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METHODOLOGY

# Valvular Aortic Stenosis: A Proteomic Insight

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**Abstract:** Calcified aortic valve disease is a slowly progressive disorder that ranges from mild valve thickening with no obstruction of blood flow, known as aortic sclerosis, to severe calcification with impaired leaflet motion or aortic stenosis. In the present work we describe a rapid, reproducible and effective method to carry out proteomic analysis of stenotic human valves by conventional 2-DE and 2D-DIGE, minimizing the interference due to high calcium concentrations. Furthermore, the protocol permits the aortic stenosis proteome to be analysed, advancing our knowledge in this area.

**Summary:** Until recently, aortic stenosis (AS) was considered a passive process secondary to calcium deposition in the aortic valves. However, it has recently been highlighted that the risk factors associated with the development of calcified AS in the elderly are similar to those of coronary artery disease. Furthermore, degenerative AS shares histological characteristics with atherosclerotic plaques, leading to the suggestion that calcified aortic valve disease is a chronic inflammatory process similar to atherosclerosis. Nevertheless, certain data does not fit with this theory making it necessary to further study this pathology. The aim of this study is to develop an effective protein extraction protocol for aortic stenosis valves such that proteomic analyses can be performed on these structures. In the present work we have defined a rapid, reproducible and effective method to extract proteins and that is compatible with 2-DE, 2D-DIGE and MS techniques. Defining the protein profile of this tissue is an important and challenging task that will help to understand the mechanisms of physiological/pathological processes in aortic stenosis valves.

**Keywords:** proteomics, human aortic valves, aortic stenosis

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## Introduction

Degenerative aortic valve stenosis (AS) is the most common of the valvular diseases and its prevalence increases with age.[1,2] Calcified aortic valve disease ranges in severity from sclerosis to stenosis, and it is characterised by irregular areas of increased thickening on the aortic side of the valve. Microscopically, these areas display evidence of chronic inflammation, with infiltration of macrophages and T-lymphocytes. There is also an accumulation of plasma lipoproteins, including LDL and Lp(a) lipoprotein, and it was recently shown that these lipoproteins are oxidatively modified.[3,4] In addition, bone proteins have also been identified in diseased heart valves[5–7] and there are several reports of bone formation in calcified cardiac valves.[8–10]

The pathogenesis of AS shares a number of features with atherosclerosis, such as endothelial dysfunction, increased leukocyte adhesion/infiltration and calcification[11–13] and both pathologies are associated with the same clinical risk factors.[14,15] However, despite the high prevalence and increasing morbidity and mortality of this condition, very little is known regarding the cellular and molecular factors involved in the development of AS and their possible relation with coronary artery disease.

In this sense, adopting a proteomic approach to study the pathogenesis of AS might provide useful information to understand the development of AS and help define its possible relation with coronary atherosclerosis.

Here we present an improve protocol to extract proteins from AS valves for their subsequent proteomic analysis. This protocol efficiently extracts most of the proteome of calcified valves and it is compatible with 2-DE and 2D-DIGE, so that future clinical comparative studies can be performed.

## Materials and Methods

### Patient selection

Heart valves with degenerative aortic stenosis (n = 20) were obtained from patients of both sexes (55% male, 45% female) with an average age 75 ± 3.9, 74.16% of whom suffered hypertension, 33% diabetes and 52.1% hyperlipidemia. All patients underwent aortic valve replacement due to severe degenerative aortic stenosis or combined with ischemic miocardiopathy following the indications established by current guidelines. Patients with aortic regurjitation mitral valve disease or suspicious of rheumatic etiology were not included. The protocol was approved by the ethical committee of "Virgen de la Salud" Hospital, Toledo.

### Processing of human aortic valves: protein extraction

Aortic stenosis valves were processed within a maximum of 2 hours after surgery having maintained the tissue at 4 °C in RPMI medium. The valves were washed 3 times in PBS to reduce blood contaminants, and the aortic valve leaflet was then ground into a powder in liquid $N_2$ with a mortar and 0.2 g of this powder was resuspended in 400 µl of protein extraction buffer (Tris 10 mM [pH 7.5], 500 mM NaCl, 0.1% Triton x-100, 1% β-mercaptoethanol, 1 mM PMSF).[16] The homogenate was centrifuged at 21,000 g (5840R Eppendorf) for 15 min at 4 °C to precipitate the membranes and tissue debris, and the supernatant (E1), containing most of the soluble proteins, was collected and stored at –20 °C. The pellet was then solubilized in 7 M Urea, 2 M Thiourea, 4% CHAPS[17,18] buffer and centrifuged again at 21,000 g, and this second supernatant (E2) was separated from the pellet of tissue debris and it was rich in hydrophobic proteins (mainly membrane proteins).

The protein concentration was determined by a Bradford-Lowry method (Bio-Rad protein assay)[19] and to check the protein composition of the extracts, 25 µg of total protein from each sample (E1 + E2) was resolved by SDS-PAGE 12% (data not shown).

### Two-dimensional Electrophoresis (2-DE)

All the chemicals and instruments used in 2-DE gels have been described previously.[19,20] The protein extracts, E1, E2, E1 + E2 were dialysed against 2 mM Tris Buffer using a Mini dialysis Kit with 1kDa cut-off (GE Healthcare). Subsequently, 300 µg of each protein extract was cleaned with the Clean up Kit (GE-Healthcare) and resuspended in rehydration buffer (7M Urea, 2M Thiourea, 4% CHAPS, 1%–2% Ampholites and 1% TBP: Bio-Rad). Isoelectric focusing (IEF) was performed in a IPGphor unit on strips that were actively rehydrated at 20 °C for 12 h at 30 V to enhance protein uptake (17 cm strips; pH 3–10 or 4–7, Bio-Rad). During focusing, the voltage

was increased according to the following program: 500 V for 30 min, 1,000 V for 1 h, 1,000–2,000 V in 1 h (gradient), 2,000–5,000 V in 2 h (gradient), 5,000–8,000 V in 1 h (gradient), 8,000 V for a total 88,000 Vhr.

Following IEF, the strips were equilibrated as described previously[19,18] and the 2nd dimension SDS-PAGE was performed according to Laemmli[21] using a Protean II system (Bio-rad) at 25 mA/gel and 4 °C. The gels were then fixed overnight, stained using a Silver Staining kit (GE Healthcare), and they were then scanned on GS-800 Calibrated Densitometer (Bio-rad). Evaluation of the 2-DE gels was performed using PDQuest gel analysis software version 8.0.1 (Bio-rad).

## 2D-DIGE

All chemicals and instruments used in 2D-DIGE were purchased from GE Healthcare. Aliquots of the E1, E2 and E1 + E2 samples together (50 µg) were separately labelled with different CyDyes (Cy5, Cy3, and Cy2, respectively) at a ratio of 50 µg protein to 400 pmol CyDye. The labelled mixtures were combined and loaded onto pH 4–7 17-cm Immobiline Dry Strips (IPG) for first dimension separation by IEF over 24 h. IEF was performed following the same program as described above and the strips were then loaded onto 12% polyacrylamide gels that were run overnight and scanned at 100 ppm using a 9400 GE Healthcare Typhoon scanner. Spot image analyses, matching and spot selection were carried out using DeCyder Software (GE Healthcare).

## Protein identification by MALDI-TOF/TOF

All differentially expressed protein spots were manually excised and identified at the HNP Proteomic Unit. The proteins were automatically digested with an "Ettan Digester" (GE Healthcare) according to Schevchenko et al[20] with minor modifications,[21] and after digestion overnight at 37 °C, the peptides were extracted with 60% acetonitrile (ACN) in 0.1% formic acid (99.5% purity; Sigma Aldrich). The samples were then dried in a speed vac and resuspended in 98% water with 0.1% formic acid (FA) and 2% ACN. An aliquot of each digestion was mixed with an aliquot of the matrix solution (3 mg/mL α-cyano-4-Hydroxycinnamic acid, Sigma Aldrich) in 30% ACN, 15% 2-propanol and 0.1% TFA, and this mixture was pipetted directly onto the stainless steel sample plate of the mass spectrometer 384 Opti-TOF 123 × 81 mm MALDI (Applied Biosystems) and dried at room temperature.

The MALDI-MS/MS data were obtained in an automated analysis loop using a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems). Spectra were acquired in the reflector positive-ion mode with a Nd:YAG, 355 nm wavelength laser at a frequency of 200 Hz, and between 1000 and 2000 individual spectra were averaged. The experiments were acquired in a uniform mode with a fixed laser intensity. For the MS/MS 1kV analysis mode, precursors were accelerated to 8 kV in source 1, and they were selected at a relative resolution of 350 (FWHM) and metastable suppression. Fragment ions generated by collision with air in a CID chamber were further accelerated at 15 kV in source 2. Mass data were analysed automatically with the 4000 Series Explorer Software version 3.5.3 (Applied Biosystems). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with m/z = 842.510 and m/z = 2211.105. For the calibrations in the MS/MS mode, the fragment ion spectra obtained from Glub-fibrinopeptide were used (4700 Cal Mix, Applied Biosystems). MALDI-MS and MS/MS data were combined through the GPS Explorer Software Version 3.6 to search a non-redundant protein database (Swissprot 56.5) using the Mascot software version 2.2 (Matrix Science),[22] employing the following parameters: 50 ppm precursor tolerance; 0.6 Da MS/MS fragment tolerance; and allowing 1 missed cleavage, carbamidomethyl cysteines and methionine oxidation as modifications. MALDI-MS(/MS) spectra and database search results were manually inspected in detail using the aforementioned software.

## Results and Discussion

Understanding the nature of the biological processes that are associated with the disease state of a tissue or organism would benefit greatly by defining the changes associated in their proteome. Defining the proteome of human aortic valves is an important and challenging task that may be fundamental to understand the mechanisms and physiological/pathological processes that lead to the development of AS. For this

reason, here we have improved the established protocol for 2-DE and 2D-DIGE analysis of the human aortic valve proteome (see schematic flowchart in Fig. A), added to a sequential extraction protocol. To describe the complete proteome of an organ or tissue it is necessary to establish an efficient extraction protocol to maximize protein solubilization. Our method was based on two sequential protein extractions (Fig. 1B),[23,24] the first of which relies on a strong lysis buffer in which most of the soluble proteins from human valves were extracted (E1 extract). The second was designed to extract the membrane and hydrophobic proteins (E2 extract) while finally, the pellet of cell debris was resuspended in loading buffer. After SDS-PAGE analysis in which it was verified that it the final pellet basically contained salts and membrane remains (data not shown), this extract was discarded. This sequential protocol is very easy to carry out, it is not very time-consuming and it is very reproducible.

One of the major problems associated with the proteomic analyses of AS valves is the high concentration of calcium that interferes with further analyses. For this reason the E1, E2, and E1 + E2 extracts were de salting using a Mini Dyalisis Kit prior to 2-DE and 2D-DIGE. We tested different pH ranges (4–7, 3–10) in order to select that which was best to use in future studies to search for biomarkers. Thus, while more than 400 spots were resolved from the E1 extract in each gel after focusing in the pH 4–7 range, there were around 300 spots per gel at pH 3–10 (Fig. 2).

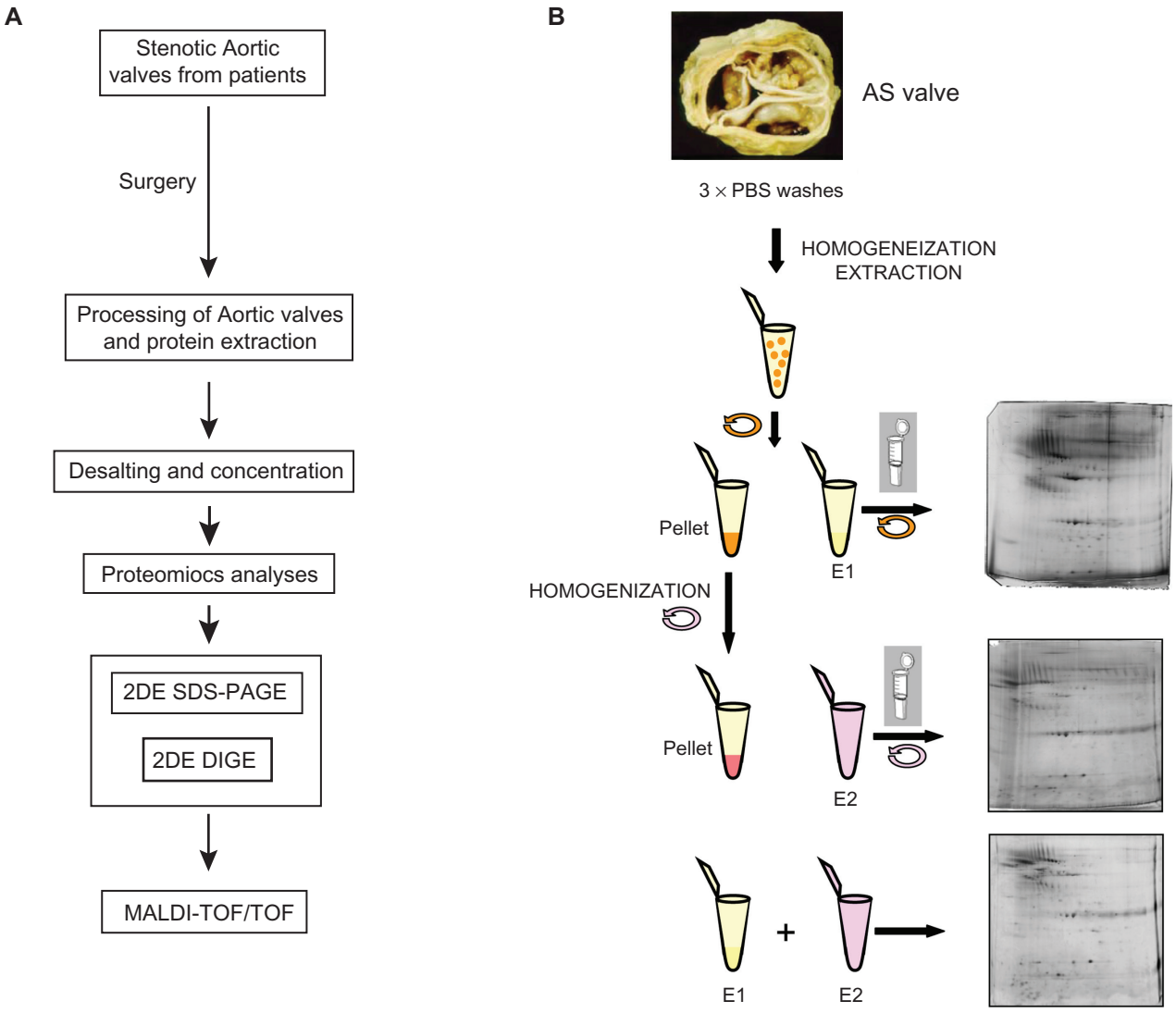



**Figure 1.** Diagram illustrating the protein extraction method employed for AS valve proteomic analysis. **A**) Flowchart representing the analysis of human AS valves. **B**) Sequential extraction protein protocol scheme.

When the E2 fraction was analysed fewer spots were identified than in the E1 extract, although new spots were detected that most likely corresponded to hydrophobic proteins. Again, while approximately 300 spots were detected when the pH 4–7 IPG strip was used, around 200 were evident when focusing was carried out across the pH 3–10 range (Fig. 2). Accordingly, when the E1 + E2 extracts together were analysed, around 500 spots were resolved well on the IPG strip of pH 4–7 when compared to the 350 spots evident from the pH 3–10 strip (Fig. 2). These data indicate that the E1 + E2 extract permitted an important pool of proteins from AS valve proteome to be visualized.

DIGE technology was employed to elucidate if the combined E1 + E2 extract really did represent a good reflection of the entire AS valve proteome. With the E1 + E2 extract we obtained a combination of all the proteins solubilized by this novel two step protocol, simplifying the analysis in a single gel (Fig. 2). Spots were analysed using DeCyder software and of the 1178 spots in E1 and 935 in E2, 535 spots coincided in both samples. Furthermore, 1346 spots were detected in E1 + E2 (Fig. 3). It is important to note that the 232 spots that did not appear in E1 + E2 extract corresponded with minor low intensity proteins that could not be identified by mass spectrometry.

Significantly, our protein extraction method was compatible with DIGE and the results obtained indicated that differential expression studies can be carried out as the sum of both extracts (E1, E2) without any significant loss of information. Indeed, analysing the E2 extract individually could generate

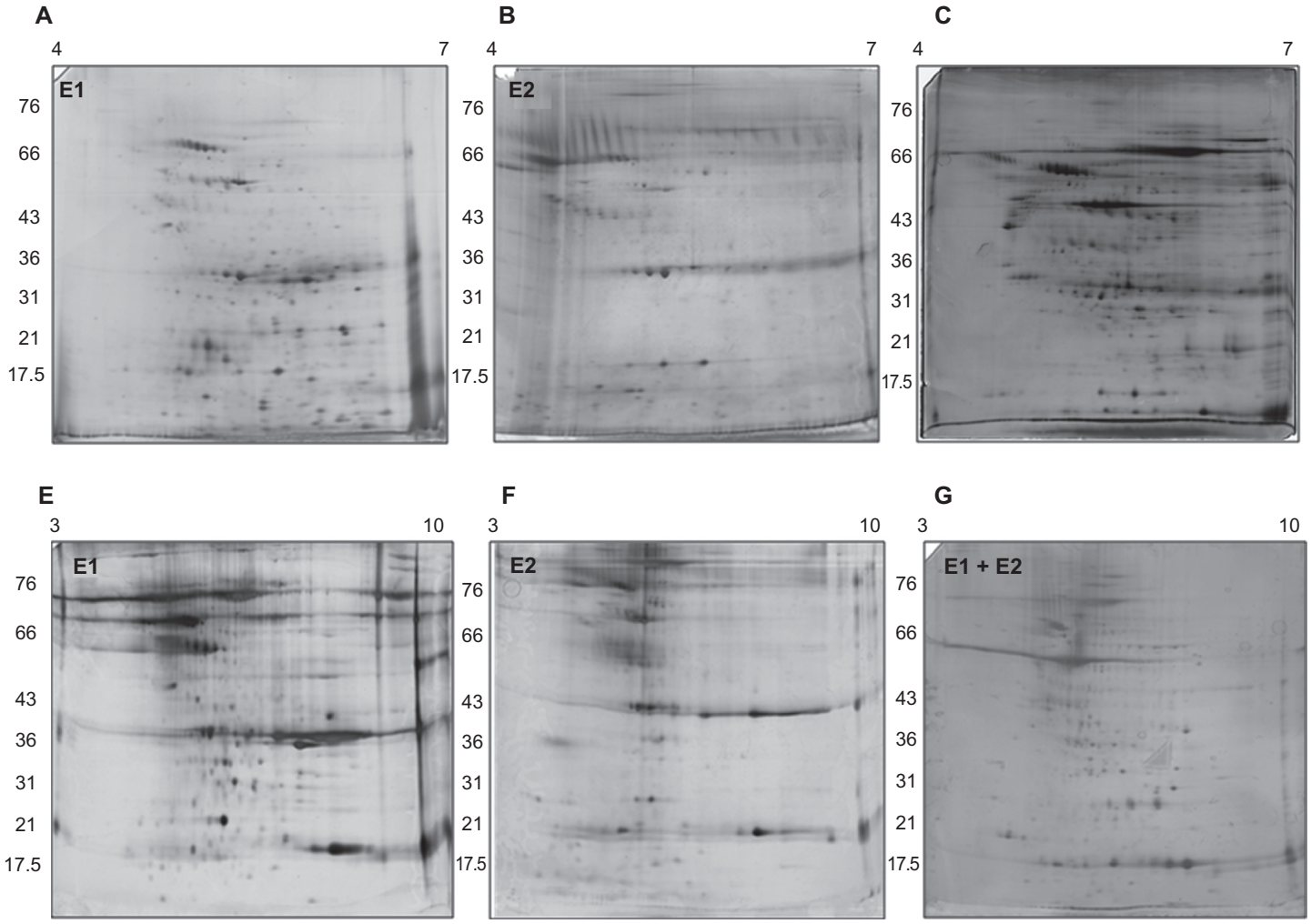
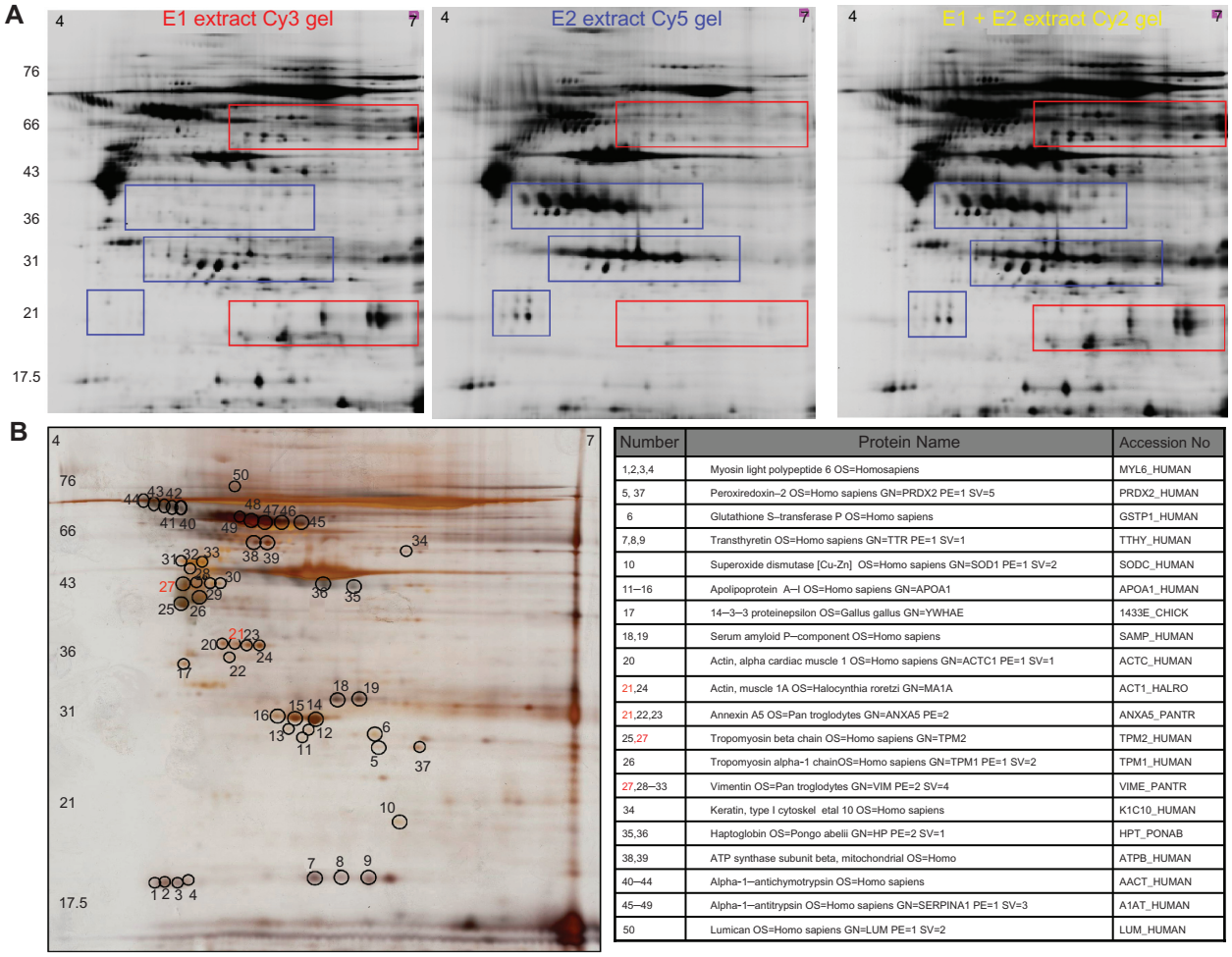



**Figure 2.** Two dimensional gel analysis of homogenates from AS valves: **A**) when focused at pH 4–7 containing the soluble protein extract (E1); **B**) the extract rich in hydrophobic proteins focused at pH 4–7 (E2); **C**) the pooled extracts focused at pH 4–7 (E1 + E2); **D**) the soluble protein extract (E1) focused at pH 3–10; **E**) the hydrophobic rich protein extract focused at pH 3–10 (E2); and **F**) the pooled extract when focused at pH3-10 (E1 + E2).




| Number | Protein Name | Accession No |
|---|---|---|
| 1,2,3,4 | Myosin light polypeptide 6 OS=Homosapiens | MYL6_HUMAN |
| 5, 37 | Peroxiredoxin–2 OS=Homo sapiens GN=PRDX2 PE=1 SV=5 | PRDX2_HUMAN |
| 6 | Glutathione S–transferase P OS=Homo sapiens | GSTP1_HUMAN |
| 7,8,9 | Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1 | TTHY_HUMAN |
| 10 | Superoxide dismutase [Cu-Zn] OS=Homo sapiens GN=SOD1 PE=1 SV=2 | SODC_HUMAN |
| 11–16 | Apolipoprotein A–I OS=Homo sapiens GN=APOA1 | APOA1_HUMAN |
| 17 | 14–3–3 proteinepsilon OS=Gallus gallus GN=YWHAE | 1433E_CHICK |
| 18,19 | Serum amyloid P–component OS=Homo sapiens | SAMP_HUMAN |
| 20 | Actin, alpha cardiac muscle 1 OS=Homo sapiens GN=ACTC1 PE=1 SV=1 | ACTC_HUMAN |
| 21,24 | Actin, muscle 1A OS=Halocynthia roretzi GN=MA1A | ACT1_HALRO |
| 21,22,23 | Annexin A5 OS=Pan troglodytes GN=ANXA5 PE=2 | ANXA5_PANTR |
| 25,27 | Tropomyosin beta chain OS=Homo sapiens GN=TPM2 | TPM2_HUMAN |
| 26 | Tropomyosin alpha-1 chainOS=Homo sapiens GN=TPM1 PE=1 SV=2 | TPM1_HUMAN |
| 27,28–33 | Vimentin OS=Pan troglodytes GN=VIM PE=2 SV=4 | VIME_PANTR |
| 34 | Keratin, type I cytoskel etal 10 OS=Homo sapiens | K1C10_HUMAN |
| 35,36 | Haptoglobin OS=Pongo abelii GN=HP PE=2 SV=1 | HPT_PONAB |
| 38,39 | ATP synthase subunit beta, mitochondrial OS=Homo | ATPB_HUMAN |
| 40–44 | Alpha-1–antichymotrypsin OS=Homo sapiens | AACT_HUMAN |
| 45–49 | Alpha-1–antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3 | A1AT_HUMAN |
| 50 | Lumican OS=Homo sapiens GN=LUM PE=1 SV=2 | LUM_HUMAN |

**Figure 3. A**) 2D-DIGE gels. The area marked in blue shows the spots obtained from the E1 extract and the area marked in red shows the spots obtained from E2. **B**) Representative image of 2-DE silver stained gel of AS valve extracts (IEF: 4–7 pH range, second dimension, 12% acrylamide) and the protein spots identified. Numbers in red corresponded to two different proteins identified in the same spot.

confusing data since this sample is prefractionated. Furthermore, the improvement in sensitivity associated with the 2D-DIGE technology with respect to conventional 2-DE was clearly evident in this analysis. The use of fluorescent labelling and the DeCyder analysis software considerably increases the number of protein spots that can be detected in the analysis when compared with more traditional methods. Of the protein spots detected, 100 have been identified and correspond to 50 different proteins, and these proteins are summarized in conjunction with their distribution on the 2DE-gels.

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## Abbreviations

AS, aortic stenosis; 2D-DIGE, two dimensional fluorescence Difference Gel Electrophoresis; LDL, low density lipoprotein; Lp(a), lipoprotein a.

## Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under

consideration by any other publication and has not been published elsewhere. The authors report no conflicts of interest.

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