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Article

# Analysis of Vascular Smooth Muscle Cells from Thoracic Aortic Aneurysms Reveals DNA Damage and Cell Cycle Arrest as Hallmarks in Bicuspid Aortic Valve Patients

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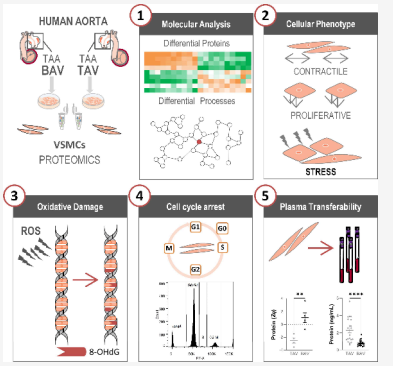
**ABSTRACT:** Thoracic aortic aneurysm (TAA) is mainly sporadic and with higher incidence in the presence of a bicuspid aortic valve (BAV) for unknown reasons. The lack of drug therapy to delay TAA progression lies in the limited knowledge of pathophysiology. We aimed to identify the molecular hallmarks that differentiate the aortic dilatation associated with BAV and tricuspid aortic valve (TAV). Aortic vascular smooth muscle cells (VSMCs) isolated from sporadic TAA patients with BAV or TAV were analyzed by mass spectrometry. DNA oxidative damage assay and cell cycle profiling were performed in three independent cohorts supporting proteomics data. The alteration of secreted proteins was confirmed in plasma. Stress phenotype, oxidative stress, and enhanced DNA damage response (increased S-phase arrest and apoptosis) were found in BAV-TAA patients. The increased levels of plasma C1QTNF5, LAMA2, THSB3, and FAP confirm the enhanced stress in BAV-TAA. Plasma FAP and BGN point to an increased inflammatory condition in TAV. The arterial wall of BAV patients shows a limited capacity to counteract drivers of sporadic TAA. The molecular pathways identified support the need of differential molecular diagnosis and therapeutic approaches for BAV and TAV patients, showing specific markers in plasma which may serve to monitor therapy efficacy.




**KEYWORDS:** *aortic aneurysm, bicuspid aortic valve, cardiovascular disease, DNA damage, oxidative stress, proteomics, vascular smooth muscle cells*



## INTRODUCTION

Thoracic aortic aneurysm (TAA) is a progressive dilatation of the thoracic aorta with at least 50% enlargement beyond the normal diameter leading to a weakening of the vessel.[1] The vast majority of TAAs are idiopathic, representing 80−95% of the cases.[2,3] Nevertheless, there is an association between TAA and the presence of a bicuspid aortic valve (BAV), being BAV the most common congenital heart defect (1−2% of the general population) and an independent risk factor in TAA.[3,4] The presence of a BAV accelerates aneurysm growth, affecting about 50% of these patients and at a younger age than those with a tricuspid aortic valve (TAV).[4,5] Current challenges in TAA are 2-fold: diagnose and treat, and most likely a third, the need to personalize treatments for BAV patients. The unique available treatment is surgery, and current drug therapy fails to avoid progression and dissection,[6−8] so there is a recognized need to define personalized therapeutic strategies based on the discovery of novel pathways and actors in TAA progression considering the particularities of the different forms of TAA.[9−12]

BAV- and TAV-associated TAAs exhibit common hallmarks participating in the aorta remodeling that include media degeneration, extracellular matrix degradation, and phenotype switch of vascular smooth muscle cells (VSMCs) from contractile to synthetic.[13−15] However, there is evidence that the pathophysiology behind TAV- and BAV-TAAs differs significantly. TAV-TAAs present severe changes in the media layer with less organized fiber structures, poor metabolic mitochondrial function associated with VSMCs, and more inflammation, while BAV-TAAs have a reduced number of VSMCs.[16] The proteins released from aneurysmal aortic tissues evidenced significantly diverging expression fingerprints in BAV or TAV patients,[17,18] partially explained by their different biomechanics that lead to TAV-TAA being associated with a greater energy loss and BAV-TAA to a greater stiffness.[19] The abnormal shear wall stress in BAV patients causes molecular distortion and conditions the response, repair

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mechanisms, and propensity for progressive dilatation of the aortic wall. However, the contribution of unknown factors other than mechanical forces cannot be discarded; thus, further knowledge of local actors in TAA progression is needed, particularly from in situ studies. As the main component of the media layer, VSMCs have a key role in the mechanical integrity and stability of the aorta, and previous findings support that VSMC alterations directly impact TAA progression differentially for BAV patients. A later study characterized sporadic TAAs by gene expression within known cellular classes, concluding that the differences are mainly found in VSMCs.[20] Recently, expression of the VSMC protein MYH10 was shown to inversely correlate with the aortic wall strength in TAV aneurysmal patients but not in BAV.[21]

Here, we investigate the VSMC protein landscape in an untargeted manner, having access to thousands of proteins identified. First, we identified those proteins and biological processes showing alteration in the VSMCs from aneurysmal aortas of BAV patients compared to TAV patients to decipher what makes the BAV aorta molecularly different. Second, we confirmed the biological alterations identified from coordinated protein behavior in DNA extracted from VSMCs and in urine from an extended cohort. Third, cell cycle analysis was performed in an independent VSMC population to evaluate potentially altered dynamics and differential cellular apoptosis. Finally, the secreted proteins differentially identified in VSMCs were confirmed in an extended cohort of BAV and TAV aneurismal plasma.

## EXPERIMENTAL SECTION

### Patient Selection and Sample Recruitment

Patients with sporadic TAA were recruited and classified according to aortic valve type as follows: BAV-TAA and TAV-TAA. Human aortic samples were collected from patients with dilated aorta who underwent ascending aorta surgery at Fundación Jiménez Díaz Hospital. Biopsies were taken during surgery from the anterior side of the ascending aorta, 1−2 cm above the sinotubular junction. CT angiography was used to measure ascending aortic diameter. The exclusion criteria were participants under 18 years old, previous cardiac surgery, neoplasia, hemodynamic instability, treatment with corticoids or immunosuppressants, active infection, and in-hospital death. Urine and blood samples were collected before any surgical or anesthetic procedure in sterile containers and EDTA tubes, respectively. Plasma was obtained by centrifugation (1400*g*, 10 min, 4 °C) and stored at −80 °C until analysis together with urine samples. The study was approved by the local Ethics Committee (PIC143-2016, PIC029-21) and adhered to the principles of the Declaration of Helsinki. All participants provided written informed consent before enrollment.

### Primary VSMC Culture

Aortic tissue specimens (2−3 $cm^2$) were obtained from TAA patients, and primary human VSMC lines were generated (one cell line per donor) as previously described.[22] In detail, the arterial segment was incubated overnight at 37 °C in a humidified atmosphere of 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM-F12; Lonza Walkersville) containing 0.1% collagenase type 1 (Gibco). The cells were seeded onto Petri dishes precoated with sterile gelatin in DMEM-F12 medium supplemented with 0.1 mg/mL heparin (Merck Life Science), 30 μg/mL endothelial cell growth factor (Merck Life Science), 1% penicillin/streptomycin (P/E; Lonza Walkersville), 1% L-glutamine (Lonza Walkersville), 10% fungizone (Lonza Walkersville), and 20% fetal bovine serum (FBS). VSMCs were negatively selected by removing endothelial cells with human CD31 antibody (BDbiosciences) and a secondary antibody bound to magnetic beads (Dynabeads antimouse IgG from the CELLection Pan Mouse IgG kit; Invitrogen). VSMCs were maintained in DMEM, 10% FBS until confluence and stored at −80 °C in FBS containing 10% dimethyl sulfoxide until analysis. VSMCs analyzed in this study were at passage 1. VSMCs were stained with specific markers of VSMC α-SMA (1:1000; ref 06198, Sigma) and calponin (1:200; ab46794, Abcam), endothelial cells (1:50 CD31; ab28364, Abcam), and fibroblast (1:50 S100A4; ab93283, Abcam) to ensure the purity of VSMC isolation methods.

### Quantitative Hypothesis-Free Mass Spectrometry-Based Proteomics of VSMCs from TAA Patients with BAV or TAV

**Protein Extraction, Digestion, and Peptide Labeling.** For quantitative proteomics analysis by isobaric labeling, $1 \times 10^6$ cells were used per biological replicate. VSMCs (TAV-TAA, $n = 4$; BAV-TAA, $n = 5$) were lysed in 50 mM Tris-HCl pH 8.5 containing 5 mM tris(2-carboxyethyl) phosphine (TCEP) and 2% SDS (Biorad), and protein extracts were digested with modified trypsin (sequencing grade; Promega) at a 1:40 ratio (trypsin:protein) using the Filter-Aided Sample Preparation method with slight modifications.[23] The resulting peptides were acidified with trifluoroacetic acid and desalted on OASIS HLB columns (Waters) prior to labeling with TMT isobaric labeling reagent (TMT-10plex kit, Fisher Scientific) following the manufacturer's protocol.

**Protein Identification and Differential Quantitation by LC-MS/MS.** TMT-labeled peptides were fractioned on C18 reversed phase columns (High pH Fractionation Kit; Thermo Scientific) into 6 fractions. High-resolution analysis by liquid chromatography coupled to mass spectrometry (LC-MS/MS) was performed with an Easy nLC 1000 nanoHPLC chromatography system (Thermo Fisher Scientific) coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto a precolumn (PepMap100 C18 LC 75 μm i.d., 2 cm, Thermo Scientific) and separated online on a NanoViper PepMap100 C18 LC analytical column (75 μm i.d., 50 cm, Thermo Scientific) in a linear acetonitrile gradient. For peptide identification, spectra were analyzed with Proteome Discoverer (2.1.0.81 version; ThermoFisher), and database searching was performed at the Uniprot database containing all sequences from humans and contaminants (UniProt_Nov, 2016; 70902 entries). Peptide identification was confirmed using the probability ratio method.[24] The false discovery rate (FDR) was calculated using inverted databases and the refined method[25] using a cutoff value of 1% FDR with an additional filtering for a precursor mass tolerance of 15 ppm.[26] Statistical differences were analyzed with the in-house software SanXoT[27] using the WSPP model for differential protein quantification.[28] Protein log2 ratios are expressed as standardized variables in units of standard deviation according to their estimated variances. Differences in protein abundance were estimated by comparing group $Zq$ medians ($\Delta Zq$).

### Coordinated Protein Alterations in BAV-TAA and TAV-TAA: Systems Biology Analysis

Coordinated protein changes and functional categories were analyzed using the Systems Biology Triangle (SBT) algorithm[29] which identifies changes in biological processes


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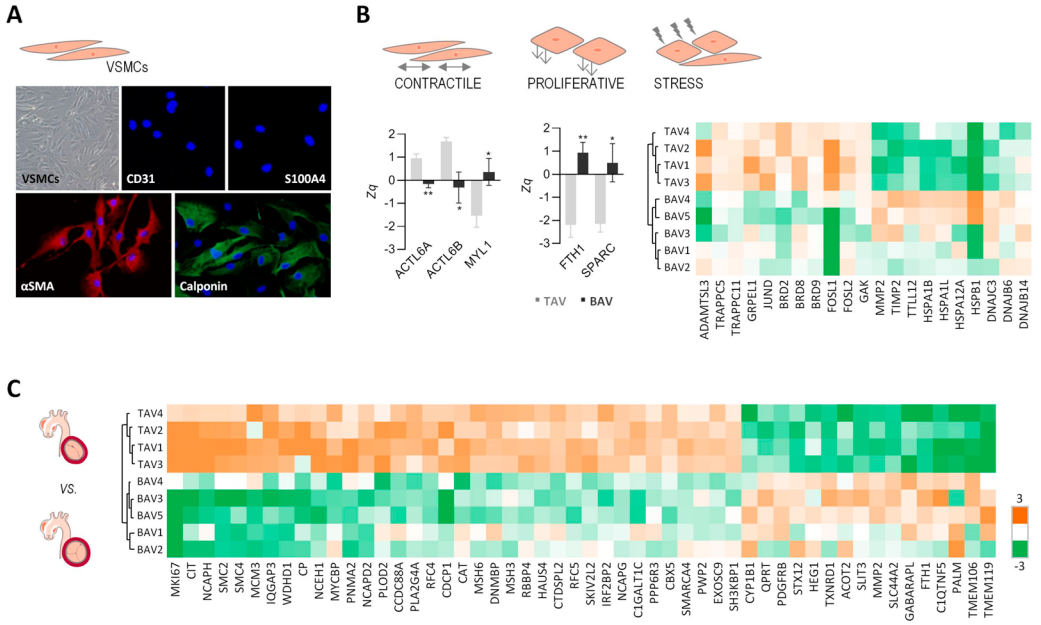


**Figure 1.** Stress phenotype and altered DNA replication and repair machinery in thoracic aortic aneurysm associated with bicuspid aortic valve. Aortic VSMCs from TAA patients were analyzed by quantitative high-throughput proteomics, and protein-abundant changes are compiled here. (A) VSMC characterization was performed by staining with $\alpha$SMA and calponin and negative staining for endothelial cells (CD31) and fibroblasts (S100A4). (B) Contractile, proliferative, and stress phenotypes were found in BAV-TAA and TAV-TAA VSMCs, but a differential stress phenotype was evident in BAV-TAA cells. (C) The differential protein profile in BAV-TAA versus TAV-TAA VSMCs includes 52 proteins with $nP \geq 3$, $-2 \geq \Delta Zq \geq 2$ and $p$ value $\leq 0.01$. Individual examination of these proteins indicates a deregulation in DNA machinery in BAV-TAA patients. Color scale: $\Delta Zq = -3$ (green), through 0 (white), to +3 (orange). * $p$ value $\leq 0.05$, ** $p$ value $< 0.01$. Heatmap proteins: $p$ value $\leq 0.05$.

far beyond individual protein responses and assigns a $Z$ score ($Zc$) to each category. Differences in biological processes were evaluated by measuring the differences in median $Zc$ values ($\Delta Zc$) between the BAV-TAA and the TAV-TAA groups, and functional categories with at least 10 proteins were further considered.

## Protein Mapping of Thoracic Aortic Aneurysms within the Cellular Structure

Specific cellular locations of the identified proteins were obtained from the subcellular map of the Human Protein Atlas (Jan 26, 2021; 18 592 entries; https://www.proteinatlas.org/). Subcellular locations included the nucleus (nucleolus, nucleoplasm, and nuclear membrane), cytoplasm (cytosol, actin filaments, intermediate filaments, microtubules, centrosome, and mitochondria), and secretory proteins (endoplasmic reticulum, Golgi apparatus, plasma membrane, secretome, and vesicles).

## 8-Hydroxy-2′-deoxyguanosine Quantitation of Oxidative DNA Damage in TAA

8-Hydroxy-2′-deoxyguanosine (8-OHdG) was measured in DNA extracted from VSMCs (TAV-TAA, $n$ = 4; BAV-TAA, $n$ = 5) and in urine samples (TAV-TAA, $n$ = 13; BAV-TAA, $n$ = 30), as urine is the best non-invasive matrix to assess 8-OHdG due to the stability of the compound in urine and because oxidative DNA damage is reflected in its excretion.[30] DNA was extracted from VSMCs with the MasterPure Complete DNA and RNA Purification Kit (Epicenter, Biosearch Technologies). Denatured DNA was digested into nucleotides by incubation for 30 min at 37 °C with nuclease P1 (New England Biolabs). Nucleotides were dephosphorylated by adjusting the sample pH to 7.5−8.5 and incubating at 37 °C for 10 min with FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher). 8-OHdG in extracted DNA from VSMC and urine samples was determined by ELISA (Abcam ab201734, Cambridge, UK).

## Cell Cycle Profiling

A total of $1.5 \times 10^5$ cells (TAV-TAA, $n$ = 5; BAV-TAA , $n$ = 8) were fixed in 70% ethanol overnight at −20 °C. Following two washes in PBS, a solution containing 0.5 mg/mL propidium iodide, 0.01% IGEPAL, and 0.1 mg/mL RNase A was added and incubated for 3 h at 4 °C. The percentage of cells in apoptosis and G0-G1, S, or G2-M phases was calculated using a BD FACSCanto II flow cytometer (BD Biosciences) and analyzed by FlowJo version X.0.7 software.

## Plasma Analysis of VSMC Secreted Proteins

Proteins in the secretome (secreted proteins) with $p$ value $\leq$ 0.05 were further investigated by inspecting the SecretomeP 2.0, Human Protein Atlas, and Uniprot databases to identify those extracellularly secreted. These altered VSMC proteins in their soluble form were quantified by enzyme-linked immunosorbent assay (ELISA) in plasma samples from TAA patients in an extended cohort: TAV-TAA ($n$ = 12) and BAV-TAA ($n$ = 28) (see Table S1 for ELISA details).

## Statistical Analysis

Values with $p$ value $\leq$ 0.05 were considered statistically significant. Continuous variables were reported as mean ± standard deviation, and categorical variables were expressed as total number and percentages. Normality was evaluated with the Shapiro−Wilk test, and the two-group comparisons were analyzed with $t$ test or Mann−Whitney test accordingly. The association of confounding variables with 8-OHdG concentration, percentage of cells in apoptosis, G0-G1, S, or G2-M phases, and plasma proteins were investigated by hypothesis testing. Then, we performed logistic regression models, adjusted for the statistically significant covariates, to correct


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the observed differences. Data were analyzed with R (RStudio Software, 2022.02.0 + 443 version) and GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA).

## RESULTS

### Bicuspid Valve-Associated Aneurysm Shows Alterations in VSMC Stress Phenotype Proteins

We investigated the influence of a BAV on the proteome of the aortic VSMCs in TAA patients to identify biological processes and protein signatures which make the aneurysmal aorta different if associated with a BAV. Figure 1A shows VSMC primary cultures from aortic tissue. The demographic and comorbidity characteristics of the clinical cohort are presented in Table 1 (VSMCs, untargeted MS). Clinical groups did not differ significantly in age or sex profiles. The prevalence rates of diabetes, hypertension, dyslipidemia, smoking or left ventricular ejection fraction do not differ either. The whole cellular proteome identified more than 8000 proteins (Table S2).

Three VSMC phenotypes have been identified in ascending aortic aneurysm: contractile, proliferating, and stress.[31] Here, we have mainly identified with differential abundance in BAV-TAA patients stress-related proteins (21 proteins, Figure 1B, Table S3). Lower levels were found for ADAMTS-like protein 3 (ADAMTSL3), trafficking protein particle complex subunit 5 and subunit 11 (TRAPPC5 and TRAPPC11), GrpE protein homologue 1, mitochondrial (GRPEL1), transcription factor jun-D (JUND), bromodomain-containing protein 2, 8, and 9 (BRD2, BRD8, and BRD9), fos-related antigen 1 and 2 (FOSL1 and FOSL2), and cyclin-G-associated kinase (GAK). Higher levels were found for matrix metalloproteinase-2 (MMP2), metalloproteinase inhibitor 2 (TIMP2), tubulin-tyrosine ligase-like protein 12 (TTLL12), heat shock 70 kDa protein 1B, 1 like, and 12A (HSPA1B, HSPA1L, HSPA12A), heat shock protein beta-1 (HSPB1), DnaJ homologue subfamily C member 3 (DNAJC3), and DnaJ homologue subfamily B member 6 and 14 (DNAJB6 and DNAJB14). These changes, based on the known protein functions, suggest that the stress phenotype in the context of a BAV is associated with changes in the ECM, protein folding and aggregation, and cell death.

### DNA Machinery Alterations in Bicuspid Aortic Valve-Associated Aneurysm

Analysis of the most altered proteins revealed a panel of 52 proteins mainly related with the DNA replication and repair machinery (16 with elevated abundance and 36 with reduced abundance) (Figure 1C, Table S4). The panel includes DNA mismatch repair (MMR) proteins Msh3 and Msh6 (MSH3 and MSH6), replication factor C subunits 4 and 5 (RFC4 and RFC5), DNA replication licensing factor MCM3 (MCM3), condensin I complex constituents structural maintenance of chromosomes protein 2 and 4 (SMC2 and SMC4), and condensin complex subunits 1, 2, and 3 (NCAPD2, NCAPH, and NCAPG). Besides, other related proteins that were also varied were MSH2, PMS1 protein homologue 1 (PMS1), polymerase $\delta$, and RFC1, RFC2, and RFC3 (Table S2), pointing all together to activation of the DNA damage response in BAV-TAA VSMCs.[32] DNA damage and cell cycle arrest is also evidenced in other subsets of the identified proteins. The repair of large-scale DNA damage requires coordination between cell cycle arrest and activation of the damage repair system through a process involving the serine/threonine-protein kinases ATR and ATM (ATR and ATM).[33]

**Table 1. Patients' Characteristics of VSMC, Urine, and Plasma Cohorts**[a]

| | untargeted MS | | | DNA oxidative damage | | | | | | cell cycle profiling | | | targeted quantification | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | VSMCs | | | VSMCs | | | urine | | | VSMCs | | | plasma | | |
| | TAV-TAA ($n$ = 4) | BAV-TAA ($n$ = 5) | BAV-TAA vs TAV-TAA | TAV-TAA ($n$ = 4) | BAV-TAA ($n$ = 5) | BAV-TAA vs TAV-TAA | TAV-TAA ($n$ = 13) | BAV-TAA ($n$ = 30) | BAV-TAA vs TAV-TAA | TAV-TAA ($n$ = 5) | BAV-TAA ($n$ = 8) | BAV-TAA vs TAV-TAA | TAV-TAA ($n$ = 12) | BAV-TAA ($n$ = 28) | BAV-TAA vs TAV-TAA |
| age (years) | 65 ± 11 | 65 ± 6 | 0.9603 | 63 ± 18 | 57 ± 18 | 0.7143 | 65 ± 12 | 60 ± 13 | 0.2043 | 68 ± 7 | 56 ± 13 | 0.0249 | 66 ± 13 | 60 ± 12 | 0.1166 |
| male sex | 3 (75) | 3 (60) | >0.9999 | 3 (75) | 3 (60) | >0.9999 | 10 (77) | 19 (63) | 0.491 | 3 (60) | 5 (62.5) | >0.9999 | 9 (75) | 17 (61) | 0.4844 |
| diabetes | 1 (25) | 1 (20) | >0.9999 | 1 (25) | 0 (0) | 0.4444 | 3 (23) | 3 (10) | 0.3455 | 1 (20) | 0 (0) | 0.3846 | 3 (25) | 3 (11) | 0.3407 |
| HTN | 3 (75) | 3 (60) | >0.9999 | 3 (75) | 3 (60) | >0.9999 | 11 (85) | 16 (53) | 0.0855 | 4 (80) | 1 (12.5) | 0.0319 | 10 (83) | 15 (54) | 0.1523 |
| DSL | 2 (50) | 1 (20) | 0.5238 | 1 (25) | 2 (40) | >0.9999 | 6 (46) | 11 (37) | 0.7357 | 1 (20) | 2 (25) | >0.9999 | 6 (50) | 10 (36) | 0.4898 |
| smoking | 0 (0) | 0 (0) | >0.9999 | 0 (0) | 1 (20) | >0.9999 | 3 (23) | 8 (27) | >0.9999 | 0 (0) | 4 (50) | 0.1939 | 3 (25) | 8 (29) | >0.9999 |
| LVEF | 0 (0) | 0 (0) | >0.9999 | 0 (0) | 1 (20) | >0.9999 | 2 (15) | 4 (13) | >0.9999 | 1 (20) | 0 (0) | 0.3846 | 2 (17) | 4 (14) | >0.9999 |
| aortic diameter (mm) | 51 ± 2 | 53 ± 2 | 0.2063 | 48 ± 5 | 54 ± 9 | 0.4444 | 55 ± 7 | 51 ± 5 | 0.0189 | 56 ± 3 | 48 ± 3 | 0.0007 | 54 ± 7 | 51 ± 5 | 0.0734 |
| AS | 2 (50) | 3 (60) | >0.9999 | 3 (75) | 3 (60) | >0.9999 | 2 (15) | 21 (70) | 0.002 | 1 (20) | 6 (75) | 0.1026 | 2 (17) | 20 (71) | 0.0021 |
| AI | 2 (50) | 0 (0) | 0.1667 | 1 (25) | 1 (20) | >0.9999 | 9 (69) | 9 (30) | 0.0225 | 5 (100) | 3 (37.5) | 0.0808 | 8 (67) | 8 (29) | 0.0367 |

[a]Values are expressed as number (%) for categorical variables and mean ± standard deviation for continuous variables. $p$ values were estimated by nonparametric test (Mann−Whitney). TAV: tricuspid aortic valve. BAV: bicuspid aortic valve. TAA: thoracic aortic aneurysm. HTN: hypertension. DSL: dyslipidemia. LVEF: left ventricular ejection fraction. AS: aortic stenosis. AI: aortic insufficiency.



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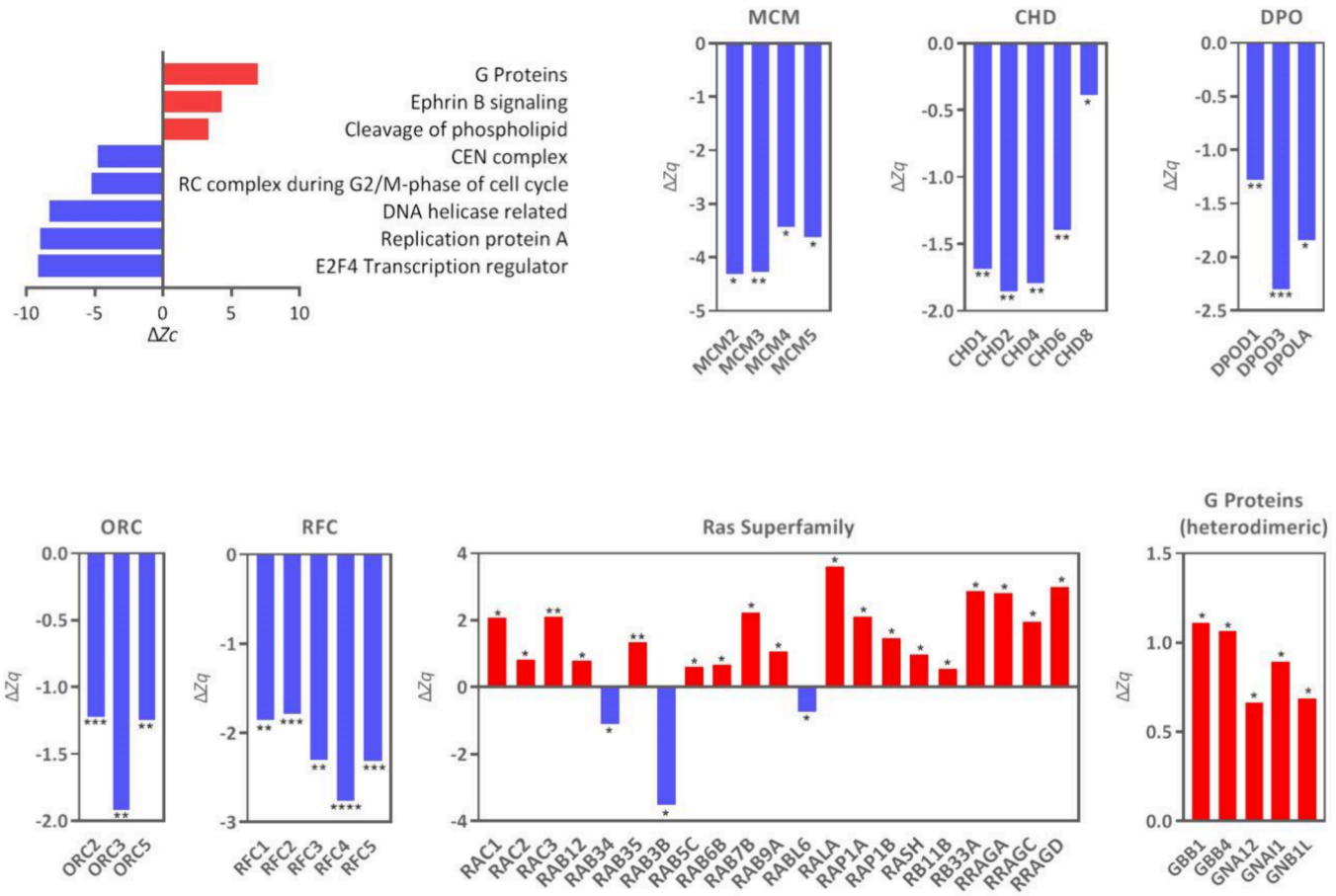


**Figure 2.** DNA replication machinery and signal transduction are altered biological processes in BAV-associated thoracic aortic aneurysm. Coordinated protein analysis was conducted with the SBT algorithm to identify biological processes altered beyond individual protein changes. Processes showing an alteration of $-3.0 > \Delta Zc > 3.0$, homogeneous intragroup behavior, and $p$ value < 0.001 were considered the most relevant altered pathways in TAA associated with BAV. The top eight altered processes are represented together with the quantitative difference between BAV-TAA and TAV-TAA patients (bar chart $\Delta Zc$). These eight processes indicate impairment of the DNA replication machinery in BAV-TAA VSMCs accompanied by an increase in G-protein-mediated signal transduction. The proteins driving these process changes were further investigated and grouped into families. The figure shows the outstanding protein abundance changes from the most representative protein families for BAV-TAA vs TAV-TAA. BAV-TAA VSMCs showed relatively lowered levels of DNA-related proteins, including MCM (minichromosome maintenance), CHD (chromodomain helicase DNA binding), DPO (DNA polymerase), ORC (origin recognition complex), and RFC (replication factor C). In contrast, several G proteins, including Ras-superfamily proteins and heterodimeric G proteins, showed significantly increased abundance. * $p$ value $\leq$ 0.05, ** $p$ value < 0.01; *** $p$ value < 0.001; **** $p$ value < 0.0001.

The content of ATM did not differ in VSMCs from TAA patients with BAV, but ATR was found to be significantly decreased, a protein which ensures the fidelity DNA replication during S phase. We also observed a generalized decrease in histones in BAV-TAA patients, with HIST1H2BM, HIST3H3, HIST1H2AD, and HIST2H3A showing the largest differences. Moreover, we also showed decreased abundance in anaphase-promoting complex subunit 1 (ANAPC1) and p53 and DNA damage-regulated protein 1 (PDRG1), both involved in cell cycle progression (Table S2). Coordinated protein responses were assessed, indicating again activation of the DNA machinery in BAV-TAA VSMCs accompanied by an increase in G-protein-mediated signal transduction type (Figure 2 and Table S5). The proteins driving these processes were further investigated and grouped into families. Relatively lowered levels of DNA and cell cycle progression-related proteins were observed, including MCM (minichromosome maintenance), CHD (chromodomain helicase DNA binding), DPO (DNA polymerase), ORC (origin recognition complex), and RFC (replication factor C). In contrast, several G proteins, including Ras-superfamily proteins and heterodimeric G proteins, showed significantly increased abundance.

## Oxidative Stress and DNA Oxidative Damage in Bicuspid Valve-Associated Aneurysm

The most altered proteins and functional categories here identified suggest an activation of the DNA damage response and cell cycle arrest in BAV-TAA patients. Further detailed analysis resulted in the identification of protein families involved in DNA repair via ATP processes, as the observed decreased expression of the CHD, ORC, and RFC families all of them ATP dependent. The limited availability of ATP is characteristic of oxidative stress conditions within aneurysm. For confirmation, we evaluated the identified functional categories involving biological redox processes, e.g. “oxidative stress response”, “synthesis and metabolism of ROS”, and “NRF2-mediated oxidative stress response”, and particularly the individual protein variations composing those biological processes. Significant abundance variation in this VSMC oxidative proteome (57 proteins) confirmed the higher ROS environment in bicuspid aortopathy compared to that of TAV-


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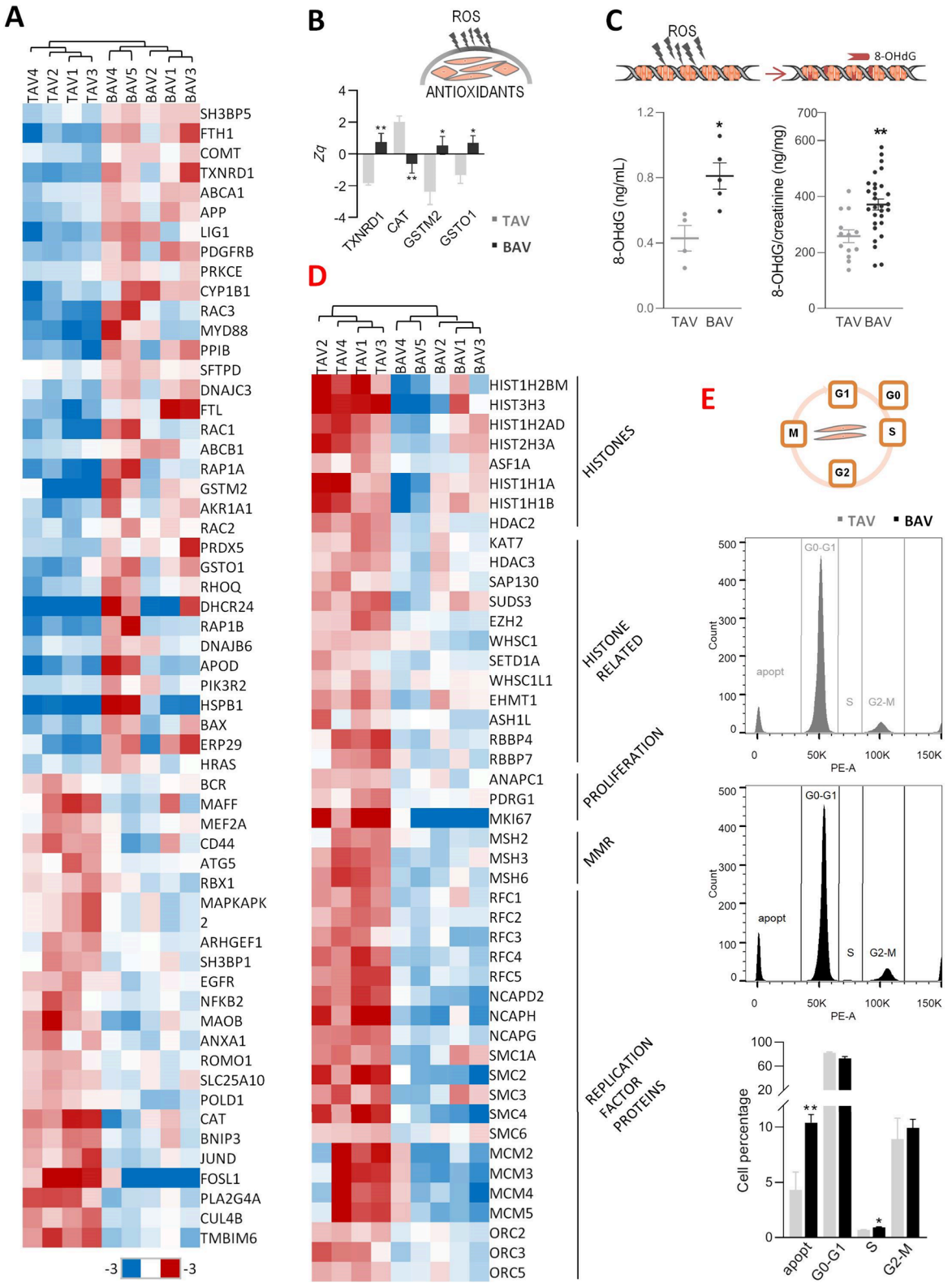


**Figure 3.** Enhanced oxidative stress environment, DNA oxidative damage, and cell cycle arrest in BAV-TAA patients. The individual protein and coordinated protein alterations in BAV-TAA patients suggest elevated oxidative stress driven by the significantly altered VSMC proteins listed. (A) Heatmap of proteins related to oxidative stress identified by coordinated response analysis. (B) Altered proteins related to antioxidant defense between BAV-TAA and TAV-TAA VSMCs. (C) 8-Hydroxy-2′-deoxyguanosine (8-OHdG) quantification from DNA extracted from VSMCs (left chart) and urine samples (right chart) in BAV-TAA patients. (D) Heatmap of proteins related to cell cycle progression: histones, histone-related proteins, proliferation markers, mismatch repair (MMR) proteins, and replication factor proteins. (E) Cell cycle profiling in TAV-TAA patients (gray histogram) and BAV-TAA patients (black histogram); cell percentage in apoptosis, G0-G1, S, and G2-M phases is shown in the bar chart. Color scale: $\Delta Zq = -3$ (blue), through 0 (white), to +3 (red). * $p$ value $\leq 0.05$, ** $p$ value $< 0.01$.



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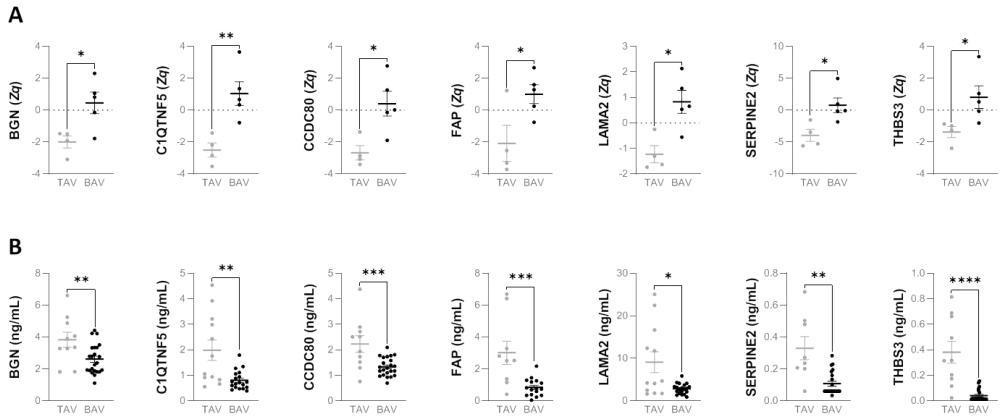


**Figure 4.** Transferable secreted proteins in VSMCs to plasma. VSMC proteins showing altered abundance in the proteomics analysis and identified as extracellularly secreted were quantified and further investigated in their soluble form in human plasma from TAV-TAA and BAV-TAA patients by ELISA. (A) Changes in protein abundance in VSMCs for C1QTNF5, LAMA2, FAP, BGN, CCDC80, SERPINE2, and THBS3 showing an increase in BAV-TAA. (B) Respective plasma alteration showing significantly diminished levels in plasma from TAA patients. Protein differences between clinical groups were analyzed by *t* test or Mann−Whitney test according to the results of the normality test. * $p$ value ≤ 0.05, ** $p$ value < 0.01, *** $p$ value < 0.001, **** $p$ value < 0.0001.

TAA (Figure 3A). VSMCs from TAA patients with a BAV or TAV showed similar levels in the content of the antioxidants superoxide dismutase, glutathione reductase, xanthine oxidase, and glutathione peroxidase. However, VSMCs from BAV-TAA patients showed increased levels of thioredoxin reductase 1 (TXNRD1), glutathione *S*-transferase Mu 2 (GSTM2), and glutathione *S*-transferase omega-1 (GSTO1) and decreased levels of catalase (CAT) (Figure 3B).

To evaluate if the elevated oxidative stress is extended to the DNA causing DNA oxidation, we quantified the DNA oxidative damage in DNA extracted from VSMCs and in urine by measuring 8-hydroxy-2′-deoxy guanosine (8-OHdG), an established marker of oxidative stress produced by the action on DNA of reactive oxygen and nitrogen species. Table 1 shows the clinical characteristics of TAA patients included in these confirmation analyses classified according to valve type (VSMCs and urine; DNA oxidative damage). No significant differences between groups were observed in clinical variables except for aortic stenosis and aortic insufficiency, which were considered as comorbidities. 8-OHdG was elevated in the VSMC DNA ($p$ value = 0.0159) and urine ($p$ value = 0.002) of BAV-TAA patients independently of the comorbidities, thus confirming a higher level of DNA damage in TAA patients with BAV due to an extended oxidative stress situation (Figure 3C).

### Cell Cycle Profiling in Bicuspid Valve-Associated Aneurysm

The observed DNA damage in BAV-TAA patients activates a DNA damage response. As previously detailed, this is reflected by decreased expression of MMR and replication factor proteins (e.g., ORC, RCF, MCM) and histones (Figure 3D), which are associated with cell cycle progression into S and G2 phases,[34] suggesting alterations in the cell cycle. Cell cycle profiling was evaluated in VSMCs from TAA patients. Table 1 (VSMCs; cell cycle profiling) shows the clinical characteristics of the TAA patients. Figure 3E shows significantly increased S-phase arrest in BAV-TAA patients, confirming activation of the DNA damage response regardless of the comorbidities. The enforcement of the corresponding checkpoint and the consequent cell cycle arrest aligns with increased damage-induced apoptosis in the BAV group.

### VSMC Protein Changes Observed Are Transferable to Plasma

A total of 21 proteins were found differentially expressed in VSMCs, which may be secreted extracellularly (Table S1). The concentrations of these proteins were estimated in an extended plasma cohort (Table 1; plasma; targeted quantification). No significant differences between groups were observed in clinical variables except for aortic stenosis and aortic insufficiency, which were considered as comorbidities. Complement C1q tumor necrosis factor-related protein 5 (C1QTNF5) ($p$ value = 0.0073), laminin subunit alpha 2 (LAMA2) ($p$ value = 0.0359), prolyl endopeptidase FAP (FAP) ($p$ value = 0.0009), biglycan (BGN) ($p$ value = 0.0071), coiled-coil domain-containing protein 80 (CCDC80) ($p$ value = 0.0007), thrombospondin-3 (THBS3) ($p$ value < 0.0001), and glia-derived nexin (SERPINE2) ($p$ value = 0.0001) showed significantly diminished levels in plasma from TAA patients with a BAV (Figure 4).

## DISCUSSION

Studying the molecular landscape of each TAA form, TAV- and BAV-associated aneurysm, is crucial to identify therapeutic targets that could lead to drug development as an alternative to prophylactic surgery and to identify in situ changes which may be reflected in blood for monitoring.

### Stress Phenotype, Protein Homeostasis, and Oxidative Stress in BAV-Thoracic Aortic Aneurysm

Aortic valve type determines a different stress in the ascending aorta in response to TAA, and abnormal wall shear stress is a hallmark in BAV patients.[35] Alterations in 44 flow-associated gene expressions were identified in BAV aortas (intima/media biopsies) compared to TAV aortas independently of dilatation, including *CEBPD* and *PKD2*.[36] PKD2 is a sensor of shear stress whose differential expression in BAV aorta had been hypothetically attributed to VSMCs in the referred study. Despite the fact that mRNA expression usually correlates poorly with protein expression, our data confirm, on one hand, their significant variation at the protein level in aneurysmal patients and, on the other hand, the role of VSMCs in this protein alteration (Table S2). Differently, 20 out of the 44 flow-associated reported genes were here detected but without


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significant variation in association to valve type. This may be due to the translation mechanism itself, but it may also evidence that not all mechanical forces acting on the endothelium affect the media and its main cellular component; in addition, there are molecular alterations at the vessel that cannot be attributed to mechanical forces.

Related to mechanical stress, a study focusing on mechanical-stress-induced ER stress highlights that ER stress participates in the onset and formation of TAA.[37] We found here an increase in the ER stress marker DnaJ homologue subfamily C member 3 (DNAJC3) and an increased abundance in ER proteins in BAV-TAA VSMCs in agreement with increased levels of ER stress (Table S6). This points to ER stress playing a key role in BAV-TAA aneurysms.

We observed here a different stress response in BAV-TAA patients. Our data show that VSMCs from BAV-TAA patients have elevated levels of stress-response proteins, mainly affecting protein homeostasis. This was the case for the HSP70 family, HSPA1B and HSPA1L, which ensures correct protein folding and clearance of defective proteins. The combination of HSPB8 and its chaperone DNAJB14 also increased in BAV-TAA patients, working to promote protein folding, preventing protein aggregation, and degrading misfolded proteins. One study focusing on amyloidosis comparing aortic tissue from TAV and BAV aneurysmal patients concluded that BAV patients did not present significant protein aggregation while TAV-TAA patients do, suggesting a differential mechanism operating in both TAA forms.[38] Our results suggest that this differential mechanism may be mediated by VSMCs since we find an increment in proteins avoiding this protein aggregation in BAV-TAA. Protein misfolding could be due to the enhanced oxidative stress present in BAV. We observed elevated VSMC expression of the antioxidant enzymes glutathione *S*-transferase (GSTO1, GSTM2) and thioredoxin reductase and significantly reduced expression of catalase. Catalase plays a key role in protecting cells from ROS-mediated damage, increased catalase expression in VSMCs protects against abdominal aortic aneurysm,[39] while catalase deficiency is linked to oxidative stress and age-related disease.[40] The recorded alterations in BAV-TAA patients suggest a chain of events in which diminished catalase levels in VSMCs are unable to neutralize ROS.

Increased oxidative stress also triggers cell signaling pathway activation with G proteins playing a key role.[41,42] Both, small GTPases and heterodimeric G proteins, showed increased abundance in the VSMCs of BAV-TAA patients vs TAV-TAA ones. The increment of the heterodimeric G proteins (GNB1, GNB4, GNAI1, and GNA12) suggests an imbalance in GTPase activity. Similarly, we detected a general increase of Ras, Rho, Rap, and Rab members of the Ras superfamily of small G proteins. Overactivation of Ras-family GTPases has been linked to disease in the cardiovascular system, and particularly, Rho GTPases (RAC1, RAC2, and RAC3) relate with ROS production during aortic aneurysm formation and mediate VSMC migration and accumulation in arterial walls.[43,44]

## DNA Damage, Cell Cycle Arrest, and Cell Death

The higher DNA oxidation observed here in BAV-TAA patients, in DNA extracted from VSMCs, and in urine also confirmed the higher oxidative stress environment causing DNA damage, evidenced by the individual and coordinated protein variations identified and also supporting previous data.[45] Effective activation of the oxidative DNA damage response appears to be one of the key mechanisms in TAA patients associated with BAV. Specifically, we found that VSMCs are arrested in S phase, which is associated with DNA replication. For this purpose, histones are synthesized during S phase, and in agreement with the observed S phase arrest, we found a generalized decrease in histones and histone-related proteins in BAV-TAA patients. A decrease in histone availability compromises DNA copying and exposes the DNA to damage.[46] In this regard, we observed that markers of cell cycle progression are decreased in BAV. ANAPC1 is the largest subunit of the E3 ubiquitin ligase APC and orchestrates cell cycle progression into S and G2-M phases.[47] Studies in tumor cell lines highlight that PDRG1 is also associated with DNA damage and cellular progress, since its silencing promotes programmed cell death and cycle arrest.[48] We also observed increased abundance of proteins associated with cell death in VSMCs from BAV-TAA patients: HSPB1, which works together with HSPB8, and CRYAB contribute to cell death regulation; HSPA12A is involved in cellular senescence, and DNAJC3 and HSPA1B are involved in apoptosis. This is in line with the reported diminished number of cells in BAV-TAA[16] and the observed increase in damage-induced apoptosis found here. VSMC loss is more pronounced in BAV compared to TAV-TAA patients, likely promoting faster aortic degradation and a reduced capacity of tissue repair.

## Plasma Reflects Protein Variations Locally Identified in VSMCs

Our VSMC results confirm the existence of different mechanisms operating in thoracic aortic dilatation in TAV and BAV subjects as do the soluble forms of the proteins found in plasma. We have found increased levels in VSMCs and decreased plasma concentrations in BAV-TAA subjects for BGN, C1QTNF5, CCDC80, FAP, LAMA2, SERPINE2, and THBS3. In tissue, an increase of BGN acts as a danger signal of stress.[49] In physiological conditions, BGN stays in the matrix, but under inflammatory conditions, it is released into the blood,[50] similarly to CCDC80, which also enhances its secretion in inflammatory conditions.[51] These increased levels of the soluble forms of BGN and CCDC80 in TAV-TAA plasma point to an increased inflammatory state in TAV, in line with previous proteomics analysis in aortic tissue, concluding that TAV aneurysms are inflammation driven.[17] FAP is a negative regulator of cardiac repair whose inhibition has been proved to enhance cardiac repair. An increased in the FAP level in cardiac cells together with a decrease in the concentration in plasma have been found after cardiac injury.[52] These trends (cell increased, blood decreased) are what we evidence in BAV-TAA, pointing again to the poor repair capacity of the BAV aortic wall.

The differential stress of BAV and TAV aneurismal arteries has been described here at individual and coordinated VSMC protein levels. The plasma alterations are, again, a reflection of this differential stress. LAMA2 has been found overexpressed in hyperplasia, and hyperplastic cellular remodeling is thought to be an adaptative response to wall stress, which is a known feature of BAV arteries.[53,54] We also identified a reflection of ER response to stress. In this line, we found increased VSMC levels of FAP and THSB3. On one hand, FAP expression has been associated with ER stress response.[55] On the other hand, THSB3 has been found to be increased in cardiac diseases in


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relation to ER stress and aiming to diminish misfolding protein accumulation.[56] In plasma, the THSB3 concentration is, on the contrary, diminished in acute myocardial infarction subjects. This is similar to what we found here for BAV-TAA subjects, confirming the enhanced stress and the deregulation of protein homeostasis. SERPINE2 (also GDN or Protease nexin-1 (PN-1)) has a protective role to maintain or restore homeostasis during damage or stress.[57] During aneurysms, intense remodeling occurs mediated by an imbalance in favor of proteolytic degradation of the extracellular matrix.[58] SERPINE2 has been shown to be overexpressed in human VSMCs from TAA patients mediated by an epigenetic reprogramming of SMAD2 activation maintained in the cells. This overexpression modulates proteinases activities, favoring their chronic progressive action. They suggested all of this to be in part due to changes in mechanotransduction.[59] We found SERPINE2 increased in VSMCs and decreased in plasma in BAV-TAA, which could be related with the marked VSMC G proteins imbalance found and with the known glycosaminoglycan retention of SERPINE2 on the cell surface, respectively.

Oxidative stress was also found to be significantly altered, as a hallmark of BAV-TAA. In BAV patients, we observed an increase in oxidative stress, associated DNA damage, and DNA damage response with cell cycle arrest, in line with repair processes being ATP dependent. In conditions of low ATP cellular levels, the AMPK alpha signaling pathway is activated by an overexpression of C1QTNF5 (also CTRP5). This is a compensatory mechanism aiming to increase the ATP level.[60] We found decreased levels of C1QTNF5 in BAV-TAA plasma samples. Previous research shows different plasma trends in different cardiovascular cohorts,[61,62] and here, we show again a contrary trend to that observed in VSMCs, suggesting a primary local action.

## Clinical Perspectives for BAV Patients

The results presented here show how the biopathology of TAA differs according to aortic valve type, revealing biological pathways and individual proteins that could form the basis of targeted clinical research and highlighting the value of specific treatments for TAA patients with a BAV vs a TAV (Figure 5). Changes in protein homeostasis, cell cycle arrest, and limited ATP availability are here reported to be more prominent in BAV-TAA patients and have been linked to aging.[63,64] Patients' groups included do not show a significant difference in age; thus, these findings point to a prematurely aged aortic wall for BAV patients. Our results complement previous research that found shortened telomeres in SMCs from BAV-TAA subjects, concluding, as we do, that aging is a relevant factor in BAV-TAAs.[16,45] A recent study demonstrated that aging modulates the aortic proteome of TAA and suggested that the treatment of these patients should be modified according to age.[65] Our results may extend this recommendation to BAV patients, who should perhaps be treated as older than their chronological age.

The evidenced differential mechanisms here in BAV- and TAV-associated TAAs point to the need for tailored therapies for both TAA forms. Nowadays, there are no established treatments to slow aortic dilatation and prevent dissection; first-line drug therapies are undefined, and by BAV patients are regarded as a target group with particular treatment needs. Due to their capacity to decrease hemodynamic force across elastin-contractile units, beta-blockers are the long-standing therapeutic recommendation to slow dilatation and reduce dissection risk. Beta-blockers and angiotensin-converting enzyme inhibitors could also help to reduce ROS-induced DNA damage,[66] thus being particularly indicated for BAV-TAA patients according to our results. Statins have been reported to inhibit TAA and dissection through different mechanisms.[67,68] Statins can upregulate specific small G proteins that protect against phenotype switching[8] and reduce DNA oxidative damage,[66] potentially providing benefits for BAV-TAA patients. Statins also reverse the increase in minichromosome maintenance (MCM) proteins in aortic disease.[69] In our cohort, the levels of MCM2, MCM3, MCM4, and MCM5 were diminished in VSMCs from TAA patients with a BAV compared to those with a TAV. Thus, statin therapy may prove to be contraindicated for BAV-TAA patients.

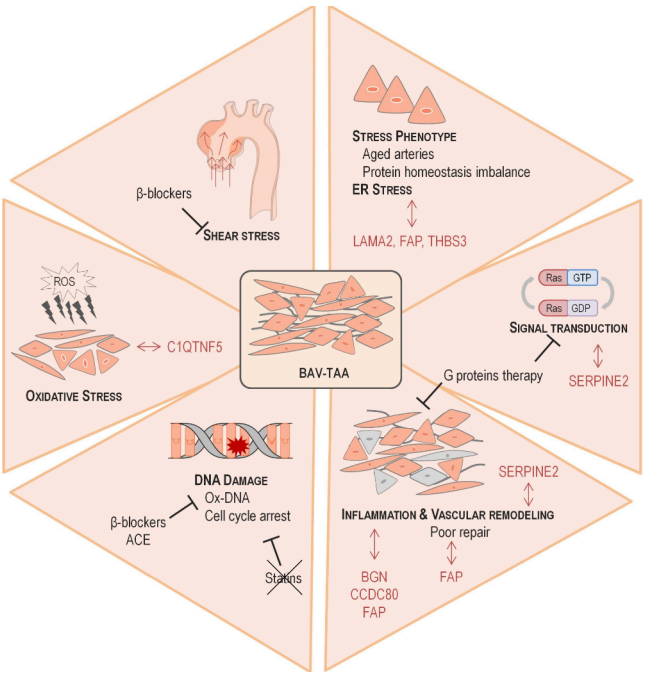


**Figure 5.** Bicuspid valve-associated thoracic aortic aneurysm is driven by specific mechanisms and should be treated as a distinct clinical entity. Individual and coordinated protein changes in TAA patients with BAV provide evidence for alterations to six biological pathways that could be potential therapeutic targets. The aortic wall is subject to greater shear stress in BAV-TAA than in TAV-TAA. This is accompanied by exposure to more severe oxidative stress, resulting in enhanced DNA oxidative damage. DNA damage response is one of the most evident mechanisms operating in BAV-TAA according to our analysis of human VSMCs. Treatments used in other cardiovascular conditions limit DNA damage and thus may help in the management of BAV-TAA patients. Plasma analysis of the soluble form of altered VSMC proteins confirmed an increased stress phenotype (LAMA2, FAP, and THBS3) and increased oxidative stress (C1QTNF5) in BAV-TAA patients. Vascular remodeling is a hallmark of aneurysm generally. THSB3, FAP, BGN, CCDC80, and SERPINE 2 evidence the differential vascular remodeling in the aorta during TAA, pointing to inflammation in TAV-TAA and to protein homeostasis and diminished repair in BAV-TAA subjects. Our analysis reveals vascular remodeling, protein homeostasis, cell cycle arrest, and enhanced cell death, partly mediated by G proteins. Because G proteins are involved in so many processes, their use as therapeutic targets is challenging and requires further research.


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## CONCLUSIONS

The arterial wall of the nonsyndromic, nonfamilial TAA shows an enhanced DNA damage response in patients with BAV evidencing a stress phenotype, DNA oxidation, cell cycle arrest, protein homeostasis, and aging hallmarks, which ultimately supports BAV patients being considered as a discrete clinical group with specific needs and who may benefit from intensified or adapted treatments.

## STUDY LIMITATIONS

The main limitation of this study is the reduced number of aortic tissue samples, which may be justified by their human origin and the difficulty to isolate primary VSMCs from aortic tissue. Another limitation is that cell culture-based alterations cannot be fully excluded, but to minimize them, VSMCs analyzed in this study were at passage 1 and the time in culture was maintained as short as possible. These issues could be partially overcome by the use of high-throughput proteomics techniques and established workflows, the analyses of four independent patients' cohorts along the study, and the performance of functional analyses confirming the untargeted omics data. Pending further analysis in larger cohorts, the individual changes identified here reinforced by the shown coordinated protein behavior toward common mechanisms and further supported by DNA oxidation functional assays endorse and clarify previous findings and open new directions in setting a diagnosis and treatment specific for BAV-TAA patients.

## ASSOCIATED CONTENT

### Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[70] partner repository with the data set identifier PXD043062. Project Name: Aortic smooth muscle cells in bicuspid valve patients. Project accession: PXD043062.

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00649.

Extracellularly secreted proteins; total identified proteins; altered proteins associated with different VSMCs phenotype; most altered proteins; most consistently varied functional categories and their related proteins; subcellular altered proteins (RAR)

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### Author Contributions

‡A.M.-B. and M.M.-L.: These authors contributed equally as first authors.


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### Notes

The study was approved by the local Ethics Committee (PIC143-2016, PIC029-21) and adhered to the principles of the Declaration of Helsinki. All participants provided written informed consent before enrollment.
The authors declare no competing financial interest.

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