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ORIGINAL RESEARCH

Matrisome and Immune Pathways Contribute to Extreme Vascular Outcomes in Williams–Beuren Syndrome

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BACKGROUND: Supravalvar aortic stenosis (SVAS) is a characteristic feature of Williams–Beuren syndrome (WBS). Its severity varies: ~20% of people with Williams–Beuren syndrome have SVAS requiring surgical intervention, whereas ~35% have no appreciable SVAS. The remaining individuals have SVAS of intermediate severity. Little is known about genetic modifiers that contribute to this variability.

METHODS AND RESULTS: We performed genome sequencing on 473 individuals with Williams–Beuren syndrome and developed strategies for modifier discovery in this rare disease population. Approaches include extreme phenotyping and nonsynonymous variant prioritization, followed by gene set enrichment and pathway-level association tests. We next used GTEx v8 and proteomic data sets to verify expression of candidate modifiers in relevant tissues. Finally, we evaluated overlap between the genes/pathways identified here and those ascertained through larger aortic disease/trait genome-wide association studies. We show that SVAS severity in Williams–Beuren syndrome is associated with increased frequency of common and rarer variants in matrisome and immune pathways. Two implicated matrisome genes (*ACAN* and *LTBP4*) were uniquely expressed in the aorta. Many genes in the identified pathways were previously reported in genome-wide association studies for aneurysm, bicuspid aortic valve, or aortic size.

CONCLUSIONS: Smaller sample sizes in rare disease studies necessitate new approaches to detect modifiers. Our strategies identified variation in matrisome and immune pathways that are associated with SVAS severity. These findings suggest that, like other aortopathies, SVAS may be influenced by the balance of synthesis and degradation of matrisome proteins. Leveraging multiomic data and results from larger aorta-focused genome-wide association studies may accelerate modifier discovery for rare aortopathies like SVAS.

Key Words: adaptive/innate immune system ■ elastin (ELN) ■ extreme phenotype ■ pathway analysis ■ supravalvar aortic stenosis ■ Williams–Beuren syndrome

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CLINICAL PERSPECTIVE

What Is New?

- Variation in genes in several pathways, including matrisome and adaptive/innate pathways, is associated with supravalvar aortic stenosis severity in people with Williams–Beuren syndrome.
- Unbalanced expression of genes controlling extracellular matrix synthesis and degradation is common in aortopathies, including aneurysm and bicuspid aortic valve, suggesting overlapping mechanisms for supravalvar aortic stenosis and these conditions.

What Are the Clinical Implications?

- New methodologies enabling identification of genetic modifiers in rare conditions may improve risk stratification for newly diagnosed individuals and identify novel pathway-based targets for therapeutics.
- In rare diseases, where sample size is small, fine phenotyping, extreme phenotype cohorting, and pathway-based analyses are viable strategies for modifier discovery.
- Leveraging multiomics data and accumulated knowledge from larger aortopathy genome-wide association studies may accelerate discovery of targets and treatments for rare aortic diseases like supravalvar aortic stenosis.

Nonstandard Abbreviations and Acronyms

SVAS	supravalvar aortic stenosis
WBS	Williams–Beuren syndrome

Williams–Beuren syndrome (WBS, MIM # 194050), caused by deletion of 1.5 to 1.8 Mb on human 7q11.23, is a multisystem disorder characterized by distinctive facies, a typical neurodevelopmental profile, and cardiovascular disease.[1] It occurs in 1 of 7500 live births[2] and is de novo in almost all cases. The cardiovascular disease in WBS is primarily mediated by the deletion of elastin (*ELN*) from this region[3–7] and consists of large and medium artery stenosis in the setting of a more global decrease in arterial caliber. Supravalvar aortic stenosis (SVAS), which is the narrowing of the ascending aorta above the aortic valve, commonly complicates WBS.[8–10] It can be focal or may consist of a more gradual narrowing along a longer segment of the aortic arch. Although more than 95% of individuals with WBS share the same basic deletion on chromosome 7q11.23, their outcomes for focal SVAS vary: about 20% have clinically significant discrete SVAS requiring surgical intervention in infancy or childhood; in contrast, about 35% of individuals with WBS never develop significant discrete SVAS, although varying levels of long segment stenosis may be present.[9,11,12] It has been unclear what features (genetic or otherwise) predispose to these extreme outcomes.

The application of genome-wide association studies (GWAS) to the identification of modifiers for rare conditions such as WBS has been challenging because most existing GWAS methods were developed for studies with thousands of participants. These numbers are unattainable for most rare disease studies, including WBS. Likewise, techniques to improve power, such as paired expression quantitative trait loci analysis[13] of affected tissues, are challenging in difficult-to-access tissues such as the aorta. As such, existing studies have primarily focused on correlation of phenotype with variants within the disease-specific locus or region.[14,15] Therefore, alternative computational and analytic strategies are needed for the study of rare diseases using genome sequencing data.

To overcome this challenge and to identify modifiers contributing to SVAS severity, we devised a set of strategies centered on the question of whether those with extreme SVAS phenotypes exhibit a relative burden of nonsynonymous variants (hereafter variants) that are enriched in a small number of biological pathways. The concept of pathway enrichment, which has been widely used in mRNA expression studies,[16] has been recently incorporated into GWAS analysis.[11,17,18] For application in our smaller sample size WBS study, we aimed to increase power for discovery by prioritizing the most influential common and rarer variants—those with greater likelihood of a functional impact[19]—and variants with allele frequency (AF) differences between the extreme phenotype groups, thereby reducing the total number of variants to be considered for downstream pathway enrichment.[20,21] The pathways identified give us a bird's-eye view of the molecules and processes that synergize to influence disease outcomes for SVAS.

Once pathways are identified, we sought additional evidence to confirm the relevance of the candidates to disease by examining tissue-specific expression of the genes using public data sets. Then, by harnessing existing GWAS data on common aortic conditions like aneurysm,[22–28] bicuspid aortic valve,[29,30] calcific valve stenosis,[31–33] and aortic size,[34–37] we examine overlap between the modifier pathways discovered for SVAS and these more commonly studied conditions. Of particular interest is the notion of an imbalance between extracellular matrix accumulation and destruction at the hands of immune system players in a host of aortic conditions.[38–41] Such synergies should allow future investigators to drill down further into the pathways uncovered by our methods to determine how they affect the aorta.

METHODS

Data Availability

Variant data are made available as part of the data supplement (Data S1-S3).

Consent

All participants alive at the time of enrollment or their caregivers signed informed consent forms to participate in research that included genome evaluation. One hundred and eighty participants signed consent approved by the National Institutes of Health (NIH) Institutional Review Board (NCT02706639), 197 signed consent approved by the Reno Institutional Review Board of the University of Nevada, 20 signed consent approved by the University of Toronto Health Sciences Research Ethics Board (those 217 were shared under the umbrella of the Nevada-Toronto collaboration), 10 signed consent approved by the Boston Children's Hospital Institutional Review Board, and 64 consented to participate in the Telethon Biobank in Italy and were approved by Fondazione IRCCS Casa Sollievo della Sofferenza Ethics Board. Two additional NIH samples were derived from tissue donated after death and were considered exempt. The data were analyzed under the NIH-approved protocol.

Sequencing and Quality Assessment

See Supplemental Methods for sequencing and sample quality details. Briefly, samples were evaluated for relatedness and genomic sex was compared with family-reported sex. Overall genomic variation within the cohort, with the genotyping matrix of 142 829 autosomal nonsynonymous single-nucleotide variants (SNVs), was assessed with a principal component analysis and visualized in a plot of principal components 1 and 2 (PC1–2) using the "bigstatsR" package.[42] By using clustering information of the individuals, as shown in Figure S1, along with available self-reported race and ethnicity data as a proxy for continental-level ancestry, we imputed missing race and ethnicity data. The race and ethnicity-linked clusters in Figure S1 are similar to those generated by the larger UK Biobank study,[43] suggesting appropriate representation of genotypes.

Extreme Phenotyping of Individuals With WBS

Participants with WBS were classified based on severity of their SVAS into 4 groups: (1) clinically significant/surgical as defined by a history of surgical intervention in the supravalvar aorta ("surgical SVAS"), (2) mild-to-moderate (defined as presence of any SVAS for which surgery was neither recommended nor performed), (3) no SVAS, meaning no appreciable "discrete" stenosis, and (4) unclassified. A combination of parental report and available medical records (cardiologist note, echo, cardiac catheterization report, or surgical reports) was used to assign phenotype. Because the degree of SVAS may increase over the first few years of life, we required that a participant be at least 3 years of age to be listed as "no SVAS." Consequently, an additional category of unclassified participants who were either too young to classify as "no SVAS" or did not have adequate data for the clinician to confidently assign a phenotypic designation was created. In the classified surgical and no SVAS cases, records were determined to be adequate to justify the classification. Parental report was not used in isolation to assign the SVAS outcome.

Our modifier evaluation focused on comparisons of those with extreme phenotypes, that is, those with surgical SVAS (n=88) and those with no SVAS (n=137). We assessed these 225 individuals for differences in variant burden (defined as the sum of 0s, 1s, and 2s for genotypes 0/0, 0/1, and 1/1, respectively, for the set of variants of an individual, among the 100 744 autosomal nonsynonymous SNVs) based on research cohort membership (Boston, NIH, Nevada-Toronto, Telethon), chromosomal sex (XX, XY), sequence batch (year 2017, year 2020), or sample type (blood, saliva, immortalized cells) with separate Wilcoxon tests. See Supplemental Methods for a detailed description of our 7-point variant prioritization strategy.

The summary statistics of the variants that support the findings of this study are available in Data S1-S3.

Statistical Analysis

The Wilcoxon test implemented in JMP16 software (SAS Institute Inc., Cary, NC) was used for all comparisons in the supplemental figures. R software (https://www.R-project.org/) implemented through Rstudio (http://www.rstudio.com) was used for generating the principal component plot. The *P* values calculated from pathway enrichment and association tests were adjusted using the Benjamini and Hochberg method. A cutoff of adjusted *P* value (false discovery rate [FDR] value)<0.05 was used for selection of enriched pathways and associated pathways.

RESULTS

Demographic Information

The 473 participants with WBS were classified into 4 categories: no SVAS (n=137), mild–moderate SVAS (n=189), surgical SVAS (n=88), and unclassified (n=59). Demographic information is presented in Table. The relative proportions of participants in the no SVAS, mild–moderate SVAS, and surgical SVAS categories

Table. Demographic Information for the 473 Participants With WBS in the Study

Variable	All patients n=473	No SVAS n=137	Mild SVAS n=189	Surgical SVAS n=88	Unclassified SVAS n=59
Sex					
Female	236	73	98	35	30
Male	237	64	91	53	29
Ancestry					
European	421	119	170	80	52
Asian	5	3	0	2	0
African	14	7	5	1	1
Mixed	33	8	14	5	6
Age					
Median	9	13	7	10	2.6
Age range, y	0.01–62.6	3.73–62.6	0.01–46	0.1–45	0.12–60

SVAS indicates supravalvar aortic stenosis; and WBS, Williams–Beuren syndrome.

are similar to those previously reported in the literature.[9,11,12] The median age at the last phenotyping event was 9 years, with an interquartile range from 4 to 18 years. Based on self-report and PC1–2 based imputation for those missing self-identified race and ethnicity, 421 are of European ancestry, 14 have African ancestry, 5 are of Asian ancestry, and the remaining 33 individuals represented in orange in the PC1–2 of Figure S1 are likely an admixture of European, Asian, and Latine/admixed American ancestry. The percentage of surgical SVAS in each of the 4 cohorts is Boston 20%, Telethon in Italy 25%, NIH 16%, and Nevada-Toronto 20% (including unclassified participants).

Consistent with previous reports,[44,45] the proportion of male participants to female participants was significantly higher in the surgical SVAS group than in the no SVAS group (P=0.048 by χ^2 test). Each of the ancestry- and sex-based subgroups had ratios of 1.2 to 2.1 individuals with no SVAS to each person with surgical SVAS. The only exception was the African ancestry subgroup in which 7 had "no SVAS" and 1 had "surgical SVAS," leading to a 7:1 ratio.

For the extreme phenotype cohort (only those with surgical SVAS and no SVAS, n=225), variant burden did not vary by sample collection site (Figure S2A, P=0.36), chromosomal sex (Figure S2B, P=0.34), sequencing batch (Figure S2C, P=0.46), or sample type (Figure S2D, P=0.21). We noticed, however, that 8 individual samples in the Nevada-Toronto and NIH collections exhibited increased variant numbers. Those 8 were mixed in terms of sample type, year of sequencing, and chromosomal sex but all belonged to the PC 1–2 cluster ascribed to those of African ancestry. Relatively increased variation is a well-known feature of the genomic structure of this subgroup.[46] Because our analysis relies on differences in AF between members of the 2 extreme phenotype groups, skew in alleles related to ancestral background (in this case 7 in the no SVAS group and only 1 in the surgical SVAS group) could be conflated with disease outcome. As such, we performed our subsequent analyses using the 217 individuals (87 with surgical SVAS and 130 with no SVAS) without skew, as shown in Figure 1A. To show how our method performs in the presence of ancestry-associated phenotype skewing, comparisons of these findings (n=217) to the findings when the 8 individuals with African ancestry (1 surgical SVAS, 7 no SVAS; n=225) are included are reported in Supplemental Analysis.

Prioritization of Variants

Because our sample size is too small to power discovery of SNV/gene-level association using established statistical packages, we instead focused on pathway-level association with SVAS severity in the 217 extreme phenotype participants (Figure 1A). The scatter plot of combined annotation dependent depletion (CADD) score versus AF difference between the surgical and no SVAS groups for the 81 039 variants (after excluding 7671 SNVs with missing CADD scores) is shown in Figure 1B. The maximum difference in AF between the extreme phenotype groups is 18.7%. The variants with extremely high CADD score (CADD >30) were generally rare and showed little difference in AF between the extreme phenotype groups.

We applied our 7-step strategy, shown in Figure 1C, to perform 3 separate analyses (for steps *i–iv*, see Supplemental Methods for further details) on variants with CADD_phred score (CADD score) >10 and a clear difference in AF between the 2 extreme phenotype groups. These analyses included (1) "common variants" that are present at a higher rate in 1 of the 2 groups (|AF(surgical)-AF(no SVAS)|>5%); (2) "surgical variants" present only in the surgical group (AF>1% in the surgical and AF=0 in the no SVAS group); and (3) "no SVAS variants" present only in the no SVAS group (AF=0 in the surgical and AF>1% in the no SVAS group). This

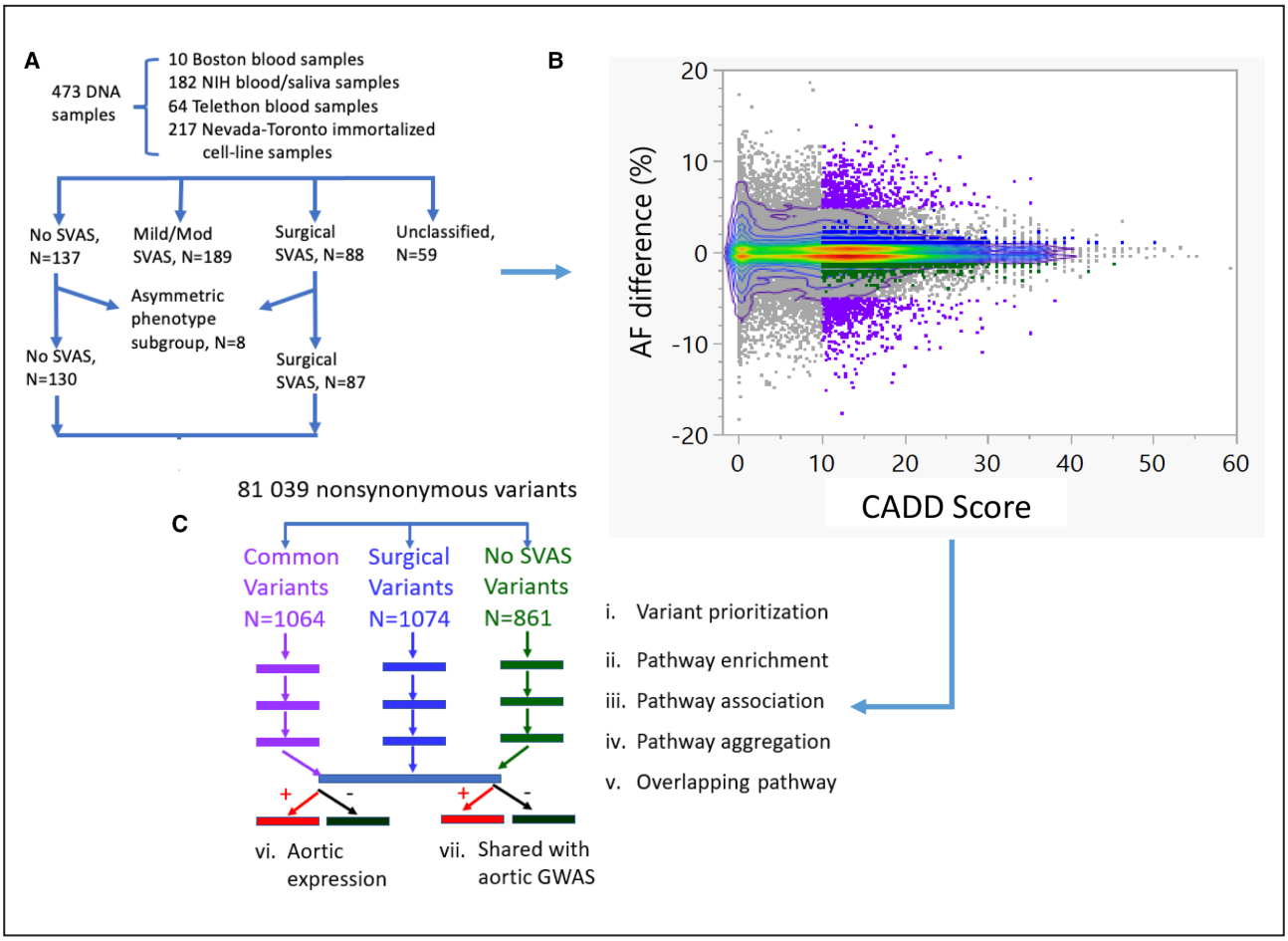

Figure 1. Flow chart of fine phenotyping of SVAS in 473 individuals with WBS and schematic flow chart outlining modifier identification and validation.

A, Phenotypes were assigned as described in the methods. The asymmetric phenotype subgroup includes 8 individuals with African ancestry: 7 with no SVAS and 1 with surgical SVAS. **B**, 81039 nonsynonymous variants were plotted based on putative pathogenicity (CADD score, *x* axis) and AF differences between the surgical minus the no SVAS groups (*y* axis). The color gradient depicts the density of the variants. Ninety percent of the 81 039 variants are inside the outer-most bivariate smoothed contour (purple) in the 2-dimensional plot. **C**, Steps *i–iv* are repeated 3 times for the subsets of variants with CADD score>10: (1) "common variants": those with AF difference between surgical SVAS and no SVAS >5%, (2) "surgical variants" present only in the surgical group (AF>1% in the surgical group and AF=0% in the no SVAS group); (3) "no SVAS variants" present only in the no SVAS group (AF=0% in the surgical group and AF>1% in the no SVAS group). The prioritization process yields 1064 common variants (purple), 1074 "surgical SVAS variants" (blue), and 861 "no SVAS" variants (green). Subsequent pathway enrichment, association, and aggregation revealed 13 key pathways of interest. Validation of genes from key pathways was performed using publicly available data sets (*vi* and *vii*). AF indicates allele frequency; CADD, combined annotation dependent depletion; GWAS, genome-wide association studies; NIH, National Institutes of Health; SVAS, supravalvar aortic stenosis; and WBS, Williams–Beuren syndrome.

prioritization method yielded 1064 "common" variants in 914 genes, 1074 "surgical" variants in 995 genes, and 861 "no SVAS" variants in 816 genes.

Among the 1064 common variants (Data S1), 15 SNVs were stopgain, stoploss, and startloss (see Table S1). Of the 914 genes, 792 have 1 variant each; 104 genes carry 2; and *ZAN*, *CDH23*, and *ZNF568* have 5 common variants each. No significant differences in the per-individual burden of the 1064 variants were observed by collection location (P=0.95), chromosomal sex (P=0.07), sequencing batch (P=0.52), sample type (P=0.89), or SVAS status (P=0.70) (Figure S3A through S3E).

Pathway Enrichment and Association Tests of Common Variants in Pathways as a Function of SVAS Severity

We hypothesized that variants with larger AF differences between phenotype groups may be part of the same pathways and may work together to influence physiologic or cellular functions. To identify pathways with an increased burden of candidate variants, we performed pathway enrichment using the 914 common variant genes; this identified 44 pathways (Table S2; step *ii* in Figure 1C).

We then formally tested each of the 44 enriched pathways for association with SVAS severity (step *iii* in Figure 1C). The results from the 3 methods, RQT, sequence kernel association test (SKAT), and sequence kernel association test-optimal (SKAT-O), are shown in Table S3. The results from SKAT and SKAT-O are similar. Thirty-nine of the 44 pathways met the cutoff of FDR<0.05 on both the RQT test and the SKAT or SKAT-O test (Figure S4). Some overlap exists across the 39 pathways. Based on this observation, we manually consolidated the 39 original pathways to 13 key pathways (Figure 2A) by grouping pathways with similar functions and overlapping genes, taking as the representative pathway the 1 with the greatest number of variant-affected genes (step *iv* in Figure 1C). The 13 key pathways include extracellular matrix (ECM; here we maintained both NABA_CORE_MATRISOME[47] (core matrisome) to represent the structural ECM and NABA_MATRISOME[47] (matrisome), which includes both ECM and ECM-associated proteins like proteases and growth factors), sensory/olfactory signaling, innate immune, developmental biology, polymerase II transcription, metabolism of lipids, transport of small molecule, ciliopathies, adaptive immune, PI3KAKT, disease of metabolism, and endocytosis.

Pathway Enrichment and Association Tests Using the "Surgical" Variants

As in the common variant analysis, we performed pathway enrichment using the 995 genes (1074 variants, Data S2) prioritized in the "surgical variant" analysis. Variants from 496 of the 995 gene were statistically enriched (FDR value <0.05) into 71 pathways (Table S4). The association tests by RQT and SKAT/SKAT-O yielded significant results for 58 out of the 71 pathways with FDR value <0.05 (Table S5). In addition to enrichment in ECM pathways, as seen in the common variant analysis, we also observed enrichment for pathways including apoptotic cleavage of cellular protein/apoptotic execution phase, cell cycle/M phase, ciliopathies, developmental biology, mitotic spindle, RHO-GTPASE, and PI3KAKT. The 58 pathways in Figure S5 were once again manually consolidated to 17 key pathways with overlapping gene content, as shown in Figure 2B. Interestingly, 28 of the 87 individuals with surgical SVAS possessed at least 1 gene with a less common variant among the 18 genes in the mitotic spindle pathway, and 47 exhibited at least 1 gene with a less frequent variant among the 36 genes in the cell cycle pathway. In total, 59 of the 87 individuals possessed a variant in one or both pathways.

Pathway Enrichment and Association Tests Using the "No SVAS" Variants

We identified 25 enriched pathways (with FDR value <0.05) from the 816 genes (861 variants, Data S3) identified in the "no SVAS" variant analysis. Twenty-three (Figure S6) of the 25 were confirmed by RQT and SKAT/SKAT-O (Table S6). The 23 pathways were

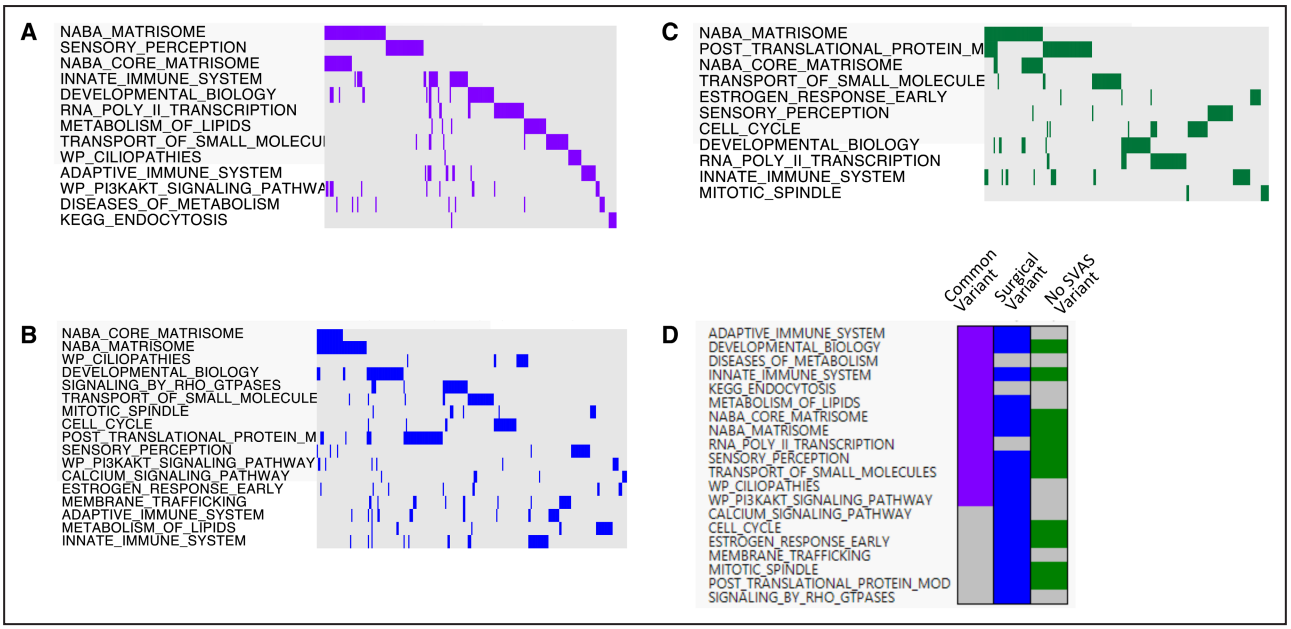

Figure 2. Depiction of SVAS modifier pathways identified through gene set enrichment and association testing.
A, Thirteen aggregated common variant pathways. The most enriched pathways are presented in order of statistical significance. The colors represent the presence (purple) and absence (gray) of genes in that pathway (genes are represented by columns across the top of the image). **B**, Seventeen aggregated surgical SVAS variant pathways in blue. **C**, Eleven aggregated no SVAS variant pathways in dark green. **D**, Overlapping of 20 pathways in the 3 sets of pathway analyses. See Figures S4, S5, and S6 for the full (preaggregation) pathways. SVAS indicates supravalvar aortic stenosis.

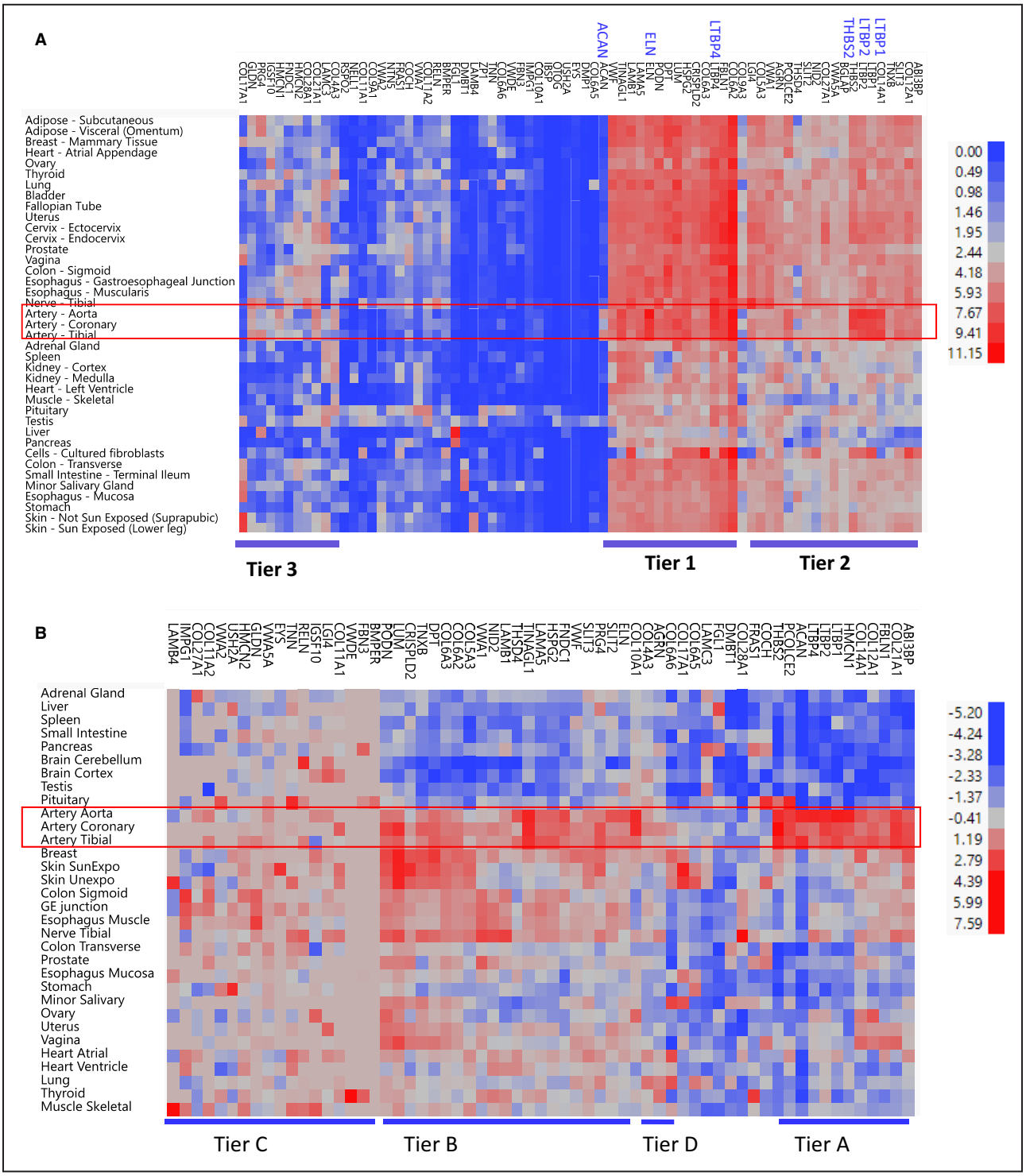

Figure 3. mRNA and protein from core matrisome modifier genes found in vascular tissues.
A, Clustering of log2 transformed expression of the 74 core matrisome genes in 37/54 human tissues (the expression in the 15 brain tissues, cell-EBV, and whole blood are not shown to improve visibility of the remaining 37 tissue names) from GTEX v8 database reveals 3 tiers of expression: Tier 1 contains 15 genes, including *ELN*, *LTBP4*, and *ACAN* that are highly expressed in aorta; Tier 2 contains 19 genes with more moderate expression; and Tier 3 contains 10 genes with lower (but still positive) expression in aorta and other tissues. Of note, 2 of 76 genes identified in our modifier screen were not assessed in the GTEX mRNA database. **B**, Two-way clustering of 63 protein levels present in 32 normal human tissues also reveals varied levels of expression: Tier A contains 12 genes, including *ACAN*, *FBLN1*, *HMCN1*, and *LTBP4* uniquely expressed in aorta, coronary, and tibial tissues; Tier B contains 22 genes including *ELN* highly expressed in aorta and other tissues; Tier C contains 17 genes with moderate expression in aorta and other tissues; Tier D contains 2 genes with lowest expression in aorta and artery coronary and artery tibial. Of note, 2 of 76 genes identified in our modifier screen were not assessed in the GTEX mRNA database. Similarly, 13 of the 76 genes were not queried in the protein database. EBV indicates Epstein-Barr virus.

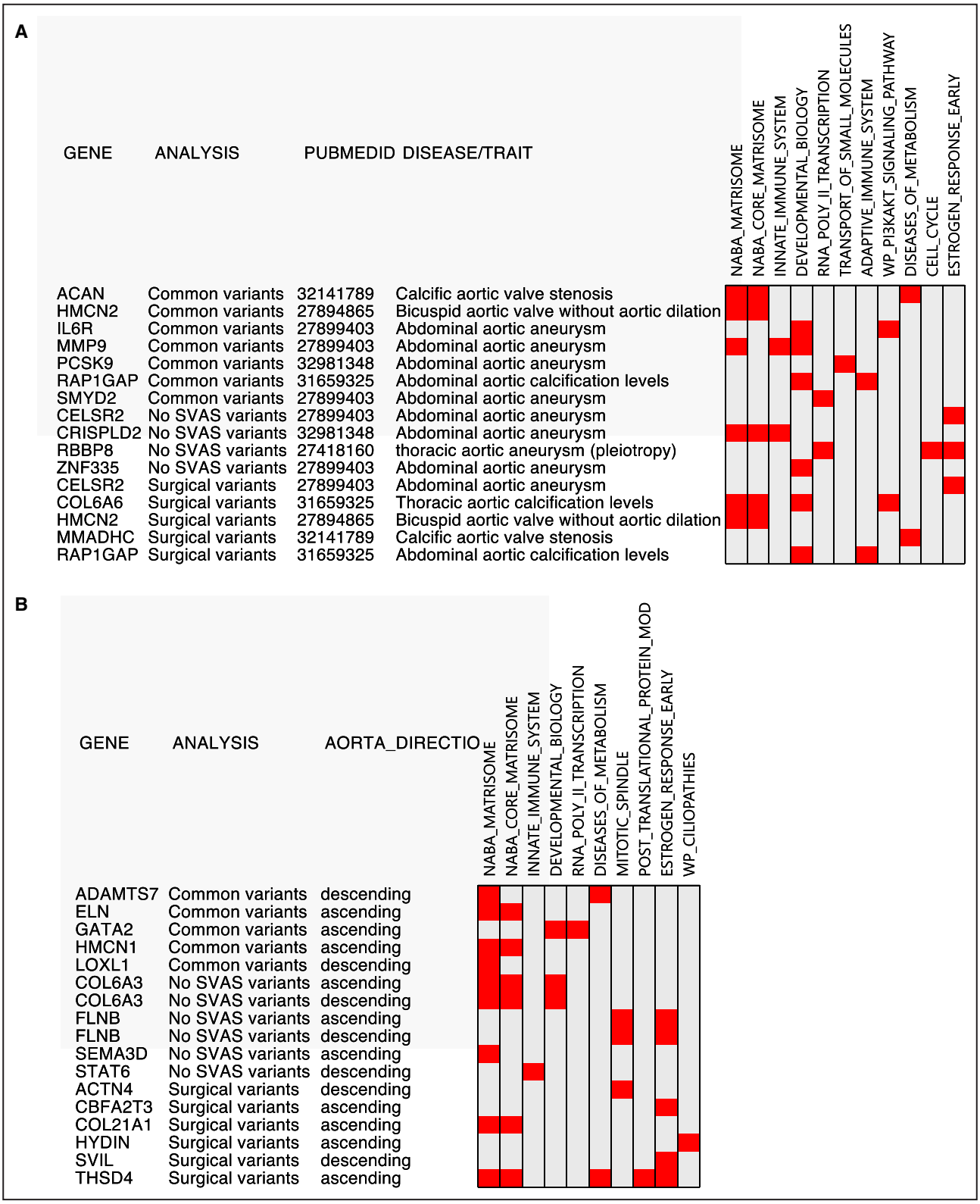

Figure 4. Overlap of genes identified in this study with published aortic disease/trait GWAS.
A, Comparison of variants identified in aortic disease GWAS (NHGRI-EBI GWAS catalog as of September 23, 2021). Thirteen genes involved in 11 pathways in our study were identified in the 13 previously published studies. **B**, Fifteen genes overlapped between the list of 117 genes in Pirruccello et al's study[36] of aortic size and our SVAS study. Notably, both *ELN* and *HMCN1*, identified from ascending aorta, are human aorta specific. GWAS indicates genome-wide association studies; NHGRI-EBI, National Human Genome Research Institute-European Bioinformatics Institute; and SVAS, supravalvar aortic stenosis.

aggregated to 11 key pathways: ECM, posttranslational protein modification, transport of small molecule, developmental biology, RNA polymerase II transcription, cell cycle, sensory, innate immune, estrogen response, and mitotic spindle, shown in Figure 2C.

Overlapping the Pathways Enriched by Common Variants, Surgical Variants, and No SVAS Variants

We then compared the 3 sets of key pathways (Figure 1C, *step v*, and Figure 2D). The developmental biology, ECM, innate immune, sensory, and transport of small molecule pathways were discovered in all 3 analyses, whereas the others were present in only 1 or 2 sets.

Influence of Ancestry and Phenotypic Skew on Pathway Selection

To determine the impact of skew in a genetic background subgroup, we repeated the 3 sequential allele frequency-based analyses in the cohort of 225 individuals with WBS, including the additional 8 individuals with African ancestry. Inclusion of these 8 participants had a mild impact on the number of significant pathways from association tests for the common variants analysis and little impact on the number of pathways from the surgical SVAS analysis In contrast, we noted a dramatic increase in the number of pathways identified through the no SVAS analysis. The details of the comparisons are provided in Supplemental Analysis. Of note, the top pathways remained consistent in both analyses.

Core Matrisome Pathway Genes With Modifier Variants Are Expressed in Human Aorta

Because the pathways discovered by this strategy are key to SVAS outcomes, the genes should be expressed by tissues relevant to that pathology. Although some gene products (like those in immune-mediated or endocrine pathways) are not predicted to be produced by native vascular cells, other products like ECM proteins are expected to be generated and deposited locally. As such, we assessed which of the 76 genes in the core matrisome pathway from our 3 analyses' mRNAs could be detected in human aorta. mRNA from 44 of the 74 genes for which data were available in GTEX (59%) were detected in large arteries (see Figure 3A for tiers of expression). Notably, *ACAN* (aggrecan), a previously described serum biomarker for detection of aorta dissection,[48] was uniquely expressed in adult human aorta.

We next investigated the protein levels of the core matrisome gene products in 32 human tissues. Overall, 84% (53/63) of the protein products present in the https://www.proteinatlas.org/[49] database from the 76 core matrisome genes identified in our study were present in adult aortic tissue and thus able to have an impact on aortic outcomes. Twelve proteins, including ACAN, FBLN1, HMCN1, and LTBP4, were highly and uniquely expressed in adult aorta (Tier A, Figure 3B).

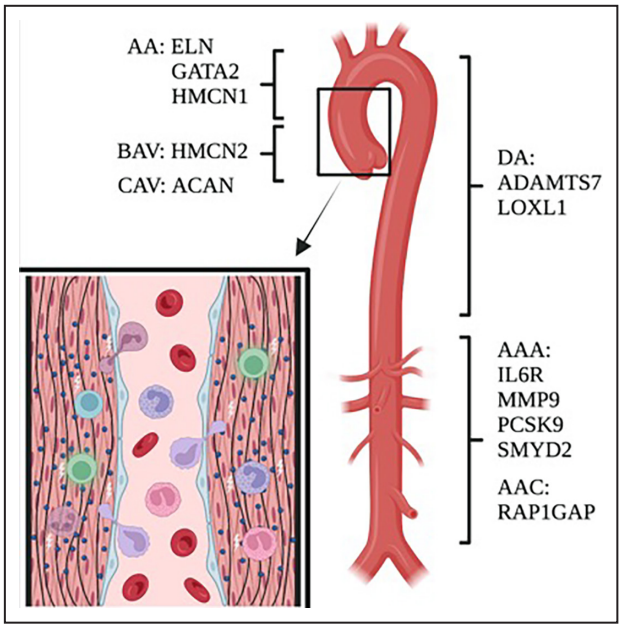

Figure 5. Summary of overlapping genes affected by common variants in our SVAS study and previous GWAS and depiction of interactions between key pathways inside the aortic wall.
Immune cells, shown in a variety of colors, circulate in the blood and may enter the vessel wall to participate in vascular remodeling. Many of the protein products of the genes shown here are known to participate in this process. AA indicates ascending aorta; AAA, abdominal aortic aneurysm; AAC, abdominal aortic calcification; BAV, bicuspid aortic valve; CAV, calcific aortic valve; DA, descending aorta; GWAS, genome-wide association studies; and SVAS, supravalvar aortic stenosis.

Innate Immune Pathway Genes With Modifier Variants in Human Aorta

We also looked at the expression of the 45 genes with common variants present in the innate immune pathway in the GTEx v8 database and the proteomics database.[49] As expected, the majority of these genes are expressed in immune tissues (white blood cells, spleen, appendix) as evidenced by mRNA (Figure S7A) and protein (Figure S7B). Interestingly, several of the 45 genes, including *ICAM3*, *ITGAL*, *MMP9*, *MMP25*, and *TLR1*, are highly expressed in immune cells with little to no expression elsewhere (including the aorta).

SVAS Modifiers Overlap With Genes Identified Through GWAS of Aortic Disease and Size

Variants in the ECM pathway predicted to modify phenotypic outcomes in WBS may perform similar

functions in other aortopathies as well. To evaluate this possibility, we addressed 2 primary questions: (1) Is the matrisome/ECM pathway enriched in other GWAS on aortic diseases or aortic caliber? and (2) Do any aorta-specific genes identified in those studies overlap with the specific matrisome/ECM pathway genes we identified in the present study?

First, we examined the 86 genes identified in the 13 aortic disease studies found in the National Human Genome Research Institute-European Bioinformatics Institute GWAS catalog as of September 23, 2021 (see Table S7 for a description of those studies). We found enrichment for the NABA_MATRISOME pathway in those studies (FDR value=2.7E-04) and additionally noted that 13 of the 86 genes found were included in 11 of our 20 SVAS modifier pathways (Figure 4A). The strongest overlap was seen with genes identified in aortic aneurysm studies (n=9), whereas 2 were ascertained in GWAS of aortic valve stenosis and 4 were noted in studies of aortic vessel or valve calcification. The 5 matrisome/ECM pathway genes: *ACAN*, *COL6A6*, *CRISPLD2*, *HMCN2*, and *MMP9*, were found in 4 aortic disorders. Three genes, *IL6R*, *PCSK9*, and *SYMD2*, are of particular interest due to existing clinical studies showing the potential for therapeutic intervention.[50–52] *MMP9*, found in the matrisome, innate immune, and developmental biology pathways in our study (Figure 4A), has been extensively studied in cancers, aging, and vascular diseases.[53–55]

We similarly applied these 2 questions to studies evaluating biomorphic traits of the aorta. Recently, Pirruccello et al[36] identified 117 genes associated with variation in ascending and descending thoracic aortic caliber in ≈40 000 adults enrolled in the UK Biobank (median age ≈64 years). As in the disease-driven studies, we found enrichment of the NABA_MATRISOME pathway (FDR value=4.7E-04) in this data set. Likewise, we found that 15 of their 117 genes overlapped with 10 of our 20 pathways (Figure 4B). Of these, 8 genes are part of the NABA_Matrisome pathway. Notably, both *ELN* and *HMCN1*, both human aorta specific genes/proteins on the matrisome pathway, were identified in the ascending thoracic aorta analysis.[36] Although not genetic markers per se, low-density lipoprotein direct and apolipoprotein B are the top 2 clinical features inversely associated with ascending thoracic aorta diameter in Pirruccello et al's study, highlighting a potential role for lipids in affecting outcomes related to aortic dimensions. The summary of genes with common variants present in both our SVAS study and the previous large GWAS studies on aortic disorders is shown in Figure 5.

DISCUSSION

WBS, like many diseases of haploinsufficiency, exhibits wide variability in outcomes. SVAS, a common vascular feature of the condition, varies from life-threatening to not appreciable in people with the typical 7q11.23 deletion. Although previous mouse and human studies have shown the potential for background genetic variation[56] or environmental exposures[57] to influence vascular outcomes in the setting of elastin insufficiency, the only feature repeatedly shown to be associated with more severe vascular outcome is male sex,[44,45] a finding further replicated in the present study. By validating (and expanding) the findings of our earlier proof-of-concept exome study[11] in this, the largest WBS genome study to date, we now confirm the importance of background variation in matrisome, immune, and other pathways for influencing vascular outcomes in WBS.

Our approach included 3 major steps: (1) identification of pathways in which gene variation is associated with extreme outcomes, (2) interrogation of the identified genes for expression in tissues relevant to SVAS, and (3) assessment of overlap of the genes identified here with those ascertained in larger aortic GWAS. A similar approach can be undertaken to identify modifiers in other rare conditions.

Matrisome Pathway Variants Confirmed as Key Modifiers of SVAS Outcomes

As in Parrish et al,[11] we detected a strong association between variation in core matrisome and matrisome-associated pathway genes and extreme SVAS outcomes. *ELN*, the gene within the WBS deletion that drives the vasculopathy,[6] encodes a smooth muscle cell-produced extracellular matrix protein that imbues aortic tissue with elasticity. Elastin is deposited in the extracellular space following interactions with other ECM molecules[58,59] such as collagens, fibrillins, and fibulins.[60–63] Elastic fibers interact with the cell through integrin and proteoglycan interactions and are remodeled in response to changes in vascular mechanics and inflammatory processes by matrix metalloproteases[54,55,64–67] and other proteases in the extracellular space (Figure 4C). As such, the finding that variation in the genes that make up the matrisome may influence SVAS outcomes is not surprising.

Because components of the ECM are expressed in many tissues, we sought additional confirmation that the modifier genes we identified were relevant to aortic outcomes. Review of publicly available data from GTEX and https://www.proteinatlas.org/[49] confirmed expression in the aorta (Figures 3A and 3B), with a subset (including *ACAN*, *ELN*, *HMCN1*, and *LTBP4*) being preferentially expressed there. Because these collections are limited to adult tissues, it is possible that inclusion of developing/pediatric tissues could further increase this percentage. Additionally, we also found significant enrichment in matrisome variants in genomes from individuals with other aortopathies (Figure 4A) and in

studies evaluating aortic caliber (Figure 4B). Together, these findings support the role of the matrisome in a variety of aortic outcomes and highlight the validity of a pathway-based approach in identifying relevant modifiers.

Immune Pathways Highlight the Potential Influence of Inflammation on Mediating SVAS Outcomes

Although it is reassuring that our methods identified expected modifier pathways like the matrisome, the identification of less obvious pathways may hold greater potential for advancement in the field. Review of the literature suggests a growing association between immune regulation and aortic disease. For example, *TLR3*, a gene on the innate/adaptive immune pathways identified in this study, was recently identified as a central regulator of calcification of the aortic valve.[68] Likewise, researchers recently showed that inhibition of the mTOR (mechanistic target of rapamycin) pathway (PI3KAK) alters tissue biomechanics and cell function in mouse and iPS models of elastin insufficiency.[63,69] Additionally, our group previously showed an increase in aortic diameter for *Eln*$^{+/-}$; *Rag1*$^{-/-}$ mice that lack B and T cells,[11] and more recently Lin et al[70] showed an influx of monocytes to the area developing stenosis in a new model of elastin insufficiency, the TagInCre; Eln$^{Fl/Fl}$. Correspondingly, although healthy aorta exhibits relatively few inflammatory cells and secretion products, a review of published SVAS pathology images notes a neointima with immune cell accumulation and concomitant expression of MMPs (matrix metallopeptidases), including MMP2, 7, and 9, as well as their inhibitors, in some patient specimens.[65,71] MMP9 controls the access of monocytes and T cells to the vascular wall in large vessel vasculitis.[55] As such, it is thought that MMP9 contributes to the degradation of ECM proteins during the development of SVAS[65] and aneurysms.[72] These studies suggest complex interactions between ECM molecules and immune cell produced matrix modifiers in aortic media in patients with aneurysmal and stenotic aortopathies.

Pathways Underlying SVAS, Aneurysm, and Bicuspid Aortic Valve Disorders

The concept that modifiers of aortic outcomes may be shared across diseases was recently discussed in an editorial that posited that phenotypes like vascular stenosis and aneurysm may exist on a spectrum[73] and disorders on both ends of the spectrum may share common modifiers. Intriguingly, many of the matrisome genes identified as modifiers in our study have also been reported in aneurysm studies in humans and mice (reviewed in Jana et al[38,74]). Likewise, immune actors are commonly implicated in the pathologic aortic remodeling phenomenon that precedes aneurysm development.[38,75,76] Our analysis of GWAS on aortic aneurysm[22–28] and bicuspid aortic valve[29,30] suggest that matrisome and innate immune pathways are key modifiers of multiple aortopathy types (Figure 5). MMP9[25] and CRISPLD2[26] were identified in studies of aortic aneurysm whereas *ACAN*,[32] an aortic-specific gene, and *HMCN2*[30] were moderately associated with biscupid aortic valve in 2 separate studies. Although biscupid aortic valve is a valve disease, the aortas of such individuals often bear the stigmata of aneurysm, including elastic fiber fragmentation and increased MMPs,[77] as has been described for SVAS. Together, these findings suggest that health of a tissue is dependent on its ability to balance the rate (or total quantity) of ECM protein synthesis with matrix degradation. When this balance is disturbed, ineffective or destructive remodeling occurs. Although not specifically tested in this study, genetic variation that further perturbs this balance may, therefore, be reasonably expected to influence outcomes. Further ranking of variants based on weighted impact on SVAS outcomes as part of a polygenic score may inform future targeted models aimed at testing the relationship between the primary WBS deletion and background gene variation.

Additional Pathways Identified by "Surgical SVAS Only" and "No SVAS" Analyses

Enrichment analyses performed on recurrent variants unique to either the surgical or no SVAS subset revealed an association with cell cycle/mitotic spindle apparatus and estrogen responsiveness pathways, among others. The cell cycle pathway is intriguing, considering the known increase in smooth muscle cell proliferation seen in SVAS lesions.[63,71] Likewise, estrogen signaling pathways could underlie the reported increase in stenosis severity in men relative to women.[11,45] Sex hormone effects have also been shown to affect outcomes in other vascular diseases such as vascular Ehlers-Danlos syndrome[78] and Marfan syndrome.[79] More than 67% of the 217 individuals in the surgical SVAS or no SVAS groups had at least 1 variant in genes in cell cycle pathway, and 34% of those with surgical SVAS had a rarer variant in the estrogen pathway, suggesting that even rarer variants within a pathway could cumulatively occur frequently enough to be considered viable modifiers. With growing information from phased haplotypes from long-read sequencing, the net effect of rare and common variants of a gene on a haplotype can be studied and will likely be a driving factor in future genomic research.

Limitations of the Study

In this study, we found a difference in extreme outcome frequency in our African ancestry subgroup (7 no SVAS versus 1 surgical) that differed from the other cohorts (1.2 SVAS versus 1 surgical). The differences in the pathway sets are driven by variants that are common (AF >5%) in the individuals of African descent (whose representation in the extreme phenotypes is asymmetric) but rare in those of European, Asian, and Latine/admixed American backgrounds. Because our method is driven by differences in AF between extreme phenotype groups, attention to this limitation in future applications of this method should be considered. Currently, the literature contains no population genetic or cardiovascular study on people with WBS of African descent, and further efforts are needed to increase diversity in rare disease reports.[80] Broad representation is needed to employ robust statistical models that can incorporate samples of multiple ancestries.[81]

In addition, we limited our focus to nonsynonymous variants with moderate or higher impact. In future work, we will extend our approaches to consider noncoding variants including those in 3' and 5' UTRs as well as more distant enhancer sites, and we will improve our SVAS classification methods for no SVAS versus mild SVAS and mild SVAS versus surgical SVAS. Multiple layers of statistical testing may increase the rate of false pathway discovery. Although we have used orthogonal methods to substantiate and replicate our top findings, lower tier pathways will need to be similarly validated in future research.

Future Studies

Our study raises new questions to be addressed in future work. First, mechanistic studies are needed to better understand how variation in the matrisome and inflammatory genes/pathways directly contributes to differences in SVAS outcomes. Animal models may be useful in this regard, but further prioritization of variants/pathways will be needed to make this technically tenable. Application of more recent methodologies, such as single cell RNAseq in affected tissues, can allow identification of key cell types within the vessel wall that are most relevant to stenosis severity. Likewise, focusing on the genes identified in the present study may accelerate hypothesis-driven analyses aimed at the detection of relevant genetic (polygenic risk score calculation) and serum-based biomarkers for identification of patients with the propensity for surgical SVAS. Key targets could include *ACAN*,[48] *HMCN1*, *LTBP4*, or macrophage/monocyte-specific gene/protein *MMP9*.[64,66] Additionally, one of the most important aspects of modifier identification is the potential for implementation of novel therapeutics. Given the overlap we found between genes relevant to SVAS and other aortopathies, future efforts leveraging therapies under investigation for those conditions may allow rapid development of therapeutics for treatment of SVAS. For example, the contributions of MMP9 to aortic aneurysm have been studied[53–55] and a variety of MMP inhibitors have been developed for vascular diseases.[82] Regulating matrix proteases during critical periods for SVAS development in children with WBS may be one promising therapeutic strategy. Likewise, enhancing ECM proteins quantity or quality in the aortic wall of those with elastin insufficiency, as was done by regulating LTBP4,[83] may be another promising direction to pursue.

CONCLUSIONS

Taken together, our findings have enabled the discovery of new pathways in which the presence of gene variation is associated with more extreme outcomes. These same strategies can easily be implemented for other rare disease applications.

ARTICLE INFORMATION

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tacatalyst.nhlbi.nih.gov/; GTEX v8 database: https://www.gtexportal.org/home/.

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Disclosures

None.

Supplemental Material

Data S1-S3
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