have been identified as the same protein, but modified potentially with post translational modifications. LC-MS/MS analysis on the other hand, identifies a number of protein isoforms that have at least one unique peptide included in their sequence. The 2D-DIGE statistical analysis was performed on 302 protein spots for which the statistics were summarized in 43 proteins in Table 1, while LC-MS/MS statistical analysis was performed on 35 proteins (a subset of the 43 proteins from 2D-DIGE experiments since eight proteins were not identified with LC-MS/MS) (Table 2). This discrepancy between the two methods nevertheless, the percentage of validated proteins turned out to be very high which clearly strengthens the results of the 2D-DIGE analysis (Table 2).

Literature search of the identified proteins indicated that the function of differentially expressed proteins in medial degeneration of aorta in patients with TAV was dominated by inflammatory processes. On the other hand, the corresponding function in patients with BAV was most probably due to the impaired repair capacity in these patients. The functional repertoire of all the identified

proteins is summarized in Table 3. Two proteins found to be associated with BAV were the plasma protein transthyretin (TTR) and the highly conserved plasma glycoprotein, Serum Amyloid P component (SAP/APCS). The function of these two proteins in the aortopathy of BAV patients is not clear. However, TTR protein expression was reduced in dilated aorta of BAV patients as compared to BAV patients with non-dilated aorta whereas APCS protein expression was increased. Hence, both proteins may function in the repair processes of the damaged vessels in BAV since the association of increased TTR with facilitated wound healing (13) and inhibition of fibroblasts differentiation by elevated level of APCS in wound healing has been reported (14). TTR is also associated with senile systemic amyloidosis (SSA), in which wild-type TTR forms amyloid deposits in various tissues, and is an age-related nonhereditary systemic amyloidosis affecting mainly cardiac functions in elderly (Reviewed in (15)). The role of TTR in amyloidosis is complex and although TTR itself has been associated with fibril formation in amyloidosis, it is also capable of acting as a protease cleaving amyloid beta peptide thereby exerting a protective role in the pathology of Alzheimer's disease AD (16). Reductions of TTR concentration in cerebrospinal fluid of AD patients (17) and hippocampus of AD mouse model (18) was shown to be negatively correlated with disease severity. Similar to TTR, APCS is also universally found associated with amyloid depositions independently of the protein origin (19). The possibility of BAV dilatation being related to proteinopathies and the function of these two amyloidosis-related proteins in dilatation of BAV patients deserves further clarification. Furthermore, the expression of three myosin light chains, MYL6, MYL9 and MYL12B have significantly increased in dilatation of BAV while only MYL6 expression is also increased in TAV dilated patients. Myosin light chains are phosphorylated and thereby promote actomyosin contraction which generates pulling force between the adjacent cells and widening of the intercellular gap (20). This may imply that the process of vascular barrier breakdown is activated more significantly in BAV dilatation. This is further supported by an increased albumin expression in dilated BAV.

The only protein where one of the protein spots (isoforms) was up regulated in dilated BAV aorta and the other protein spot (isoform) down regulated in dilated TAV is filamin binding LIM protein (FBLIM1/migfilin). FBLIM1 has several important interactions which makes it a very important molecular switch for cell-ECM and cell-cell interactions. It localizes to both cell-extracellular matrix and in endothelial cells adherens junctions via its C-terminal end LIM binding domain. However, its recruitment to ECM is via interaction with another protein, fermitin family member 2 (FERMT2/MIG2) that is not required for the FBLIM1 localization to adherens junctions. The N-terminal part of the protein binds filamin through which it regulates the cytoskeleton (Reviewed in(21)). Furthermore, it acts as a molecular switch, disconnecting filamin from integrin for regulating integrin activation and dynamics of extracellular matrix-actin linkage (22). It has been proposed that since the C-terminal domain is required for both localization of FBLIM1 to cell-ECM adhesions as well as cell-cell junctions there will be a competition between the two activities of FBLIM1 and this will dictate the relative distribution of FBLIM1 between the two pathways (Reviewed in(21)).

Interestingly, we have observed a higher expression of FERMT2/MIG2 mRNA in BAV relative to TAV irrespective of the dilatation state (Maleki et al, (23)), implying that the balance between the two cellular pathways may have changed in BAV dilatation relative to TAV. This observation may be very important with respect to the differences between the dilatation in BAV and TAV and may separate the two events mechanistically. Another relevant observation in this regard is that the distribution of FBLIM1 is barely detectable in normal smooth muscle cells (SMC), but abundant in neoplastic transformed SMC, implying that its expression in SMC is related to the pathological changes (24).

Up regulation of transglutaminase 2 (TGM2) was only detected in TAV patients. This is interesting since transforming growth factor  $\beta$  (TGF $\beta$ ) is known to upregulate TGM2 (25). Previous data have indicated differences in TGF $\beta$  signaling pathway between BAV and TAV patients (4, 26). Furthermore, vimentin (VIM), one of the components of intermediate filament, is a major substrate for transglutaminases (27). Hence, medial degeneration in TAV patients may be the result of vimentin

crosslinking by TGM2, as has been proposed for arterial remodeling (27). Moreover, most of the proteins specifically associated with dilatation in TAV *i.e.* FGG, VIM, MFAP4, and HSPA1L have also been cited in association with either inflammatory diseases or inflammatory processes (Table 1, Table 3) which is in line with our previous observation of an increased medial inflammation in TAV but not in BAV patients (2). We propose that medial degeneration in TAV may be the result of a direct attack on the medial layer by pro-inflammatory molecules, while in BAV it may be secondary to the higher permeability and diminished capacity of vascular endothelium for regeneration. One of the factors which may contribute to higher permeability of endothelial layer can be the constant exposure of BAV aorta to abnormal hemodynamics as has been demonstrated (28, 29). In support of this argument, we have recently shown that in BAV aorta, there is an angiostatic profile of gene expression, containing several EC-specific-genes, even in non-dilated aortas (23).

Although both mRNA expression and protein expression could separate patients with dilated aorta from patients with non-dilated aorta, it was clear that there were very few differentially expressed genes showing the same pattern of differential expression at protein level (Supplemental Table 8, Table 1 and 4). This is not unexpected and, in accordance with our data, other studies have shown that correlation between mRNA expression and protein expression is overall low (30). An explanation to this may be that individual half-lives of proteins are influenced by several post-translational factors which will influence the mRNA-protein expression correlation. The life time of a protein is dependent upon a number of different processes, *e.g.* protein stability, post-translational processing (phosphorylation, and ubiquitination), as well as localization of the protein (31) (30), processes that can lead to discrepancy observed in the expression at transcript and protein levels. Furthermore, as shown from our analyses, there were several spots that could be detected representing the same protein. The different spots probably represent different post-translational modifications that will influence the comparison between mRNA level, representing the total gene expression, with the expression of a subset of the protein. Another possibility could be that the different spots represent different splice forms of the protein. We have previously analyzed differential splicing in the TGF $\beta$

signaling pathway in the same cohort (4). From this analysis it was clear that differential splicing is a common phenomena but the study also showed that the expression of the differentially spliced mRNAs were low compared with the wild-type transcript. Therefore, it is unlikely that the protein spots that could be detected by the 2D gel analyses represent different protein isoforms as a result of differential splicing. This is further supported by the lack of clear association between the presence of splicing and the number of detected protein spots.

Several previous studies have performed proteomic studies to identify protein expression associated with TAA. In only one of the studies, TAV and BAV patients were analyzed separately (32). However, in that study, only patients with dilated aorta tissues were included. By analyzing 16 patients in total, the authors showed that heat shock protein 27 was significantly lower in dilated aorta of BAV patients compared to dilated aorta of TAV patients. In the present study, heat shock protein 27 was significantly higher in dilatation in both TAV and BAV samples (Table 1), while there is no significant difference observed between TAV and BAV in neither dilated nor non-dilated samples (data not shown). Furthermore, a study of aneurysm in human ascending aorta showed that ENO1 variant (fragment) had significantly higher expression while VIM shows lower expression in aneurysm tissues compared to controls (33). In the present study, ENO1 protein expression was higher in dilated aorta in both TAV and BAV patients (Table 1) thus showing similar pattern as the study performed by Schachner et al. VIM, however, was higher in dilated samples only in TAV, but not in BAV in the present study (Table 1).

Since non-dilated samples were obtained from patients with TAV and BAV undergoing valve repair it is important to evaluate their status as control samples. We have previously analyzed the global mRNA expression profiles of these samples together with control samples taken from heart transplantation donors. Based on total mRNA expression, the transplant control samples clustered close to non-dilated aorta media tissue samples in a PCA thereby showing that the non-dilated tissue

samples have similar mRNA expression profile as the transplant control samples (2). The divergent mRNA expression and protein fingerprints further strengthen the notion that dilated samples from patients with BAV need to be compared to non-dilated aorta from patients with BAV.

In summary, the present study suggests that the dilatation and medial degeneration evolves by two different mechanisms in patients with BAV and TAV. In particular, the importance of components of the wound healing machinery and inflammation for the observed differences in aortopathy between patients with BAV and TAV is highlighted.

Running title: Proteomics in thoracic aortic aneurysm

### **Funding Sources**

This work was supported by the Swedish Research Council, the Swedish Heart-Lung Foundation, the European Commission [FAD, Health F2 2008 200647], a donation by Fredrik Lundberg, VINNOVA [Shohreh Maleki], and Swedish Research Council post-doctoral fellowship [21629 to S.K.].

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## Figure legends

### *Figure 1*

Representative gel figure showing internal standard. Spots chosen for identification are marked in circles and numbers. The isoelectric point range is 3-10 non-linear, as indicated on top of the figure.

### *Figure 2*

PCA (A-B) and OPLS-DA (C-D) of protein spot expression data. The analyses were performed on 44 patients and 302 protein spots. The Hotelling's T<sup>2</sup> (based on 95% confidence level) tolerance ellipsoid and ellipse are shown in the scores plots of PCA and OPLS-DA, respectively. Three-dimensional scores plot showing the PC1-PC3 plane of non-dilated (black) and dilated (red) thoracic aorta tissue samples with TAV (A) and BAV (B) patients separately. Two-dimensional scores plot of an OPLS-DA showing the first predictive component (tp1) and orthogonal component (to1) plane of non-dilated (black) and dilated (red) thoracic aorta tissue samples with TAV (C) and BAV (D) separately.

### *Figure 3*

Venn diagram and OPLS-DA of protein spot expression data. The significantly differentially expressed protein spots have been calculated with OPLS-DA where  $ABS(\text{loadings}) - ABS(\text{jack-knife confidence interval})$  if positive indicates significance. The model has been applied on 302 protein spots of dilated versus non-dilated samples (44 patients with and without TAA) in TAV and BAV separately. The bar plot of loadings and jack-knife confidence intervals contributing to the first PC for significant spots common for TAV and BAV models, red bars (A), statistically significant only in TAV, blue bars, and BAV, green bars (B) and (C) respectively. (D) Venn diagram based on  $ABS(\text{loading}) - ABS(\text{jack-knife confidence interval})$  significant protein spots of dilated versus non-dilated samples in TAV and BAV patients separately (derived from Supplemental Table 2), color coded according to (A)-(C). (E) Combined model scatter plot where loadings from TAV model of dilated versus non-dilated aorta

samples is plotted against loadings from BAV model of dilated versus non-dilated aorta samples, color coded according to (A)-(C). Proteins that are significantly differentially expressed only in TAV and BAV are marked in blue and green respectively, while proteins that show statistically significant differential expression in both TAV and BAV are marked in red. 109 protein spots chosen for identification by MALDI-TOF are marked with numbers in the combined model scatter plot. The black diagonal is aimed for the interpretation purposes.

*Figure 4*

Western blot validation of differentially expressed protein LDH. Eight patients with TAV, four with dilated and four with non-dilated aorta tissues, were chosen for Western blot validation. (A) Western blot gel showing LDH and the internal standard TF. (B) Box-plot of TF normalized LDH expression (y-axis) in four dilated and four non-dilated aorta tissue samples from (A).

**Table 1.** Proteins identified by MALDI TOF MS from 2D-DIGE experiments. Significant proteins according to multivariate statistics indicated. <sup>a</sup>Dark green and blue indicate statistical behavior of the majority of protein spots in a particular protein cluster, while light blue and green on the other hand indicate the behavior of at least one of the spots for a particular protein. In the cases where all protein spots are not-significant, the colour is white. Dilated (D) and non-dilated (C) thoracic aortic samples were examined. See Supplemental Table 2 for complete statistical analysis for each protein spot.

Gene name	Protein name	Protein accession number	No of protein spots**	TAV		BAV	
				CvsD <sup>b</sup>	Fold (D-C) <sup>c</sup>	CvsD <sup>d</sup>	Fold (D-C) <sup>c</sup>
ACTA2	actin, aortic smooth muscle	P62736	6	0.056	1.40	0.081	1.36
ACTN1	alpha actinin 1	P12814	2	0.051	1.44	0.049	1.34
ACTN4	alpha actinin 4	O43707	5	0.063	1.44	0.086	1.35
ALB	Serum albumin	P02768	11	0.040	-1.23	0.011	1.17
ANXA1	annexin A1	P04083	2	0.030	1.42		
ANXA4	annexin A4	P09525	1				
ANXA5	annexin A5	P08758	1				
APCS	serum amyloid P-component precursor	P02743	1			0.035	1.22
CLIC4	chloride intracellular channel protein 4	Q9Y696	1				
ENO1	alpha-enolase isoform 1	P06733	3	0.025	1.30	0.050	1.25
FBLIM1	filamin-binding LIM protein 1	Q8WUP2	2	0.012	-1.17	0.021	1.13
FGB	fibrinogen beta chain, isoform CRA_d	P02675	1				
FGG	fibrinogen gamma	P02679	1	0.078	1.79		
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	P04406	2	0.027	1.14	0.037	1.15
GSN	gelsolin	P06396	3	0.024	1.63	0.039	1.38
HBA1	hemoglobin subunit alpha 1	P69905	1	0.042	-2.45	0.044	-1.70
HBB	Hemoglobin subunit beta	P68871	7	0.053	-3.06	0.057	-2.00
HBD	hemoglobin subunit delta	P02042	1	0.044	-2.58	0.045	-1.76
HSP90AB1	heat shock protein HSP 90-beta	P08238	1	0.083	1.80	0.063	1.28
HSPA1L	HSP70-1 (heat shock protein)	P34931	3	0.035	-1.25		
HSPB1	heat shock protein 27	P04792	2	0.042	1.64	0.044	1.29
KIAA1429	KIAA1429 protein	Q69YN4	1	0.025	1.31	0.015	1.18
LDHA	lactate dehydrogenase A variant	P00338	1	0.022	1.39		
LGALS1	galectin-1	P09382	1	0.021	1.38	0.049	1.27
MFAP4	microfibrillar-associated protein 4	A8MVM2	2	0.021	-1.20		
MYL6	myosin light polypeptide 6	P60660	1	0.026	1.47	0.031	1.28
MYL9	myosin regulatory light polypeptide 9	P24844	2			0.026	1.22
MYL12B	Myosin regulatory light chain 12B	O14950	2			0.012	1.14
OGN	osteolectin OG	Q7Z532	3	0.046	-1.27	0.027	-1.35
PARK7	protein DJ-1	Q99497	1	0.030	1.27	0.037	1.19
PDLIM3	PDZ and LIM domain protein 3 isoform b	Q53GG5	1				
TAGLN	transgelin	Q01995	5	0.030	1.49	0.030	1.30
TAGLN2	Transgelin 2	P37802	1				
TF	transferrin	P02787	4				
TGM2	protein-glutamine gamma-glutamyltransferase 2 isoform a	P21980	1	0.004	1.28		
TINAGL1	tubulointerstitial nephritis antigen-like isoform 3	Q9GZM7	2	0.032	1.25	0.031	1.14
TPM2	tropomyosin beta chain	P07951	3	0.039	1.27	0.082	1.34
TTR	Transthyretin	P02766	1			0.005	-1.21
TUBB	Tubulin, beta	P07437	3	0.037	1.23	0.045	1.16
VIM*	vimentin	P08670	8	0.014	1.31		
YWHAH	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3 protein beta/alpha)	P31946	2	0.076	1.43	0.001	1.12
YWHAQ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3 protein theta)	P27348	1	0.050	1.80		
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3 protein zeta/delta)	P63104	2	0.065	1.38	0.029	1.12

<sup>a</sup> The statistical analyses are based on non-dilated versus dilated thoracic aorta samples in TAV and BAV.

<sup>b</sup> ABS (loading)-ABS(jack-knife conf intervall) protein expression data, dilated higher (green), non-dilated higher (blue), TAV

<sup>c</sup> Difference in average mean protein spot expression between the groups indicated

<sup>d</sup> ABS (loading)-ABS(jack-knife conf intervall) protein expression data, dilated higher (green), non-dilated higher (blue), BAV

\* Previously published

\*\* Number of protein spots identified on the gel

**Table 2.** Comparison between proteins identified by MALDI TOF MS from 2D-DIGE experiments including 44 patients and from LC-MS/MS validation experiments including 21 patients (a subgroup of patients included in the 2D-DIGE experiments). Significant proteins according to multivariate statistics indicated. <sup>a</sup>Dark green and blue indicate statistical behavior of the majority of protein spots in a particular protein cluster, while light blue and green on the other hand indicate the behavior of at least one of the spots for a particular protein. In the cases where all protein spots are not-significant, the colour is white. Dilated (D) and non-dilated (C) thoracic aortic samples were examined. See Supplemental Table 4 for identification details on the LC-MS/MS analysis.

Gene name	Protein accession number	No of protein spots**	TAV (CvsD <sup>b</sup> )		BAV (CvsD <sup>d</sup> )	
			2D-gel	LC-MS/MS <sup>c</sup>	2D-gel	LC-MS/MS <sup>c</sup>
ACTN1	P12814	2	0.051		0.049	0.115
ACTN4	O43707	5	0.063		0.086	0.161
ALB	P02768	11	0.040		0.011	
ANXA1	P04083	2	0.030			
ANXA4	P09525	1				
ANXA5	P08758	1				
APCS	P02743	1			0.035	
CLIC4	Q9Y696	1				
ENO1	P06733	3	0.025	0.108	0.050	0.117
FBLIM1	Q8WUP2	2	0.012		0.021	0.082
FGB	P02675	1				
FGG	P02679	1	0.078			
GAPDH	P04406	2	0.027	0.029	0.037	
GSN	P06396	3	0.024		0.039	0.049
HBA1	P69905	1	0.042	0.202	0.044	0.198
HBB	P68871	7	0.053	0.146	0.057	0.123
HBD	P02042	1	0.044	0.136	0.045	0.113
HSPB1	P04792	2	0.042		0.044	
LDHA	P00338	1	0.022	0.126		
LGALS1	P09382	1	0.021	0.005	0.049	0.163
MYL6	P60660	1	0.026	0.059	0.031	0.194
MYL9	P24844	2			0.026	0.152
PARK7	Q99497	1	0.030	0.191	0.037	0.158
PDLIM3	Q53GG5	1				
TAGLN	Q01995	5	0.030		0.030	0.184
TAGLN2	P37802	1		0.183		0.091
TF	P02787	4				
TGM2	P21980	1	0.004			
TINAGL1	Q9GZM7	2	0.032		0.031	
TPM2	P07951	3	0.039		0.082	0.039
TTR	P02766	1			0.005	
TUBB	P07437	3	0.037	0.162	0.045	
VIM*	P08670	8	0.014			
YWHAB	P31946	2	0.076		0.001	
YWHAZ	P63104	2	0.065	0.082	0.029	0.103

<sup>a</sup> The statistical analyses are based on non-dilated versus dilated thoracic aorta samples in TAV and BAV (2D gel results are derived from Table 1, with eight proteins not found in LC-MS/MS validation excluded).

<sup>b</sup> ABS (loading)-ABS(jack-knife conf intervall) protein expression data, dilated higher (green), non-dilated higher (blue), TAV

<sup>c</sup> LC-MS/MS validation of 21 patients (a subgroup of patients included in the original 2D-DIGE analysis)

<sup>d</sup> ABS (loading)-ABS(jack-knife conf intervall) protein expression data, dilated higher (green), non-dilated higher (blue), BAV

\* Previously published

\*\* Number of protein spots identified on the gel in the 2D-DIGE experiments

**Table 3.** Functional characteristics of differentially expressed proteins between dilated TAV and BAV. (↑↓) statistically significant up or down regulated proteins in dilated aorta samples in all or the majority of spots (dark green and blue color in Table 1). (↑↓) statistically significant up or down regulated proteins in dilated aorta samples in at least one of the spots in the same group (light green and blue color in Table 1). (---) No statistically significant change in any of the spots identified (white color in Table 1).

<b>(i) Proteins that show different expression pattern between TAV and BAV in dilated aorta tissues.</b>			
<b>Gene Symbol</b>	<b>TAV</b>	<b>BAV</b>	<b>Function( Literature search)</b>
<b>FBLIM1</b>	↓	↑	Links ECM to cytoskeleton (1), binds to integrin (2), interacts with regulatory proteins of vasodilation (3).
<b>ALB</b>	↓	↑	Is a marker for vascular permeability (4),(5), ALB fragmentation increased during wound maturation (6).
<b>TGM2</b>	↑	---	Up regulated in many inflammatory diseases, induces constant activation of NF-κB (7) and in a complex with NF-κB induces epithelial-mesenchymal transition (EMT) (8). It is activated by TGFβ in response to oxidative stress, leading to vimentin aggregation (9).
<b>YWHAQ</b>	↑	---	This protein family play a role in protein localization and trafficking, are associated with human diseases such as cancer and neurodegenerative diseases (10).
<b>LDHA</b>	↑	---	Glycolytic enzyme, inducible by Hypoxia-inducible Factor 1(HIF1) (11).
<b>FGG</b>	↑	---	γ-Fibrinogen produces clots that are stiffer than the more common fibrinogen-α, is a key regulator and a marker of inflammation (12), (13) and functions during wound healing.
<b>VIM</b>	↑	---	Intermediate filament is up regulated in connection to inflammation (14),(15), in EC exposed to hypoxia is redistributed through interaction with HSP27 (16).
<b>ANXA1</b>	↑	---	Anti-inflammatory, modulates myoblast cell differentiation, regulation of cytoskeleton and extracellular matrix integrity. (17).
<b>MFAP4</b>	↓	---	Involved in innate immune system in vertebrate (18), macrophage specific in zebrafish (19).
<b>HSPA1L</b>	↓	---	Involved in systemic inflammatory response (20), affects cytokine concentration (21), polymorphism in the gene is associated with systemic lupus erythematosus (22). Together with HSP90 is induced both in chronic and acute inflammation (23).
<b>APCS</b>	---	↑	It is a constitutive serum protein synthesized by hepatocytes, binds to ECM (24) and basement membrane (25) components. It has a role in wound healing (26) and formation of amyloid fibres (4).
<b>MYL12B</b>	---	↑	Interacts with MYL9 and MYL6, is critical for maintenance of myosin heavy chain stability and cell integrity (27).
<b>MYL9</b>	---	↑	Associated with vascular permeability, and was strongly associated with arterial injuries in aged versus young mice at different time points studied (28).
<b>TTR</b>	---	↓	Associated with amyloidosis, (29), and wound healing (30).

**(ii) Proteins that show the same expression pattern in TAV and BAV in dilated aorta tissues**

<b>TUBB</b>	↑	↑	Component of SMC cytoskeleton (31).
<b>GSN</b>	↑	↑	Actin binding protein, highly expressed in cardiac injuries and remodeling via a HIF-1 $\alpha$ dependent mechanism (32).
<b>TPM2</b>	↑	↑	One of the four tropomyosin isoforms binding to and regulating the function of actin filaments (33).
<b>MYL6</b>	↑	↑	Associated with the breakdown of the barrier integrity of corneal epithelial cells (34).
<b>ACTA2</b>	↑	↑	A smooth muscle actin, mutation in which is responsible for aneurysms (35).
<b>ACTN1</b>	↑	↑	Cytoskeletal $\alpha$ -actinin, interacting with ACTN4 to regulate cytoskeleton in activating the barrier function (36).
<b>ACTN4</b>	↑	↑	Cytoskeletal $\alpha$ -actinin more involved in cell motility (37), essential for spreading and contractility of fibroblasts (38).
<b>LGALS1</b>	↑	↑	Ubiquitously expressed mammalian lectin, is regulated by NF- $\kappa$ B (39), is responsible for incorporation of some ECM component in SMC (40) and SMC proliferation (41), interacts with integrin (42). Is induced by HIF-1 (43).
<b>KIAA1429</b>	↑	↑	Not very well known proteins, was shown to be associated with YWHAB in a proteomic study(44).
<b>HSP90AB1</b>	↑	↑	an ATP-dependent molecular chaperone regulated by NF- $\kappa$ B (45), required for interferon signalling (46). Functions in generation of vasodilator endothelial nitric-oxide synthase (eNOS) (47).
<b>PARK7</b>	↑	↑	Cause recessively inherited Parkinson disease (PD), anti-oxidant, acts as a chaperone to prevent misfolding and aggregation of proteins under conditions of oxidative stress (48), interacts with HSP70 and 90 (49), cleaves and degrades TTR (50).
<b>GAPDH</b>	↑	↑	Regulated by Hypoxia-inducible Factor 1(HIF1), together with HSP90 (51).
<b>ENO1</b>	↑	↑	Induced in hypoxia and developing heart exposed to (52), inducible by Hypoxia-inducible Factor 1(HIF1) (11).
<b>YWHAB</b>	↑	↑	This protein family play a role in protein localization and traffickin, are associated with human diseases such as cancer and neurodegenerative diseases (10). YWHAB is involved in the regulation of cytoskeleton organization and fibronectin assembly (53) ,regulates myosin light chain phosphorylation via binding to myosin phosphatase (54).
<b>TAGLN</b>	↑	↑	Component of SMC and cytoskeleton, its down regulation promotes arterial inflammation via NF- $\kappa$ B related mechanisms (55), up-regulated in human pulmonary artery smooth muscle cells exposed to hypoxia (56).
<b>TINAGL1</b>	↑	↑	Matricellular protein localized in ECM of arterial SMC, binds to fibronectin and collagen ,promotes adhesion and spreading via interaction with integrins (57).
<b>HSPB1</b>	↑	↑	Involved in the regulation of actin filament formation in SMC (58),signaling hypoxia induced EC permeability (59).
<b>YWHAZ</b>	↑	↑	Belongs to the same protein family as YWHAB, Q. Is associated with injuries and hemorrhagic shock. (60), functions as heat shock protein (61).
<b>HBA1</b>	↓	↓	Hemoglobin expressed in adults. Marker for diabetes diagnosis (62).
<b>HBD</b>	↓	↓	The delta (HBD) and beta (HBB) genes are normally expressed in adults.
<b>HBB</b>	↓	↓	Buffering the heme released during clot resolution in hemorrhage (63).
<b>OGN</b>	↓	↓	Component of vascular matrix, involved in neointima formation and vascular remodeling (64).

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**Table 4.** Gene expression analysis of proteins identified by MALDI TOF MS from 2D-DIGE experiments. Significant genes according to multivariate statistics indicated. <sup>a</sup>

Gene name	Meta probe set ID	Protein accession number	No of protein spots**	TAV		BAV	
				CvsD <sup>b</sup>	Fold (D-C) <sup>c</sup>	CvsD <sup>d</sup>	Fold (D-C) <sup>c</sup>
ACTA2	3299504	P62736	6		1.06		1.03
ACTN1	3569814	P12814	2	0.070	1.30	0.096	1.23
ACTN4	3832643	O43707	5	0.178	1.41	0.163	1.23
ALB	2731192	P02768	11		-1.01		1.00
ANXA1	3174816	P04083	2		-1.08		-1.04
ANXA4	2487412	P09525	1		1.02		-1.04
ANXA5	2784027	P08758	1		1.09		1.01
APCS	2362651	P02743	1	0.015	-1.10		-1.00
CLIC4	2325593	Q9Y696	1		-1.00		-1.02
ENO1	2395490	P06733	3	0.122	1.28		1.13
FBLIM1	2322036	Q8WVUP2	2		1.06	0.067	1.17
FGB	2748542	P02675	1		-1.04		-1.02
FGG	2790652	P02679	1	0.031	-1.04	0.129	-1.05
GAPDH	3402625	P04406	2		-1.08		-1.05
GSN	3187686	P06396	3		1.04		1.06
HBA1	3642675	P69905	1		-1.13		1.02
HBB	3360401	P68871	7	0.211	-3.91	0.253	-3.29
HBD	3360417	P02042	1	0.023	-1.15	0.017	-1.11
HSP90AB1	2908474	P08238	1		1.11		-1.02
HSPA1L	2949450	P34931	3		-1.04	0.017	1.04
HSPB1	3009399	P04792	2		-1.02	0.063	1.08
KIAA1429	3145020	Q69YN4	1		1.09		1.03
LDHA	3322775	P00338	1	0.041	1.12		-1.02
LGALS1	3944882	P09382	1		-1.20	0.201	-1.12
MFAP4	3748798	A8MVM2	2	0.224	1.36	0.090	1.32
MYL6	3417457	P60660	1	0.030	-1.23	0.134	-1.19
MYL9	3883921	P24844	2		1.13		1.16
MYL12B	3776449	O14950	2		1.05		-1.01
OGN	3214800	Q7Z532	3		-1.07		-1.01
PARK7	2318736	Q99497	1		-1.10	0.079	-1.13
PDLIM3	2796951	Q53GG5	1		1.05		-1.05
TAGLN	3350830	Q01995	5		-1.03		1.01
TAGLN2	2439842	P37802	1	0.119	1.17		-1.01
TF	2643217	P02787	4		-1.03		1.02
TGM2	3905145	P21980	1		1.27		1.28
TINAGL1	2328320	Q9GZM7	2	0.055	1.26	0.028	1.14
TPM2	3204721	P07951	3		1.04		1.09
TTR	3783565	P02766	1		-1.02		-1.01
TUBB	2901913	P07437	3	0.020	1.24	0.102	1.12
VIM*	3236958	P08670	8		-1.02		1.01
YWHAB	3886639	P31946	2	0.030	1.16		1.07
YWHAQ	2539869	P27348	1	0.038	1.09		1.00
YWHAZ	3146898	P63104	2	0.032	-1.10		-1.07

<sup>a</sup> The statistical analyses are based on non-dilated versus dilated thoracic aorta samples in TAV and BAV.

<sup>b</sup> ABS (loading)-ABS(jack-knife conf intervall) meta probe set array expression data, dilated higher (green), non-dilated higher (blue), TAV

<sup>c</sup> Difference in average mean meta probe set expression between the groups indicated

<sup>d</sup> ABS (loading)-ABS(jack-knife conf intervall) meta probe set array expression data, dilated higher (green), non-dilated higher (blue), BAV

\* Previously published

\*\* Number of protein spots identified on the gel

**Table 5.** Exon expression analysis of proteins identified by MALDI TOF MS from 2D-DIGE experiments.Significant exons according to multivariate statistics. <sup>a</sup>

Gene name	Meta probe set ID	Protein accession No number	of protein spots**	TAV <sup>b</sup>	BAV <sup>c</sup>	Total number of probe sets/gene
ACTA2	3299504	P62736	6	2	2	9
ACTN1	3569814	P12814	2	12	5	22
ACTN4	3832643	O43707	5	6	5	20
ALB	2731192	P02768	11	1	2	16
ANXA1	3174816	P04083	2	1	1	11
ANXA4	2487412	P09525	1	3	6	15
ANXA5	2784027	P08758	1	4	0	13
APCS	2362651	P02743	1	0	1	6
CLIC4	2325593	Q9Y696	1	0	0	2
ENO1	2395490	P06733	3	1	1	6
FBLIM1	2322036	Q8WUP2	2	4	4	8
FGF	2748542	P02675	1	0	0	11
FGG	2790652	P02679	1	2	2	17
GAPDH	3402625	P04406	2	na	na	
GSN	3187686	P06396	3	13	5	20
HBA1	3642675	P69905	1	na	na	
HBB	3360401	P68871	7	4	3	5
HBD	3360417	P02042	1	2	3	4
HSP90AB1	2908474	P08238	1	na	na	
HSPA1L	2949450	P34931	3	1	1	6
HSPB1	3009399	P04792	2	2	0	4
KIAA1429	3145020	Q69YN4	1	7	8	31
LDHA	3322775	P00338	1	2	1	2
LGALS1	3944882	P09382	1	0	1	3
MFAP4	3748798	A8MVM2	2	5	4	9
MYL6	3417457	P60660	1	0	2	2
MYL9	3883921	P24844	2	2	1	5
MYL12B	3776449	O14950	2	na	na	
OGN	3214800	Q7Z532	3	6	3	12
PARK7	2318736	Q99497	1	0	2	5
PDLIM3	2796951	Q53GG5	1	4	4	13
TAGLN	3350830	Q01995	5	3	0	11
TAGLN2	2439842	P37802	1	na	na	
TF	2643217	P02787	4	3	6	21
TGM2	3905145	P21980	1	7	1	17
TINAGL1	2328320	Q9GZM7	2	4	7	15
TPM2	3204721	P07951	3	2	4	13
TTR	3783565	P02766	1	0	0	5
TUBB	2901913	P07437	3	na	na	
VIM	3236958	P08670	8	3	7	17
YWHAB	3886639	P31946	2	5	3	10
YWHAQ	2539869	P27348	1	1	1	4
YWHAZ	3146898	P63104	2	0	1	3

<sup>a</sup> The statistical analyses are based on non-dilated versus dilated thoracic aorta samples in TAV and BAV.<sup>b</sup> Number of exons based on ABS (loading)-ABS(jack-knife conf intervall) probe set array expression data, TAV<sup>c</sup> Number of exons based on ABS (loading)-ABS(jack-knife conf intervall) probe set array expression data, BAV

na - not analyzed due to not present at core level or only one exon

\*\* Number of protein spots identified on the gel

Figure 1

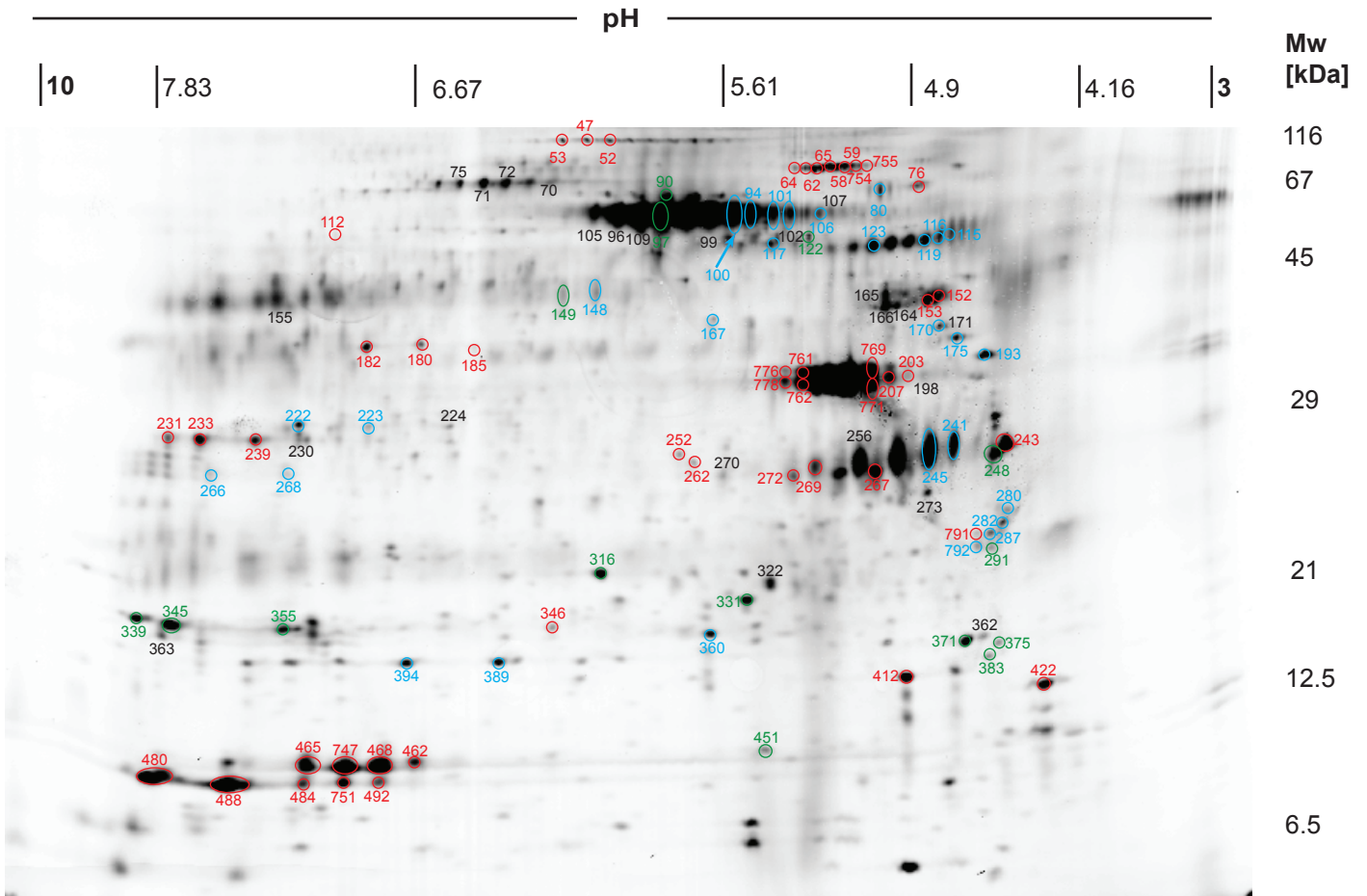
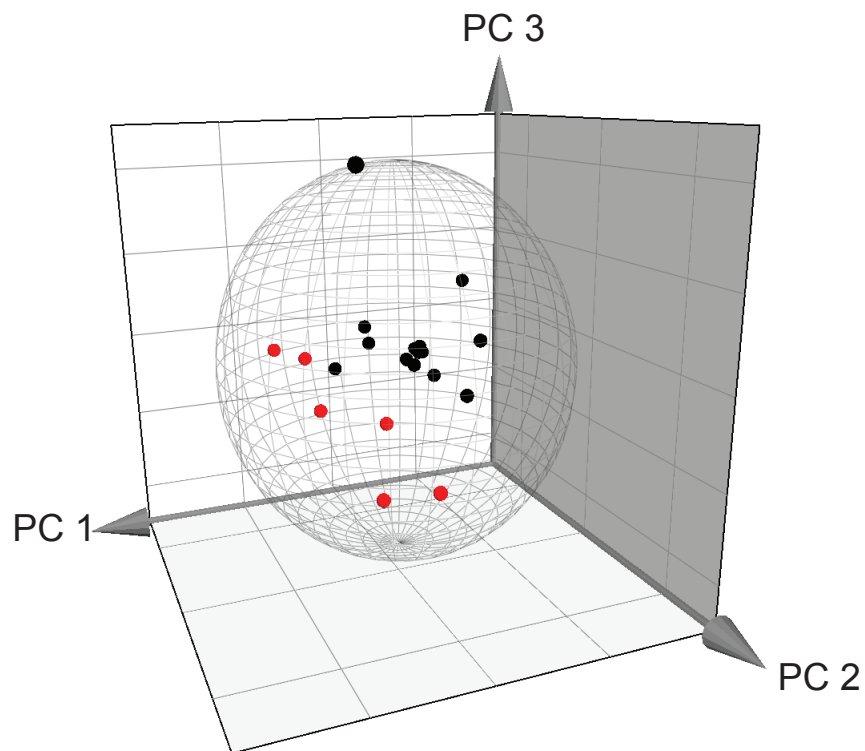
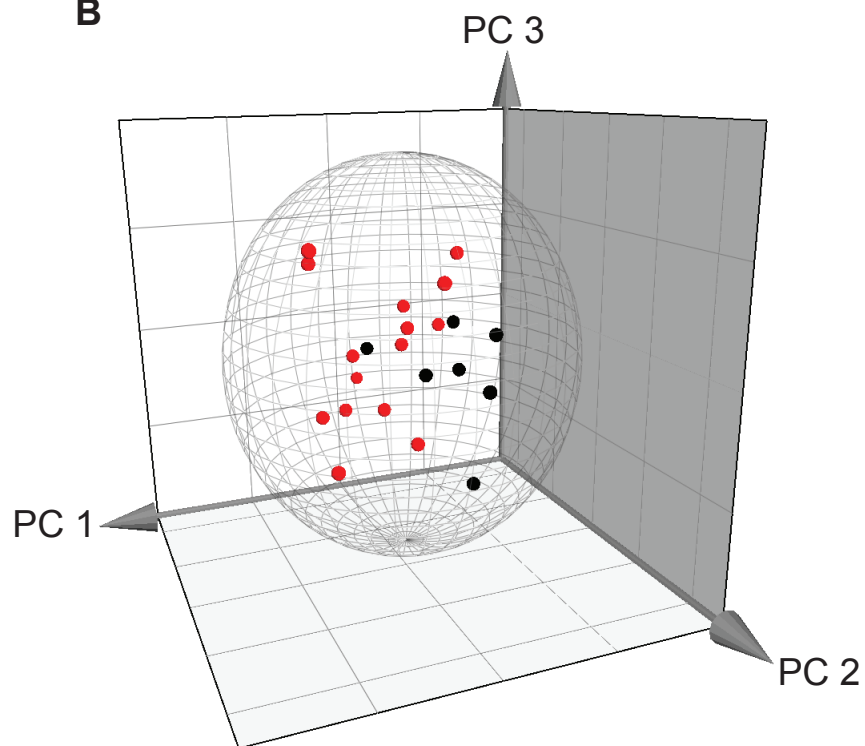


Figure 2

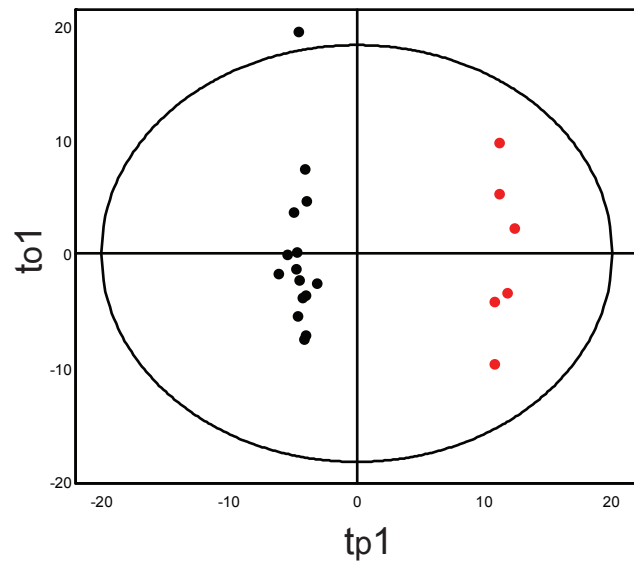
**A**



**B**



**C**



**D**

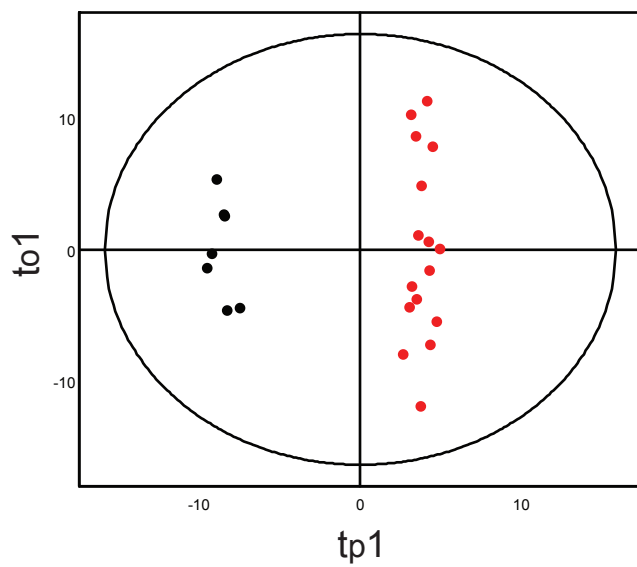


Figure 3

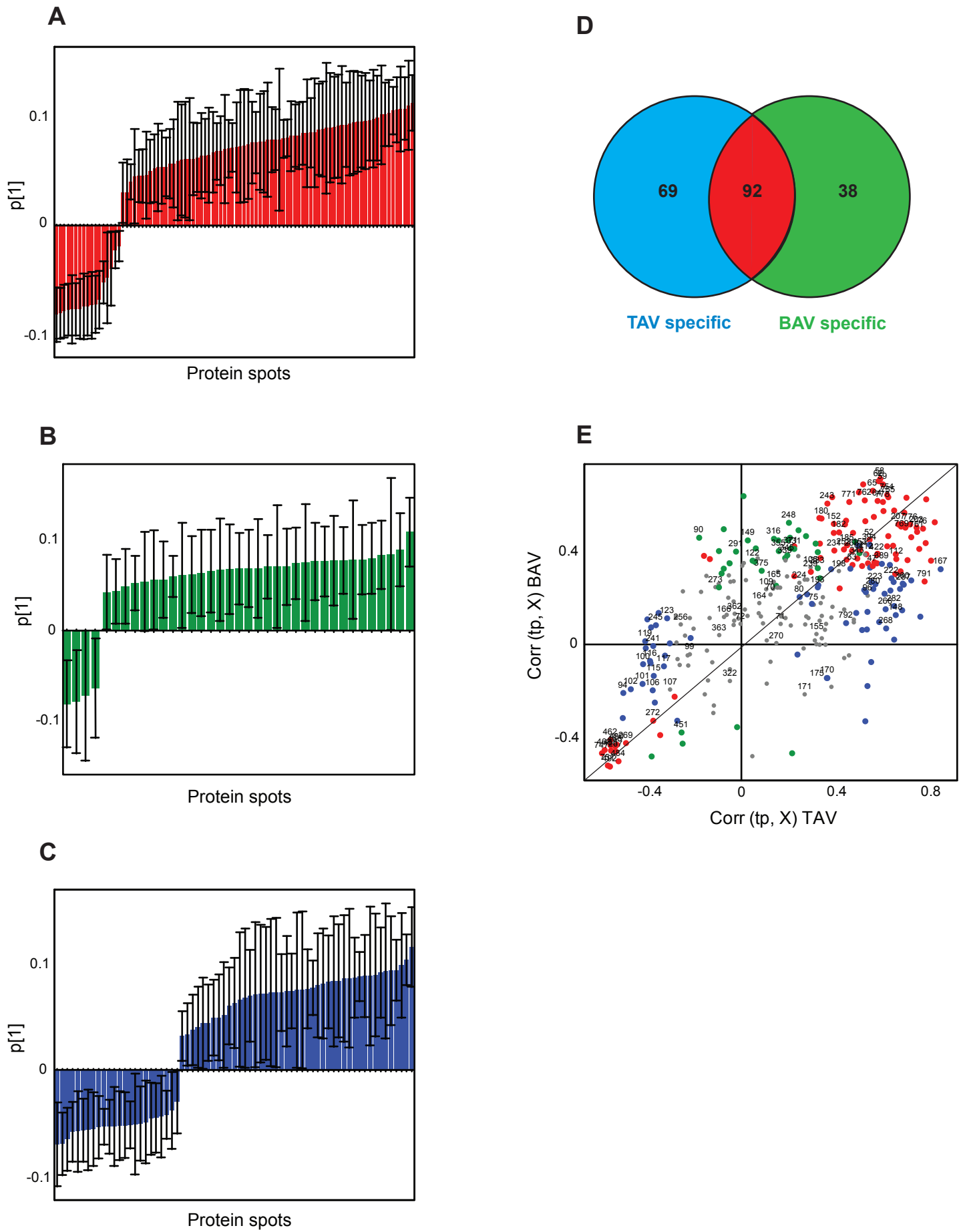


Figure 4

