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Proteomic and degradomic signatures of extracellular matrix remodeling in calcific aortic valve stenosis

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Abstract

Background Aortic stenosis is a progressive fibro-inflammatory valvular disorder with major clinical burden and no disease-modifying pharmacological therapy. Defining molecular circuits associated with thrombo-inflammatory activation to extracellular matrix remodeling may enable future therapeutic targeting and biomarker development.

Methods Human aortic valves explanted from patients with severe aortic stenosis and non-stenotic surgical controls (aortic regurgitation) were profiled using a multi-layer mass spectrometry strategy. Global protein changes were quantified by label-free proteomics (data-independent acquisition). Extracellular matrix proteolysis was interrogated using an extracellular matrix-focused semi-tryptic peptide workflow. Collagen qualitative remodeling was assessed by mapping hydroxyproline enrichment. Differential abundance was evaluated using multiple-testing correction (false discovery rate). Selected candidates were validated by targeted multiple reaction monitoring, and elastin integrity was assessed histologically.

Results We identified 594 significantly modulated proteins in severe aortic stenosis, with predominant upregulation of complement/coagulation and extracellular matrix-related pathways. Targeted Multiple Reaction Monitoring confirmed key thrombo-inflammatory and matrix-associated candidates. Semi-tryptic profiling revealed a focused extracellular matrix degradomic signature dominated by small leucine-rich proteoglycans (decorin, lumican, PRELP), fibrillin-1, and collagen VI, consistent with preferential proteolytic targeting of structural matrix scaffolds. Histology showed marked elastin fragmentation in stenotic leaflets. Collagen post-translational modification analysis revealed increased hydroxyproline-bearing peptides across selected collagen chains despite minimal changes in total collagen abundance, indicating qualitative remodeling beyond protein accumulation.

Conclusions An integrated proteomic-degradomic-post-translational modification framework reveals concomitant thrombo-inflammatory activation to matrix breakdown and qualitative collagen remodeling in severe aortic stenosis, highlighting molecular circuits that may inform future biomarker development and therapeutic target discovery.

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Keywords Calcific aortic valve disease, Proteomics, Degradomics, Extracellular matrix, Complement, Collagen post-translational modifications

Introduction

Calcific aortic valve disease (CAVD) is the most prevalent valvular heart disease in developed countries and its incidence is expected to increase with population aging. It affects up to 4% of individuals over 75 years and is associated with high morbidity and mortality if left untreated (Otto and Prendergast 2014). While congenital bicuspid aortic valve accounts for a proportion of cases in younger patients, age, male sex, hypertension, dyslipidemia, diabetes, and smoking are established risk factors for degenerative forms (Moosa and Garcia 2025). Despite its clinical relevance, no pharmacological therapies have proven effective in slowing or reversing CAVD, and surgical or transcatheter valve replacement remain the only treatment options. The pathogenesis of CAVD is an active process that shares features with atherosclerosis but involves valve-specific mechanisms. Endothelial dysfunction permits the infiltration of lipoproteins and immune cells into the subendothelial space, initiating chronic inflammation and oxidative stress (Shen et al. 2025). Apolipoprotein accumulation and oxidation within valve tissue promote macrophage and T lymphocyte recruitment, with subsequent release of cytokines, proteases, and reactive oxygen species that amplify tissue injury (Wang et al. 2025; Yeang et al. 2016; Greenberg et al. 2022). Activation of the complement cascade has been demonstrated in stenotic valves, particularly the classical and terminal pathways, mediating immune cell recruitment and tissue remodeling (Helske et al. 2008). Parallel activation of coagulation pathways, with fibrin and fibrinogen deposition, underscores the thrombo-inflammatory nature of the valvular microenvironment (Sakaue et al. 2018). Together, these immune and hemostatic processes sustain a vicious cycle of inflammation, thrombosis, and remodeling. Extracellular matrix (ECM) remodeling is a hallmark of CAVD, involving excess deposition and cross-linking of collagens, loss of elastin, and proteoglycan accumulation. These changes alter cusp biomechanics, increase stiffness, and create scaffolds for calcific nodule formation (Chen and Simmons 2011). In parallel, valvular interstitial cells (VICs) undergo osteogenic reprogramming, with RUNX2 acting as the master regulator of osteoblast-like differentiation (Konstandin et al. 2013). Recent evidence also highlights sex-specific differences in valve remodeling. Male patients tend to exhibit greater collagen I deposition, inflammation, and compact calcifications, whereas females more frequently display proteoglycan-rich ECM and less fibrotic remodeling (Tiraplegui et al. 2025). Moreover, metabolic reprogramming of VICs, characterized by suppression of

mitochondrial activity and fatty acid oxidation in favor of glycolysis, further supports the pro-calcific phenotype. Altogether, these findings establish CAVD as a multifactorial disease driven by interconnected immune, coagulant, ECM, metabolic, and osteogenic mechanisms. A deeper molecular understanding of these pathways may provide biomarkers of disease activity and novel therapeutic strategies to slow or prevent progression beyond the need for valve replacement.

In this study, comparative assays were performed between aortic valve samples explanted from patients with severe stenosis (AS) and those explanted from non-stenotic subjects. The aim was to identify, through label-free differential proteomic approaches, molecular signatures uniquely specific to the pathological state, both in terms of protein expression levels and post-translational modifications of collagen chains, the main component of the extracellular matrix. Furthermore, a molecular assessment of the degradation processes of the ECM was performed using an innovative degradomics approach integrated with biopsies analysis.

Methods

Samples' description

A total of 10 calcific aortic valves were obtained from patients undergoing aortic valve replacement for severe AS. To investigate molecular alterations associated with AS, we included a control group of 9 non-calcific aortic valves obtained from patients undergoing cardiac surgery for aortic valve insufficiency. Although these samples do not represent healthy valves, they were selected as the most appropriate surgical comparator in the absence of calcific stenotic disease, also considering the distinct pathophysiological background of aortic insufficiency and calcific AS. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All study procedures received approval by our institution's human research committee (Lang et al. 2015). All patients provided written informed consent before inclusion in the study.

Echocardiography

All patients underwent complete echocardiography before cardiac surgery (Vivid E9, GE healthcare), according to the current recommendations (Lang et al. 2015). AS was evaluated by calculating the following parameters: AV area; AV mean gradient; AV max velocity; stroke volume index, according to the current recommendations (Lang et al. 2015). Aortic valve replacement decision was based on the 2021 European Society of

Cardiology/European Association for Cardio-Thoracic Surgery guidelines for the management of valvular heart disease. (Vahanian et al. 2022) Echocardiographic data were used for clinical characterization and surgical indication, and the main parameters are summarized in Supplementary Table S1.

Tissues collection

Each patient underwent surgical aortic valve replacement through a standard median sternotomy and cardiopulmonary bypass. During cardiac surgery, the explanted AV were collected and were analyzed at proteomic analysis.

Sample preparation for proteomics analysis

All valve samples, including both severe AS and control aortic regurgitation specimens, were crushed in liquid nitrogen to obtain a fine powder. A minimum of approximately 30 mg of powder was weighed for each sample. Subsequently, 20 μ l of a decalcification solution (0.5 M EDTA pH=8 and protease inhibitors) was added and the samples were left overnight at 4 °C, to chelate Ca^{2+} cations present in the tissue.

To obtain the protein extracts, cell lysis was performed using a lysis buffer (5% sodium dodecyl sulphate, 50 mM ammonium bicarbonate) and crushing for 10 min at low temperature with a pestle. Then, the sample was sonicated and centrifuged at 13,000 rpm for 30 min at 20 °C. The supernatants, containing the protein extract, have been collected and each protein lysate was quantified with BCA assay (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer instructions. Proteins were digested using a shotgun proteomics approach onto micro S-Trap columns (Protifi), as previously reported (Cozzolino et al. 2023).

LC–MS/MS analysis for untargeted differential proteomics

Peptide mixtures were analyzed with an Orbitrap Exploris 240 instrument equipped with a Vanquish-Neo nanoLC system and Nanospray Flex ion source. Samples were fractionated on a C18 capillary reverse phase column (150 mm, 75 μ m, 2 μ m, 100 Å) at a flow rate of 250 nl/min. For Data-Dependent Acquisition (DDA) was used a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid and 2% acetonitrile in LC–MS grade water) from 2 to 90% in 73 min with the following set up: 1) scan spectrum spanned from 300 to 1800 m/z, 2) top 20 ions in each scan were selected for the fragmentation, 3) 45 s for the dynamic exclusion window. For the acquisition in Data-Independent Acquisition (DIA) mode, peptide samples were fractionated with a gradient from 2 to 90% in 72 min, the full scan range was set to 400 to 900 m/z with an isolation window of 12 m/z. The MS1 and MS2 resolution are 60000 for both, AGC%=300, and injection time was 20 ms.

Mass spectrometry data processing

The raw data obtained from the LC–MS/MS runs in DIA mode were analyzed using DIA-NN 2.0.1 (Demichev et al. 2020). The parameters selected for protein identification were very stringent: minimum 3 peptides, at least 1 unique and a false discovery rate (FDR) of 0.01 for protein identification was applied. The statistically significant differentially expressed proteins were evaluated using Perseus software 1.6.15.0 (Tyanova et al. 2016) applying a Student's t-test with 5% FDR Benjamini–Hochberg corrected p-value (Iacobucci et al. 2025).

No formal post-acquisition batch-correction procedure was applied. Instead, instrumental performance was checked before the analytical sequence by injection the standard QC of a HeLa digest used as a system suitability control, by evaluating retention time stability, chromatographic peak width at half height, and the intensity of extracted ion chromatograms corresponding to five reference peaks, and number of the total identified proteins. To determine the levels of proline hydroxylation, samples were analyzed in Data-Dependent Acquisition DDA and processed with MaxQuant (Tyanova et al. 2016) using a database containing collagen-related protein family. This targeted search space was selected because the specific aim of this analysis was to interrogate collagen-associated PTMs, while limiting search complexity and the risk of false-positive identifications when variable modifications were included. Hydroxyproline was searched for as a variable modification in two independent runs. The parameters selected for protein identification were a minimum of 4 peptides, at least 2 unique peptides, and FDR of 1%. Hydroxylation quantification was performed at the level of the modified peptides using MaxQuant Intensity values. These values were not normalized to the corresponding unmodified peptide species. Rather, only those peptides with FC>1.5 derived from proteins invariant under the two conditions (FCs~1) were prioritized. Statistically significant hydroxyproline peptides were evaluated using Perseus software version 1.6.15.0, applying a statistical filter based on Student's t-test (5% FDR). To assess the significance of modification variation between severe and control data plotted in Fig. 6, two-way ANOVA with Bonferroni multiple comparisons test were performed.

To investigate the presence of dysregulated semi-tryptic peptides, samples, analyzed in DDA mode, were processed with Proteome Discoverer (Orsburn 2021) using a human extracellular matrix protein database downloaded by MatrixDB and converted to a FASTA file with UniProt ID mapping tool. The parameters selected for protein identification were minimum 3 peptides, at least 1 unique and a false discovery rate (FDR) of 0.01 was applied. Protein and peptide groups were analyzed with Perseus software to highlight

statistically varying peptides, applying a statistical Student's t-test ($FDR < 0.05$). For candidate prioritization, semi-tryptic peptides were further filtered by retaining only those mapping to proteins with no overall abundance change in the DIA dataset ($-0.5 < \log_2 FC < 0.5$). Proteins were first required to have more than 4 semi-tryptic peptides. The corresponding peptides were then filtered for Severe/Control fold change > 1.5 , and proteins retaining at least 4 FC-filtered semitryptic peptides were selected for downstream enrichment analysis. These stringent criteria allowed us to derive a high-confidence shortlist of proteins with convergent evidence of increased proteolytic susceptibility.

Bioinformatic analysis

Principal Component Analysis (PCA) was performed on the proteomic filtered and imputed dataset based on the intensity values to assess the global variance and visualize sample clustering based on their expression profiles. PCA was computed in Python and samples were plotted as individual points in the PC1-PC2 space, and 95% confidence ellipses were calculated for each biological group using the covariance structure of the component scores. The enrichment analysis of the biological processes was performed with STRING platform (Szklarczyk et al. 2023) based on Gene Ontology terms. We reported the best 15 terms sorted by signal parameter grouping each term by similarity ≥ 0.9 .

Multiple reaction monitoring experiments

Multiple Reaction Monitoring (MRM) analyses were carried out using the Xevo-TQS mass spectrometer coupled to the nanoAcquity UHPLC equipped with the IonKey CHIP interface (Cozzolino et al. 2023; Cozzolino et al. 2020), to validate some proteins of interest identified in the untargeted proteomics analysis. Peptide mixtures were separated on Peptide BEH C18 130 Å, 1.7 µm, 150 µm x 50 mm, iKey using a linear gradient of eluent B (95% acetonitrile LC-MS grade, 0.2% formic acid) from 7 to 95% over 60 min at a flow rate of 3 µl/min. Raw data were processed using Skyline. For each protein, at least two prototypical peptides were selected and at least two transitions were monitored for each parent ion as predicted in silico using Skyline. Histone H4 was used as a practical technical reference for MRM signal normalization after prior equalization of protein input by BCA assay. Each run was analyzed in duplicate, and the total area of each peptide transition was used for relative quantification of specific proteins, normalized to the mean area of H4 peptides transitions.

Results

Clinical and echocardiographic characteristics of the study population

The main clinical and echocardiographic characteristics of the study population are reported in Supplementary Table S1. Patients with aortic stenosis fulfilled the criteria for severe disease, showing markedly reduced aortic valve area ($0.8 \pm 0.1 \text{ cm}^2$) and increased mean transvalvular gradient ($44.9 \pm 10.3 \text{ mmHg}$), whereas control subjects with aortic regurgitation showed preserved valve area ($3.3 \pm 0.3 \text{ cm}^2$). Left ventricular ejection fraction was comparable between groups, while patients with aortic stenosis were older, consistent with disease epidemiology. Additional echocardiographic parameters, including E/E' and left ventricular diameters, further reflected the distinct hemodynamic profile of the two groups.

Proteomic profiling of aortic valves

To investigate molecular alterations associated with aortic valve stenosis (AS), we performed a differential proteomic analysis on valvular tissue from patients with severe AS ($n = 10$) compared to controls ($n = 9$). Briefly, all valve samples, obtained from patients undergoing aortic valve replacement, were finely powdered in liquid nitrogen, subjected to decalcification using EDTA and protease inhibitors, and processed for protein extraction and tryptic digestion according to a shotgun protocol. LC-MS/MS analysis of peptide mixtures were acquired in DIA mode on an Orbitrap Exploris 240 system, and data were analyzed using DIA-NN software with stringent identification criteria (≥ 3 peptides per protein, at least 1 unique, $FDR < 0.01$). A total of 1971 proteins were identified using DIA-NN under the established cutoff criteria (see Supplementary Table S2). Principal component analysis (PCA) of the DIA protein abundance data revealed a clear separation between control and severe AS samples along the first principal component (PC1), accounting for 24.3% of the total variance (Fig. 1A). Statistical analysis using Student's t-test (FDR -adjusted p -value < 0.05) identified 594 proteins significantly modulated in severe AS versus controls. Among these, only 462 proteins passed the biological significant filter of $|\log_2 FC| > 0.5$. Of those, 341 proteins were upregulated, while 121 were downregulated (see Supplementary Table S2), indicating a predominant trend toward protein accumulation in the diseased tissue (Fig. 1B).

Analyzing the dataset of differentially expressed proteins we found several interesting results. To obtain a descriptive overview of the biological processes associated with the proteomic differences observed in severe aortic stenosis (AS), we performed Gene Ontology (GO) enrichment analysis using the STRING platform (Szklarczyk et al. 2023) on the set of 341 upregulated and 121 downregulated proteins identified in the DIA-based

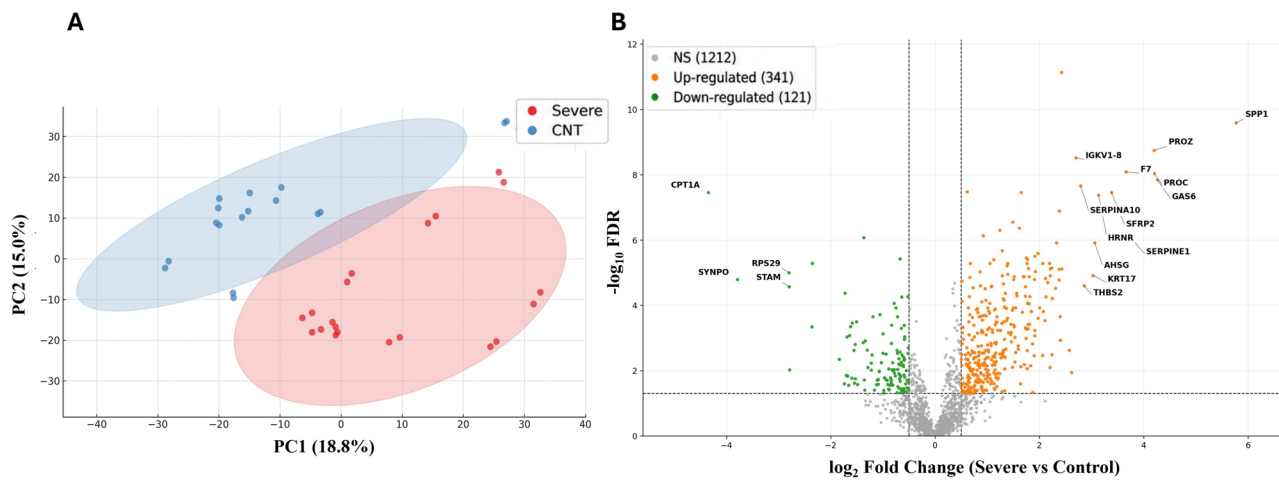


Fig. 1 Differential proteomics results. **A** PCA plot of quantified proteins separates Severe (blue dots) from Control (red dots) samples. Each dot is a sample, and shaded ellipses depict 95% confidence regions. **B** Volcano plot for Severe vs Control shows $\log_2$ fold change (x-axis) versus $-\log_{10}$ FDR (y-axis). Dashed lines mark the thresholds ($|\log_2 FC| > 0.5$, $FDR < 0.05$). Proteins are colored as up-regulated (orange, $n = 341$), down-regulated (green, $n = 121$), or not significant (grey, $n = 1212$). Gene labeling was reported for proteins with $FDR < 0.001$ and $|\log_2 FC| > 2.5$

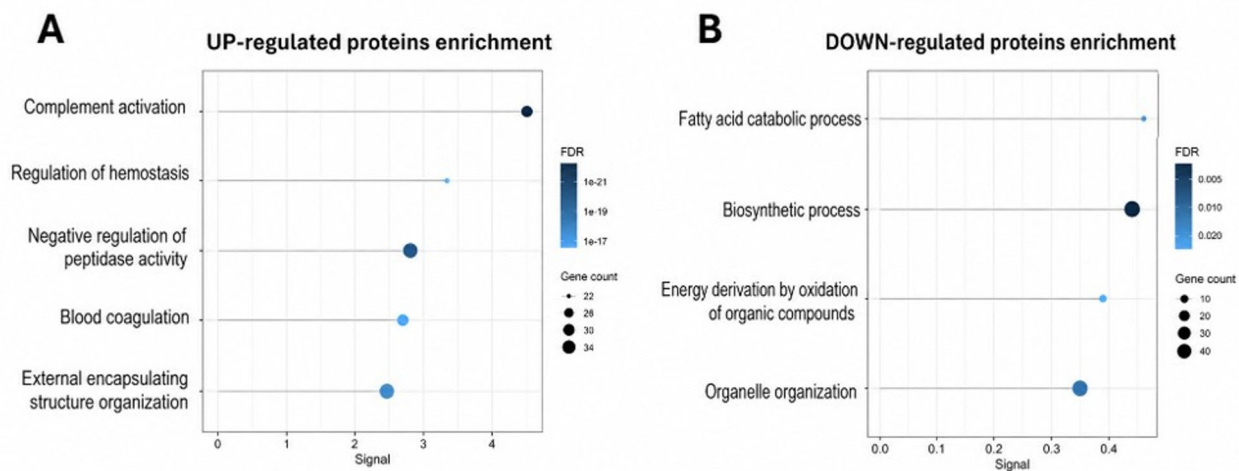


Fig. 2 Representative GO Biological Process enrichment terms associated with differentially expressed proteins. Lollipop plot of enriched processes related to **(A)** up-regulated proteins; **(B)** enriched processes regarding down-regulated proteins. For both panels, the y-axis lists enriched GO Biological Process terms; the x-axis shows the Signal. Bubble size encodes the protein count per term, and bubble color reflects the FDR as indicated (lighter = lower FDR)

differential proteomic analysis. Fig. 2 displays GO enrichment results as lollipop plots for up-regulated proteins (panel A) and down-regulated proteins (panel B). Along the y-axis are the enriched GO Biological Process terms, and the x-axis shows the Signal, i.e., the weighted harmonic mean between the observed versus expected ratio and $-\log(FDR)$. Bubble size encodes the protein count contributing to that term, while bubble color reflects the FDR according to the legend. Because GO terms are hierarchically organized and partially overlapping, Fig. 2 reports representative enriched processes that summarize the main functional themes emerging from the dataset.

The enrichment analysis of up-regulated proteins revealed strong representation of inflammation and

innate immune activation (Fig. 2A). In particular, “complement activation” was the most significantly enriched term and highest signal value, consistent with the known involvement of innate immune mechanisms in AS pathophysiology (Helske et al. 2008). Consistently, multiple complement components (e.g., C1QA, C1QB, C1QC, C2, C3, C4A, C5–C9) were upregulated, highlighting activation of both the classical and terminal pathways mediating immune cell recruitment and tissue remodeling. Another well-established mechanism enriched in our dataset involves coagulation and hemostasis. Terms such as “regulation of hemostasis” and “blood coagulation” are consistent with enrichment of thrombo-inflammatory and hemostatic processes in diseased valves. Supporting this, we observed upregulation of fibrinogen chains

(FGA/B/G), prothrombin (F2), and multiple coagulation factors (F7, F9–F13), together with protease inhibitors like SERPINE1 and SERPINF2, suggesting a pro-thrombotic environment within the valvular microenvironment (Katayama et al. 2024; Natorska and Undas 2015). Processes related to fibrinolysis were also enriched, indicating that fibrin turnover is actively modulated in stenotic tissue. Structural and repair processes such as “external encapsulating structure organization” were enriched as well, in line with extensive extracellular matrix (ECM) remodeling and fibrosis, hallmarks of AS pathology (Di Vito et al. 2021). This is further supported by the overrepresentation of several collagen isoforms (e.g., COL5A1, COL5A2, COL8A1, COL12A1, COL14A1, COL16A1, COL18A1), pointing to active matrix turnover. In line with an evident alteration of collagen components in ECM, many enzymes associated with the collagens and collagen deriving fibrils formation and maturation are found overexpressed. In particular, our dataset includes increased PLOD1 (FC=2.26), a lysyl hydroxylase involved in collagen maturation, together with increased LOX (FC=2.20), a key enzyme mediating collagen cross-linking, and PCOLCE (FC=1.69), which supports procollagen processing. Enrichment in “negative regulation of peptidase activity” suggests enhanced protease inhibition, potentially balancing ECM degradation and inflammatory protease activity, consistent with the upregulation of multiple serpins (SERPINA1, SERPINE1, SERPING1).

In addition, the increasing in proteins such as immunoglobulin heavy, light, and variable chain components (e.g., IGHG2, IGHV3–15, IGLV3–10) reflects systemic immune activation and local antibody (Aimo et al. 2017). Finally, the upregulation of multiple apolipoproteins (APOA1, APOA2, APOA4, APOB, APOC1, APOC2, APOD, APOL1, APOM) was also observed. The presence of these apolipoproteins supports the involvement of lipoprotein deposition and oxidation in early inflammation and calcification, as reported in previous studies (Thanassoulis 2016).

Together, these enrichment results provide a descriptive functional overview in which lipid-related, immune, coagulant, and ECM-remodeling pathways co-occur in CAVD. These associations should be interpreted as hypothesis-generating rather than as direct mechanistic evidence. Conversely, down-regulated proteins were predominantly enriched in biosynthetic and metabolic processes, labelled as “biosynthetic process”, “energy derivation by oxidation of organic compounds”, and “fatty acid catabolic process” (Fig. 2B). These findings are consistent with metabolic reprogramming in valvular interstitial cells undergoing phenotypic switching, where mitochondrial and anabolic activity are suppressed in favor of osteogenic and pro-calcific functions (Gu et al. 2024).

Validation of expression levels of selected proteins

To validate the proteomic results, we focused on the up-regulated proteins, selecting a panel of potential biomarkers for targeted quantification via Multiple Reaction Monitoring (MRM) (Fig. 3). The selection was driven by their biological relevance in valve remodeling and calcification, as well as their consistent differential expression in the discovery dataset. Among the validated proteins were F2, F9, F10, C3, KNG1, FGG, and KLKB1, all functionally related to coagulation and complement activation, pathways strongly implicated in CAVD pathogenesis.

The inclusion of structural and matrix-associated proteins such as ACAN and EMILIN1, as well as HTRA1, a serine protease involved in matrix remodeling, further supports the activation of extracellular matrix turnover mechanisms in stenotic valves. Overall, MRM validation strengthens the robustness of the proteomic findings and highlights a network of coagulation, complement, and matrix remodeling proteins as key contributors to CAVD.

Semitryptic signatures of extracellular matrix degradation

Given the central role of extracellular matrix (ECM) remodeling in disease progression, we further investigated whether specific ECM proteins in stenotic valves are targets of enhanced proteolytic processing by displaying an increased semitryptic peptide release upon trypsinization procedure. This strategy provided a degradomic-like snapshot of the proteolytic landscape within the valvular tissue and allowed us to prioritize ECM proteins showing a qualitative and quantitative higher abundance in semi-tryptic peptides in stenotic valves. Briefly, AS and control proteome were analyzed by LC–MS/MS in DDA mode onto an Orbitrap Exploris 240 system, and the data were processed with Proteome Discoverer v2.5 allowing semi-tryptic cleavages in the analytical parameters. A total of 14,597 peptides were identified, including both the canonical tryptic and semi-tryptic ones (Supplementary Table S3). Among all, 1,564 semi-tryptic peptides were passed the statistical filters (Student’s t-test with a FDR<5% using Benjamini–Hochberg correction) carried out using Perseus v1.6.15.0 (Supplementary Table S3). For downstream analyses, only semi-tryptic peptides mapping to proteins with no overall change in abundance ($-0.5 < \log_2 \text{FC} < 0.5$) were retained, so that differences in semitryptic peptide levels are more likely to reflect an increased proteolytic processing rather than simple protein-abundance changes. The protein set was further refined to retain only candidates supported by at least 4 distinct semi-tryptic peptides and showing a Severe/Control fold change > 1.5. This conservative filtering strategy was adopted to prioritize proteins supported by convergent semitryptic evidence at the protein level. Therefore, proteins represented by less than 4 semitryptic site were not considered absent or biologically irrelevant,

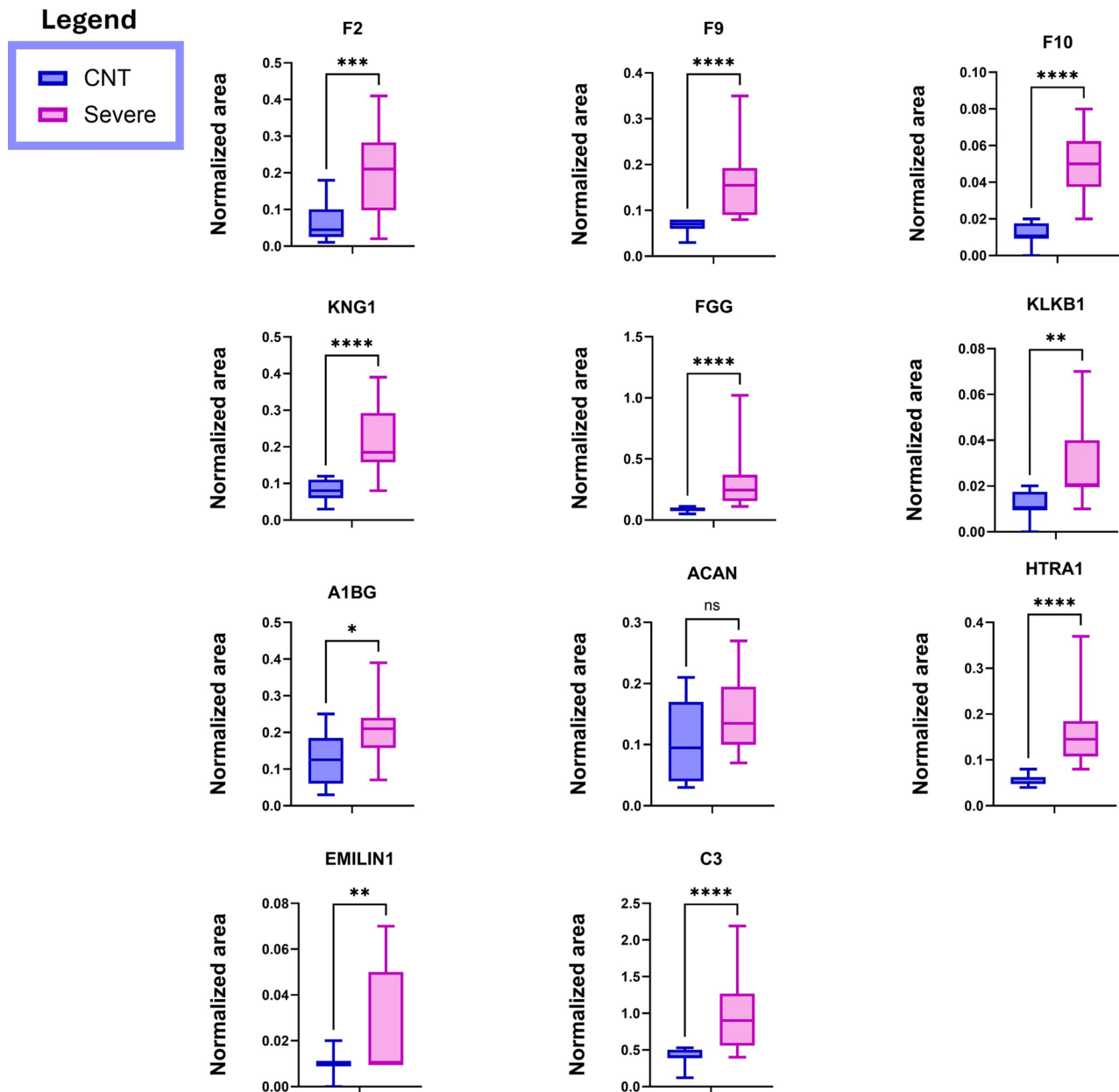


Fig. 3 Targeted MRM validation of selected proteins in stenotic valves. Box-and-whisker plots show normalized peak areas for Control (CNT, blue boxes) and Severe (magenta boxes) samples. Differential expression significance for each monitored protein is indicated as $P < 0.05^*$, $< 0.01^*$, $< 0.001^{**}$, $< 0.0001^{****}$; ns, not significant

but were not retained in the final high-confidence short-list. The analysis yielded 13 proteins that fulfill these criteria and thus represent putative, non-canonical protease substrates in stenotic valves. As shown in Fig. 4A, the enrichment analysis of the semitryptic substrate dataset is dominated by structural ECM activities. The top molecular function (MF) terms (Fig. 4B) were represented by “Structural molecule activity” (FDR $\sim 6\text{E-}08$) comprising DCN, LUM, FBN1, COL6A1, PRELP, ACTN1, ACTB, TUBB, FGA, HSPG2, “Extracellular matrix structural constituent” (FDR $\sim 1\text{E-}07$) including

DCN, LUM, FBN1, COL6A1, PRELP, FGA, HSPG2, and “Extracellular matrix structural constituent conferring compression resistance (FDR $\sim 1.7\text{E-}04$) grouping DCN, LUM, PRELP, HSPG2). These terms point to a matrix-centric degradomic signature focused on SLRPs (decorin, lumican, PRELP), microfibrils (FBN1), and collagen VI (COL6A1). Additional molecular functions terms “Collagen binding” (DCN, LUM, COL6A1) and “Glycosaminoglycan binding” (DCN, FBN1, PRELP, ITIH1) highlight substrates that scaffold collagen fibrils and interface with glycosaminoglycans (GAGs).

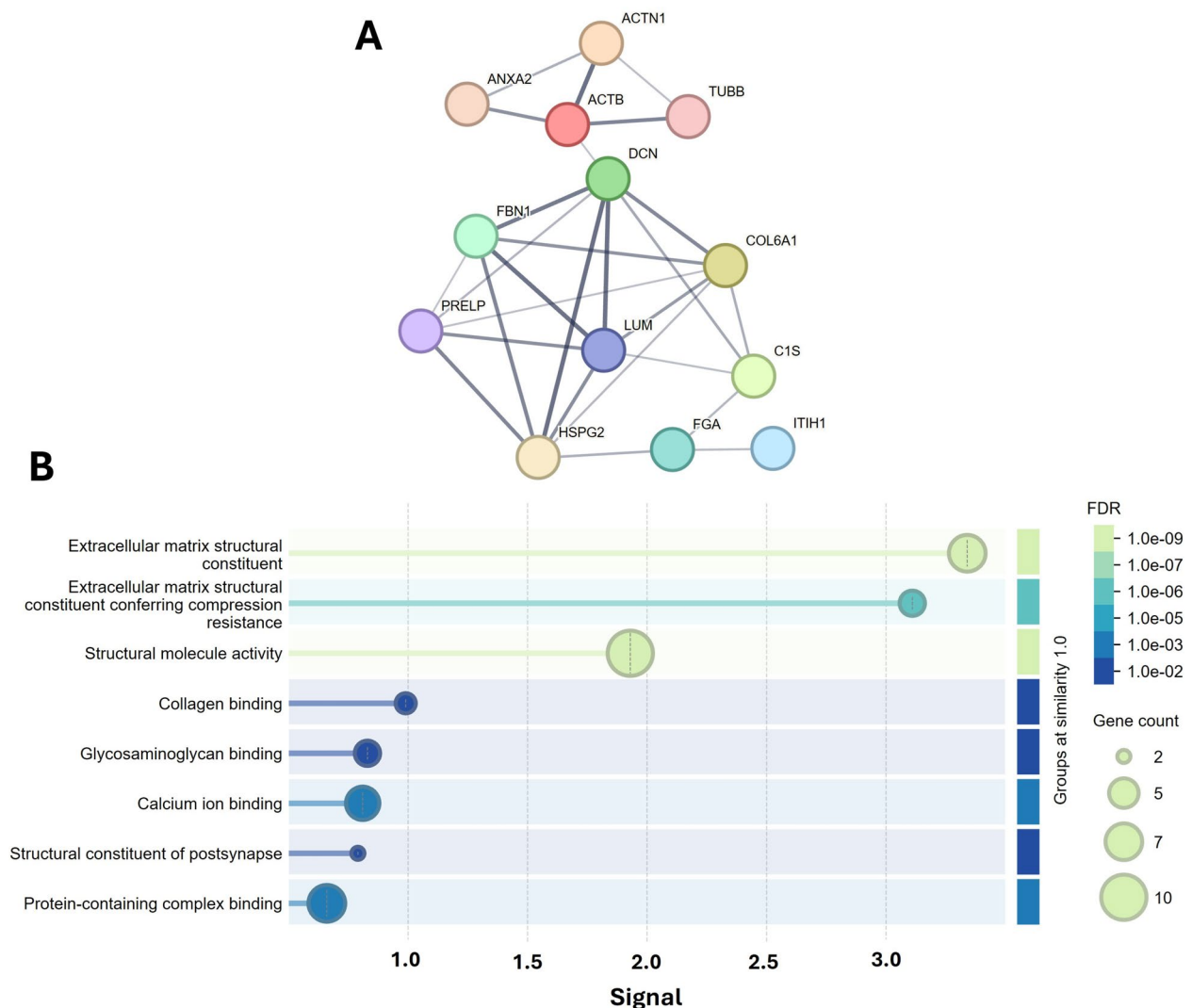


Fig. 4 ECM-centered semitryptic degradomic signature in stenotic valves. **A** STRING network of proteins showed evidence of proteolysis in severe samples. **B** GO Molecular Function. In lollipop plots, the x-axis is Signal, bubble size indicates gene count, and color encodes FDR

Although only 13 proteins emerged from the semitryptic peptide analysis, it is noteworthy that they cluster into a highly interconnected network (Fig. 4B). Indeed, the STRING network shows a compact ECM-centric hub with a PPI enrichment p -value = 9.44×10^{-10} . This means that the “degradome” signature has more interactions among themselves than what would be expected for a random set of proteins of the same size and degree distribution drawn from the genome. Such an enrichment indicates that the proteins are at least partially biologically connected, as a group. The densest subnetwork is formed by Decorin (DCN), Lumican (LUM), Prolargin (PRELP), fibrillin-1 (FBN1), Perlecan (HSPG2) and Collagen alpha-1(VI) chain (COL6A1), that are SLRP proteoglycans and microfibrillar/collagen-VI components that coordinate collagen fibrillogenesis and pericellular matrix architecture. Two blood-interface proteins, C1S

(complement C1s) and FGA (fibrinogen α -chain), are also included to this ECM core, indicating crosstalk between complement/coagulation and matrix remodeling at the valve surface. A small cytoskeletal cluster (ACTB, ACTN1, TUBB, ANXA2) connects weakly through ACTB, consistent with cell–matrix anchorage. ITIH1, a heavy chain of inter- α -trypsin inhibitor that stabilizes hyaluronan-proteoglycan complexes, links towards FGA, further supporting turnover of proteoglycan-rich scaffolds. Overall, the network highlights that degraded proteins concentrate on core structural ECM constituents, with complement/fibrin nodes bridging to the thrombo-inflammatory environment, an architecture fully compatible with active matrix degradation and stiffening in stenotic aortic valves.

Histologic assessment of elastin fragmentation in stenotic valves

Because elastin was poorly captured by our shotgun workflows and thus not quantifiable in the proteomic dataset, we evaluated cusp elastic architecture histologically using Verhoeff–Van Gieson (VVG) staining on FFPE aortic valves from severe AS ($n=8$) and controls (CNT; $n=19$). In CNT cusps, continuous elastic lamellae sharply delineated ventricularis, whereas severe AS showed rarefaction, discontinuity, and fraying of elastic fibers with collagen-rich replacement, most evident at the ventricularis–spongiosa interface (Fig. 5A–B). Quantification of elastin confirmed increased elastin fragmentation in severe AS compared with CNT (Fig. 5C).

Collagen post-translational modifications as a marker of ECM remodeling in stenotic valves

To enable triple-helix stabilization, supramolecular assembly, and higher-order network formation, collagens undergo extensive post-translational modifications (PTMs), including proline/lysine hydroxylation, glycosylation, and covalent cross-linking. In pro-inflammatory and fibrotic milieus such as aortic stenosis, ECM remodeling affects not only the amount of collagen deposited but also its “quality”, as encoded by the collagen PTM landscape (Karsdal et al. 2016). Because proline hydroxylation is a canonical hallmark of triple-helix stabilization and maturation, and hydroxyproline-rich peptide profiles have proven informative

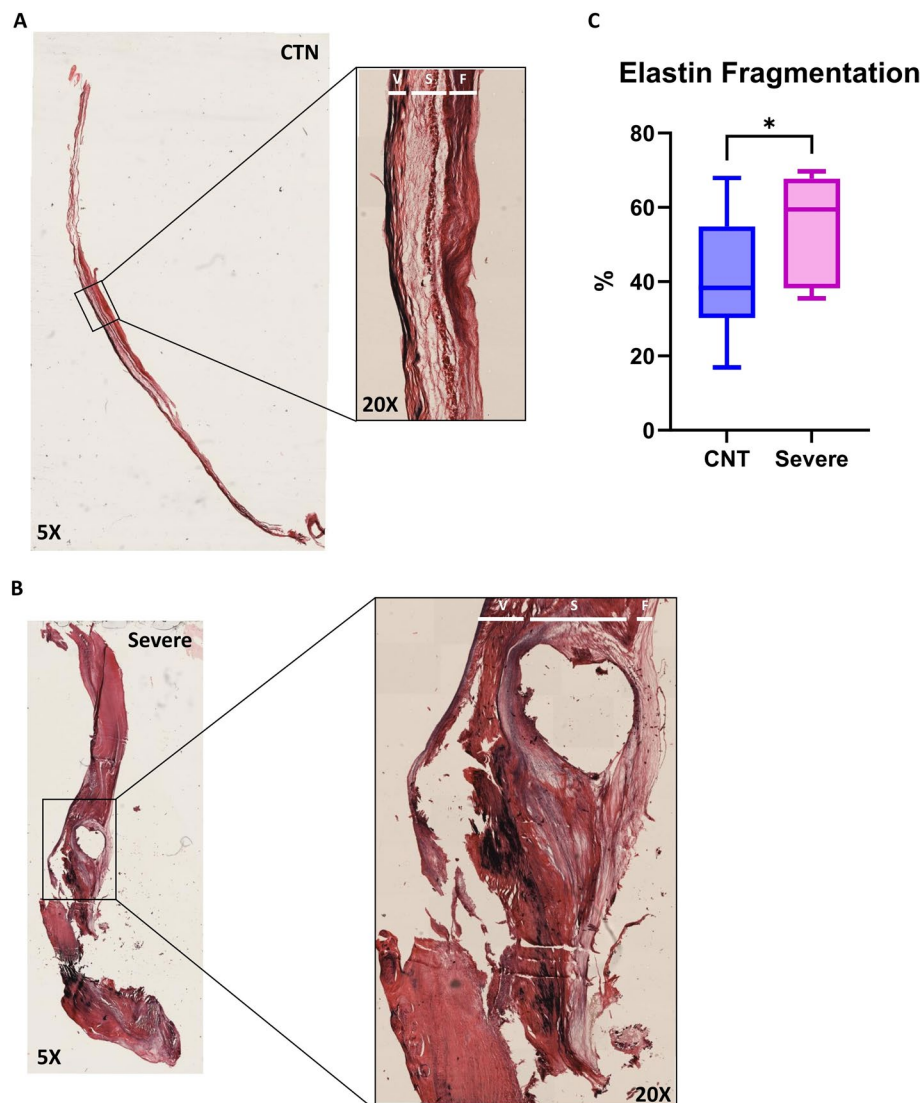


Fig. 5 Elastin fragmentation in control versus severe AS aortic valves. Representative Verhoeff–Van Gieson–stained sections from control (CNT; $n=19$; **A**) and severe aortic stenosis (AS; $n=8$; **B**) specimens are shown at low power (5x) and higher power (20x). Layer boundaries are indicated: ventricularis (V), spongiosa (S), and fibrosa (F). Elastic fibers stain black, collagen red, and background yellow. **C** The box-and-whisker plot shows increased elastin fragmentation between CNT and severe AS. * indicates a $P<0.05$

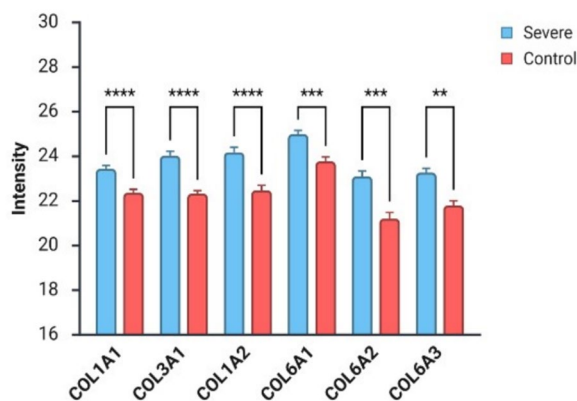


Fig. 6 Collagen PTM analysis by mass spectrometry. Intensity of hydroxyproline-containing peptides mapped to the indicated collagen chains in severe CAVD versus controls. Bars represent mean $\pm$ SEM; ns, not significant, **adjusted p -value < 0.01 , ***adjusted p -value < 0.001 , ****adjusted p -value < 0.0001

across collagen-driven pathologies (Qiu et al. 2014), we expanded our ECM remodeling analysis by specifically interrogating collagen-associated PTMs. In addition to proline hydroxylation, we investigated also other PTMs (i.e. methionine oxidation, lysine carbamylation, Asn/Glu deamidation, formylation, carboxyethylation, carboxymethylation, and aminoadipic acid modification), but no other collagen chain modifications were found significantly dysregulated.

The identification and quantification analysis was carried out with the MaxQuant and Perseus software by using Pro $\rightarrow$ Hydroxyl-Pro as variable modification (Supplementary Table S4). Hydroxyproline-modified peptides mapped to multiple collagen chains with robust significance (adjusted $p < 0.05$), with higher levels in severe CAVD than in controls. To assess whether the hydroxyproline signal could be trivially explained by changes in bulk collagen abundance, we next checked the expression of the corresponding collagen chains in the differential proteomics analysis described above, and found no significant change for COL1A1, COL1A2, COL3A1, COL6A1, or COL6A3 (all FDR > 0.05), whereas COL6A2 and COL6A6 were significantly down-regulated (FC = 0.51 and FC = 0.33, respectively). Bars in Fig. 6 summarize the intensities of modified peptides, pooled across multiple modification sites per collagen chain, that passed Perseus statistical filtering. Significance between the severe and control groups was tested by two-way ANOVA with Bonferroni correction.

Hydroxyproline-containing peptides mapped to multiple fibrillar and microfibrillar collagen chains (COL1A1, COL1A2, COL3A1, COL6A1, COL6A2, COL6A3) and were consistently increased in severe CAVD compared with controls (Fig. 6), indicating a broad shift toward hydroxylation-enriched collagen peptide patterns in

advanced disease. It has been reported that cardiac fibrosis is characterized by the excessive production of extracellular matrix (ECM) and high structured fibrillar collagens belonging to the type I and III (COL1A1 and COL3A1). In fact, the enrichment in hydroxylation suggests either enhanced collagen maturation or compensatory ECM stiffening in response to chronic mechanical stress and inflammation in calcified valves, supporting the view that these PTM changes are compatible with altered collagen state/maturation in advanced disease. In valves, collagen type VI organizes the interface between the valve interstitial cells (VICs) and the matrix. A structural alteration, likewise that induced by an increased hydroxylation level, affects the way the cells perceive mechanical stress (Rutkovskiy et al. 2017). In particular, the valve cells show an altered perception of blood pressure signals and consequently are stimulated to counteract this stress, also increasing the hydroxylation extent, which induce higher rigidity levels, thus contributing to fibro-calcified degeneration.

Notably, the finding of increased hydroxylation COL6A6 peptides despite lower overall COL6A6 abundance suggests that a reduced/reshaped collagen VI pool in severe disease may be enriched for chemically “aged” or slowly turned-over species, reinforcing the concept that CAVD progression involves qualitative ECM remodeling beyond bulk collagen accumulation.

This interpretation is consistent with the broader paradigm of cardiac fibrosis, where increased stiffness is driven not only by the amount of type I/III collagens but also by their maturation state and cross-linking density. In the aortic valve, the concomitant increase of hydroxyproline peptides in fibrillar collagens (COL1A1/COL1A2, COL3A1) and in collagen VI chains (COL6A1/2/3), supports a model in which collagen architecture and pericellular anchoring networks are chemically remodeled in advanced stenosis, potentially contributing to leaflet stiffening and maladaptive mechanotransduction in the calcified valve microenvironment. Taken together, these results suggest that the enrichment of hydroxyproline signatures is consistent with qualitative collagen remodeling that is not fully explained by bulk collagen abundance changes alone (Merl-Pham et al. 2019).

Discussion

In this study, we combined DIA-based differential proteomics, ECM-focused degradomics, collagen post-translational modification (PTM) mapping, targeted MRM, and histology to investigate the molecular mechanisms altered in calcific aortic valve disease (CAVD) and to obtain a multi-layered view of extracellular matrix remodeling. As known, unlike aortic regurgitation, the development of calcific AS is a slow and multifaceted process, spanning many years and driven by mechanical

and biological factors. We used a seemingly limited sample size (10 AS vs. 9 AR), but this has already been done in other studies (Bouchareb et al. 2021). This is an intrinsic limitation related to the availability of human surgical valve samples. This aspect was highly detrimental to the control, and the choice to use valves from patients with aortic regurgitation rather than healthy valves allowed us to recruit more patients. In our opinion, these samples constitute an appropriate non-stenotic surgical comparison, as they nevertheless exhibit characteristics of valve remodeling that should be taken into account when interpreting the molecular differences observed between the groups.

Over time, fibrosis, inflammation, oxidative stress, angiogenesis, and osteogenic differentiation contribute to the progressive remodeling of the aortic valve leaflets (Rajamannan et al. 2007; Freeman and Otto 2005). Our study focused on the molecular characterization of valvular tissue in a patient cohort in a multi-layer manner: (i) a DIA-based proteomic map that highlights enrichment of known stenotic processes (e.g. complement and coagulation), structural ECM components (e.g., multiple collagen isoforms) and regulators of protease/antiprotease balance; (ii) a degradomic signature based on semitryptic peptides that selectively targets SLRPs (Decorin, Lumican, PRELP), microfibrillar and pericellular matrix components (including FBN1, COL6A1 and HSPG2/Perlecan), suggesting that these ECM constituents act as preferential substrates of endogenous proteolytic activity in stenotic valves. Histological evidence of marked elastin rarefaction and fragmentation indicates that ECM degradation in stenotic valves involves not only SLRPs, microfibrillar structures, and Collagen VI, but also elastic lamellae; and (iii) a PTM-focused analysis showing increases hydroxyproline-containing peptides in several collagen chains in the absence of upregulation in total collagen abundance.

Using a label-free proteomics approach in DIA mode, we quantified ~2,000 proteins in human aortic valves and identified 341 proteins up-regulated in severe stenosis compared with controls, with a clear shift towards accumulation of proteins involved in inflammation, complement and coagulation, lipid handling, and extracellular matrix organization. Conversely, metabolic and biosynthetic pathways were globally suppressed, consistent with phenotypic and metabolic reprogramming of valvular interstitial cells (VICs) toward a pro-fibrotic, pro-calcific state. The DIA-based proteomic analysis recapitulated several expected features of CAVD biology, with upregulation of complement components (C3, C4, and terminal C5b–9) (Helske et al. 2008), coagulation and fibrinolysis regulators such as fibrinogen chains (Kopytek et al. 2023), osteogenic mediators including SPP1/osteopontin (O'Brien et al. 1995), structural ECM proteins such as

FN1 and LOX (Bouchareb et al. 2021; Ballester-Servera et al. 2024), and ApoB-containing lipoprotein pathway components (Mohty et al. 2008). In parallel, we observed downregulation of key enzymes of mitochondrial fatty-acid oxidation, exemplified by CPT1A (Pal et al. 2024), consistent with the metabolic remodeling previously described in AS. These findings reinforce the current view of CAVD as an active, multifactorial fibrocalcific disease driven by the interplay of immune, coagulant, ECM, metabolic, and osteogenic pathways, rather than a purely degenerative or passive calcific process (Büttner et al. 2021).

Within this global landscape, we focused on the extracellular matrix remodeling mechanisms to characterize potential hallmarks of the disease. Previous proteomic and histopathological studies have shown that CAVD is characterized by excess deposition and disorganization of fibrillar collagens, loss of elastin, accumulation of proteoglycans, and activation of fibrosis-related programs in the valve leaflets (Fondard et al. 2005; Hutson et al. 2016). More recent work has refined this view by focusing on the valvular ECM proteome and identifying matrix proteins such as FNDC1 and MXRA5, which were found up-regulated in our dataset, as candidate biomarkers and potential effectors of fibrotic remodeling (Bouchareb et al. 2021; Martin-Rojas et al. 2015; Anousakis-Vlachochristou et al. 2023).

The degradomic signature based on semitryptic peptides, obtained by restricting the analysis to proteins without major changes in total abundance and applying stringent filters on fold change and number of semi-tryptic peptides, which yielded a focused set of 13 candidates that likely act as preferential substrates of endogenous proteolytic activity in stenotic valves. Notably, most of these proteins were ECM constituents, with small leucine-rich proteoglycans (SLRPs; Decorin, Lumican, PRELP), Perlecan (HSPG2), Fibrillin-1 (FBN1) and Collagen VI (COL6A1) forming the densest subnetwork. SLRPs are key organizers of collagen fibrillogenesis, matrix hydration and fibril spacing, and they modulate growth factor availability and immune cell behavior (Iozzo and Schaefer 2010). Their selective enrichment in the semi-tryptic peptide pool therefore suggests active cleavage that may alter collagen architecture, growth factor signaling and local inflammatory cues in a manner that favors fibrocalcific remodeling, in line with previous reports of SLRP dysregulation in aortic stenosis (Martin-Rojas et al. 2015; Barth et al. 2019). Likewise, the presence of multiple semi-tryptic peptides derived from FBN1 and COL6A1 points to enhanced proteolytic pressure on the pericellular microfibrillar scaffold: fibrillin-1-containing microfibrils integrate with elastin and collagens and provide a reservoir for signaling molecules such as TGF- β (Di Vito et al. 2021;

Van Doren 2015), while collagen VI forms a microfibrillar network that links interstitial collagens to basement membranes and elastic fibers, contributing to tissue integrity and mechanotransduction (Sánchez-Cabo et al. 2020). In combination with the histological evidence of marked elastin rarefaction and fragmentation, these findings support a scenario in which the elastic-microfibrillar interface is progressively dismantled, weakening the mechanical coupling between ECM compartments and reshaping mechanosensitive and profibrotic signaling pathways. The degradomic network also included plasma-derived fibrinogen and complement component C1s, which bridge the SLRP-microfibrillar cluster to the hemostatic and innate immune systems and are consistent with an association between the thrombo-inflammatory milieu at the blood-valve interface and ECM proteolysis (Anousakis-Vlachochristou et al. 2023; Jylhävä et al. 2014).

We next asked whether our ECM-centered degradomic signature was paralleled by qualitative changes in the structural backbone of the valve matrix. Collagen is the most abundant ECM component in the aortic valve, and its biomechanical behavior critically depends on post-translational modifications (PTMs), including proline/lysine hydroxylation, glycosylation, and the formation of mature inter-fibrillar cross-links (Shoulders and Raines 2009). In fibrotic and inflammatory settings such as aortic stenosis, these PTMs are increasingly recognized as determinants of collagen quality, influencing triple-helix stability, fibril architecture and tissue stiffness beyond simple changes in protein abundance (Shoulders and Raines 2009; Neff and Bradshaw 2021). By specifically interrogating collagen-associated PTMs and focusing on hydroxyproline, a canonical hallmark of triple-helix stabilization, we found a consistent increase in hydroxyproline-containing peptides across several fibrillar and microfibrillar collagen chains (COL1A1, COL1A2, COL3A1, COL6A1, COL6A2 and COL6A3), with robust statistical support in severe CAVD compared with controls. Importantly, inspection of the DIA-based differential proteomics data showed no significant change in total abundance for COL1A1, COL1A2, COL3A1, COL6A1, and COL6A3 (all FDR > 0.05), while COL6A2 and COL6A6 were down-regulated (FC = 0.51 and FC = 0.33, respectively). This divergence indicates suggests that the signal captured by hydroxyproline-bearing peptides is not simply explained by increased collagen expression per se and is instead consistent with qualitative collagen remodeling, pointing to qualitative ECM remodeling in stenotic valves. These variations in prolyl-hydroxylation levels, however, merit comment regarding the mean age of the populations compared, as collagen changes are strongly influenced by aging processes. Both pathological samples and controls derive from patients with a mean

age between 62 and 68.6 years, with AS patients being the oldest on average, consistently with the epidemiology of the disease. However, considering that over a time span of over 60 years, all samples fall within a relatively narrow time window, we can speculate that the main reason for the observed variations in prolyl hydroxylation may be attributable to disease-specific remodeling, in addition to a smaller age-related contribution. Consistent with this interpretation, the observed enrichment of hydroxyproline-containing peptides may reflect altered collagen maturation/state and ECM remodeling (Merl-Pham et al. 2019; Neff and Bradshaw 2021). In the context of cardiac fibrosis, excessive production of highly structured fibrillar collagens type I and III is a well-established hallmark of increased myocardial stiffness. Conversely, given the well documented function of collagen type VI in maintaining the mechanical strength of elastic tissues acting as an anchor, we can speculate that the down regulation of such isoforms coupled with an altered hydroxylation profile may affect not only its stability but also the physiological interaction between cell surface receptors, such as integrins, and the matrix (Cescon et al. 2015; Rappu et al. 2019) very delicate process in following the contractile movements of the valve. Particularly in COL6A6 despite its reduced total abundance, may reflect accumulation of long-resident/damage-prone collagen VI species and/or altered turnover within the pericellular microfibrillar network, which is highly relevant to mechanotransduction and fibrosis in calcific valve disease. Overall, our data extend the concept of “collagen remodeling” beyond bulk abundance changes and support the presence of qualitative alterations in collagen state in stenotic valves, as reflected by changes in hydroxyproline-bearing peptides, even when total protein levels remain relatively unchanged.

Importantly, other PTMs were searched (i.e. methionine oxidation, Asn/Gln deamidation lysine carbamylation, formylation, carboxyethylation, carboxymethylation, and aminoadipic acid modification), but no collagen chain modifications were found significantly dysregulated.

Complementing the collagen PTM layer, histological assessment of elastin architecture by Verhoeff–Van Gieson staining revealed marked rarefaction, discontinuity and fraying of elastic lamellae in severe AS cusps, with collagen-rich replacement particularly evident at the ventricularis–spongiosa interface. Quantitative analysis confirmed a significant increase in elastin fragmentation in stenotic valves compared with controls, in line with previous ultrastructural and histopathological reports linking elastic fiber breakdown to calcification and fibrotic remodeling in native aortic valves (Hutson et al. 2016; Perrotta et al. 2011) and increased crosslinking enzyme LOX (FC = 2.2 in

our dataset) expression in CAVD (Hutson et al. 2016). When integrated with the degradomic data, these findings suggest a “double hit” on the mechanical scaffold of the valve: on one side, SLRPs, fibrillin-1 and collagen VI are preferentially targeted by proteolysis, weakening the pericellular microfibrillar network; on the other, fibrillar collagens undergo enhanced hydroxylation and presumed cross-linking, while elastic lamellae are progressively lost and replaced by more rigid collagenous tissue. Together, collagen PTM remodeling and elastin fragmentation therefore act as complementary markers of ECM stiffening in CAVD, supporting a potential relationship between molecular remodeling of the matrix and the macroscopic increase in cusp rigidity that underlies severe aortic stenosis.

Finally, to explore the translational relevance of our findings and to orthogonally validate the DIA-derived differential proteomics, we used targeted MRM to quantify a focused panel of candidates encompassing both circulating and ECM-related components, as presented in Fig. 3. The panel included coagulation and complement factors F2, F9, F10, C3, KNG1, FGG, and KLKB1, as well as the matrix proteins Aggrecan (ACAN), EMILIN1, and HTRA1. MRM data confirmed the direction and, for most analytes, also the magnitude of changes observed by DIA, with consistent upregulation in stenotic valves and good agreement between discovery and validation platforms, thereby supporting not only the analytical robustness of the panel but also the quantitative reliability of the untargeted DIA dataset (Iacobucci et al. 2025; Cozzolino et al. 2023). From a patho-biological standpoint, each protein in this panel captures a distinct facet of the CAVD network: F2, F9, F10, FGG, KLKB1 and KNG1 integrate coagulation, contact activation and kinin generation; C3 reports on complement activation at the blood–valve interface; ACAN, EMILIN1 and HTRA1 read out specific aspects of ECM architecture, elastic–microfibrillar integrity and matrix protease activity (Koch et al. 2020; Munjal et al. 2014; Adamo et al. 2022; Blaser et al. 2021; Ikawati et al. 2018). In line with the multifactorial pathogenesis of CAVD, such a multi-analytes configuration is conceptually preferable to a single biomarker, as it captures the convergence of thrombo-inflammatory, complement and ECM-remodeling processes rather than any isolated pathway (Blaser et al. 2021; Towler 2013). In the context of a thrombo-inflammatory microenvironment rich in complement and coagulation factors, this ECM-centered remodeling is consistent with increase cusp stiffness, alter local mechanotransduction, and promote fibrocalcific progression, in line with contemporary models of CAVD pathobiology (Patterson et al. 2024).

Conclusions

A key strength of this work is its multi-layer design, which combines DIA-based quantitative proteomics (supported by DDA spectral library generation), semi-tryptic degradomics, collagen PTM mapping, and histological assessment within the same well-defined pathological setting. This integrated strategy aimed to achieve a molecular characterization of several complementary facets of aortic stenosis, including immune-coagulant activation, ECM degradation, collagen qualitative remodeling, and elastin damage. Taken together, these multi-layer data support a model in which stenotic valves are not only enriched in ECM proteins, but are also characterized by qualitative remodeling of the matrix: structural collagens show hydroxyproline-enriched peptide patterns consistent with altered collagen state/maturation and likely ECM stiffening, small leucine-rich proteoglycans and microfibrillar components are actively cleaved, and elastic lamellae are fragmented and replaced by rigid collagenous tissue.

Our work therefore refines existing proteomic descriptions of stenotic valves by providing an integrated “quantitative-degradomic-PTM” framework that integrates ECM architecture, proteolysis, and collagen quality to the clinical phenotype of severe aortic stenosis.

Abbreviations

AS	Aortic stenosis
AV	Aortic valve
AVA	Aortic valve area
CAVD	Calcific aortic valve disease
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FDR	False discovery rate
MRM	Multiple reaction monitoring
PTM	Post-translational modification
SLRPs	Small leucine-rich proteoglycan(s)
VICs	Valvular interstitial cell(s)

Supplementary Information

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Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.

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Authors' contributions

Conceptualization, Investigation, Writing—Original Draft, Writing—Review & Editing, Visualization (I.I.); Investigation, Visualization, Methodology (V.M.); Formal analysis, Writing – Original Draft, Visualization (A.L.L.); Formal analysis(B.C); Formal analysis (L.B); Resources, Investigation (M.C); Investigation, Visualization (V.A.M); Investigation, Visualization (V.V); Writing—Review &

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Data availability

The datasets supporting the conclusions of this article are available in the ProteomeXchange Consortium via the PRIDE partner repository, under PXD070758 code (DIA data) and PXD073246 (Degradomic and collagen PTM data).

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board/Ethics Committee of Centro Cardiologico Monzino IRCCS (protocol/project code: R1348/20-CCM1418). Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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