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ORIGINAL RESEARCH

Differential Expression Analyses on Human Aortic Tissue Reveal Novel Genes and Pathways Associated With Abdominal Aortic Aneurysm Onset and Progression

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BACKGROUND: Abdominal aortic aneurysms (AAAs) are focal dilatations of the abdominal aorta that expand progressively, increasing their risk of rupture. Rupture of an AAA is associated with high mortality rates, but the mechanisms underlying the initiation, expansion, and rupture of AAAs are not yet fully understood. We aimed to characterize the pathophysiology of AAAs and identify new genes associated with AAA initiation and progression.

METHODS AND RESULTS: This study used RNA sequencing data on 140 samples, becoming the largest RNA sequencing data set for differential expression studies of AAAs. We performed differential expression analyses and analyses of differential splicing between dilated and nondilated aortic tissue samples, and between AAAs of different diameters. We identified 3002 differentially expressed genes between AAAs and controls that were independent of ischemic time, 1425 of which were new. Additionally, 8 genes (*EXTL3*, *ZFR*, *DUSP8*, *DISP1*, *USP33*, *VPS37C*, *ZNF784*, *RFX1*) were differentially expressed between AAAs of varying diameters and between AAAs and control samples. Finally, 7 genes (*SPP1*, *FHL1*, *GNAS*, *MORF4L2*, *HMGN1*, *ARL1*, *RNASE4*) with differential splicing patterns were also differentially expressed genes between AAAs and controls, suggesting that splicing differences in these genes may contribute to the observed expression changes and disease development.

CONCLUSIONS: This study identifies new genes and splicing patterns associated with AAAs and validates previous relevant pathways on AAAs. These findings contribute to the understanding of the complex mechanisms underlying AAAs and may provide potential targets to limit AAA progression and mortality risk.

Key Words: abdominal aortic aneurysm ■ allele-specific expression ■ alternative splicing ■ differential expression ■ transcriptomics

Abdominal aortic aneurysms (AAAs) are characterized by a local dilation of the infrarenal abdominal aorta to about 1.5 times the normal adjacent aortic diameter or >3 cm in maximum diameter.[1] AAAs are accompanied by chronic inflammation, apoptosis of vascular smooth muscle cells, and neovascularization.[2,3] Additionally, extracellular matrix degradation, microcalcification, and oxidative stress contribute to the degeneration of the aortic wall.[1,2] The disease is progressive, and most aneurysms develop without

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RESEARCH PERSPECTIVE

What Is New?

- The study identifies 1452 novel differential expressed genes in human abdominal aortic aneurysm tissue compared with aortic control tissue through robust and comprehensive analysis of the whole transcriptome and confirms associations with inflammation, immunity, ATP synthesis and intracellular calcium regulation.
- The study of differential splicing processes and allele-specific expression provides insights into the regulation of gene expression patterns and identifies genetic haplotypes associated with disease risk.

What Questions Should Be Addressed Next?

- Functional validation of the newly identified genes is warranted to support their role in the pathogenesis of abdominal aortic aneurysm and to determine their potential as future therapeutic targets.

Nonstandard Abbreviations and Acronyms

AAA	abdominal aortic aneurysm
CD	cluster of differentiation
DEGs	differentially expressed genes
FDR	false discovery rate
GO	Gene Ontology
GTEx	Genotype-Tissue Expression
MAF	minor allele frequency
RIN	RNA integrity number
TABS	Triple A Barcelona Study
TPMs	transcripts per million

causing symptoms.[1] However, in the event of AAA rupture, mortality rates can reach 80%.[4] The only effective treatment currently available for AAA is aortic tissue repair, either through open surgery or endovascular repair.[1,5]

Some AAA risk factors are known, including age, male sex, smoking, and family history of AAA.[1] Smoking, in addition, is also known to increase the risk of rupture.[6] Additionally, recent genomic studies have revealed 121 loci associated with risk of developing an AAA, contributing to the knowledge of the possible pathways leading to this disease.[7] However, there is still an insufficient understanding of the clear mechanisms that underlie the initiation, progression, and rupture of AAAs.

The study of gene expression, known as transcriptomics, is a valuable tool to understand human disease and to reveal new therapeutic targets.[8,9] Several studies have been performed to study the differentially expressed genes (DEGs) between human dilated aortic tissue and nondilated control aorta using microarray technology, detecting DEGs especially associated with the immune and inflammatory responses, extracellular matrix remodeling, and angiogenesis.[10–15] In the present study, we performed RNA sequencing of the complete transcriptome in 140 human abdominal aortic tissue samples (96 AAA aortas and 44 control aortas from deceased donors) from the TABS (Triple A Barcelona Study) cohort, to identify new DEGs and pathways associated with the pathophysiology of AAA initiation and progression, allowing for a more comprehensive analysis of the transcriptome, and becoming the largest human RNA sequencing data set for AAA tissue. Additionally, we aimed to investigate the differences in alternative splicing patterns in the context of AAA, and the role of genetic variants in gene expression in AAA tissue. The study design is described in Figure 1.

METHODS

Ethics Approval and Consent to Participate

The study was approved by the Hospital de la Santa Creu i Sant Pau Ethics Committee (IIBSP-OMI-2019-102). All patients gave written informed consent before surgery to participate in the study. The study conformed to the principles of the Declaration of Helsinki.

Data Availability

The personal data used for this study are available from the corresponding author on reasonable request for collaborations, provided it complies with the ethical permits of the study. All other data supporting the findings of this study are available within the article and its Supplementary Information. The code used for data preparation and analysis is available on Github.

Subjects

We used a total of 140 human abdominal aortic samples from 96 patients diagnosed with infrarenal AAA and undergoing open surgery for AAA repair at Hospital de la Santa Creu i Sant Pau and Hospital del Mar (Barcelona, Spain) and 44 controls. The study participants were obtained from the TABS cohort, which includes genomic, transcriptomic, clinical, and maximum aneurysm diameter data, from patients with AAA and individuals without AAA. Maximum aortic diameters were obtained

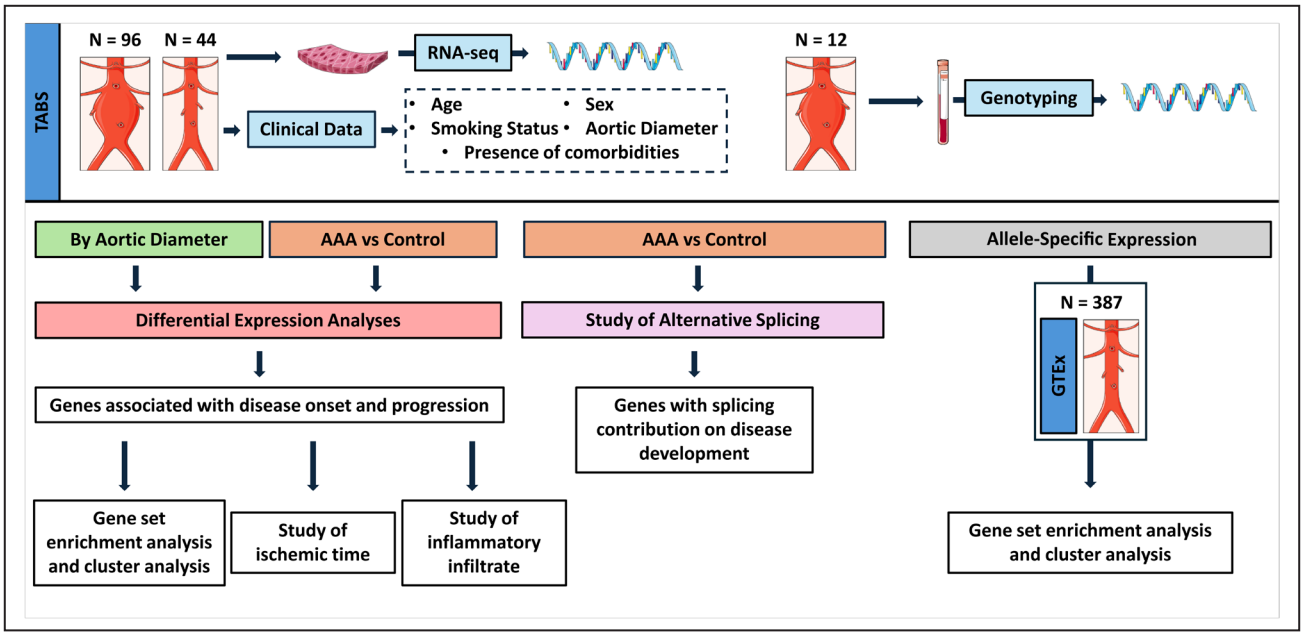

Figure 1. Study design flowchart.
AAA indicates abdominal aortic aneurysm; RNA-seq, RNA sequencing; and TABS, Triple A Barcelona Study.

from computed tomography. Genomic data were obtained through genotyping using the Infinium Global Screening Array-24 version 2.0 (Illumina, San Diego, CA) (coverage 665 608 variants) and imputation to the Trans-Omics in Precision Medicione Reference Panel. Only variants with imputation quality >0.3 were used for the allele-specific expression analyses. Healthy abdominal aortas were obtained from 21 male and 23 female multiorgan donors with confirmed absence of aortic dilatation (Table). Cause of death for the control individuals was available for 22 of the 44 control samples, which primarily included intracranial hemorrhages and cardiovascular events. A complete list of donors' cause of death is provided in Data S1.

Sample Processing

A portion of tissue sample was placed in RNAlater solution (Qiagen GmbH, Hilden, Germany) and stored for 24 hours at 4 °C before long-term storage at −80 °C until further processing. We ensured that this procedure was initiated within 3 hours after the aneurysm samples had been collected. For RNA isolation, tissues were then homogenized in 1 mL of Trizol (Ambion, Carlsbad, CA) in the FastPrep-24 homogenizer and Lysing Matrix D tubes (MP Biomedicals, Solon, OH), and RNA was purified using PureLink RNA Mini Kit (Invitrogen, Waltham, MA) following the manufacturer's recommendations. RNA concentration was measured using Nanodrop 200 (Thermo Scientific, Waltham, MA).

RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). RNA integrity number (RIN) was recorded, and only samples with an RIN >6 were used.

RNA Sequencing

We performed sequencing analyses using the NovaSeq 6000 (Illumina, San Diego, CA), with a read length of 150 bp and paired-end sequencing. Two sequencing runs were performed to reduce the variability of the technical variables. First, AAA and control samples were randomized between sequencing plates. Second, only AAAs were sequenced with as little technical variability as possible. In all sequencing runs, a minimum of 30 million reads were required, repeating the sequencing on a new plate if this limit was not reached.

Alignment, Quantification, and Quality Control

We used STAR version 2.5.3a[16] to perform the alignment on the reference genome version GRCh38 and we then used RSEM version 1.3.0[17] for gene quantification. For both analyses, we used gene models from GENCODE version 26 gene annotation file.[18] In total, 58 219 genes were quantified. Of these, we selected protein coding genes and long noncoding RNA genes that had been either experimentally validated (level 1 annotation) or manually annotated (level 2 annotation),[18] resulting in 27 290 genes (19 777 protein-coding and 7513 long noncoding RNA) for further analyses. Gene quantifications were expressed as transcripts per million (TPMs), which were obtained by normalizing for gene length first, and then for sequencing depth. This

Table. Characteristics of Study Participants

	Controls (N=44)	AAA (N=96)	*P* value	Missing values
Age, y	61.66 (21–82)	70.38 (53–87)	0.0006	0 (0)
Sex, male	21 (47.73)	92 (95.83)	1.00E-10	0 (0)
Smoking				
Current	8 (21.62)	27 (32.14)	0.3378	19 (13.57)
Never	26 (70.27)	15 (17.86)	6.52E-08	19 (13.57)
Past	3 (8.11)	42 (50)	2.81E-05	19 (13.57)
Aortic diameter, mm	NA	65.57 (38–100.12)	…	0 (0)
Hypertension	15 (40.54)	54 (64.29)	0.0256	19 (13.57)
Dyslipidemia	10 (27.03)	46 (54.76)	0.0087	19 (13.57)
Diabetes	5 (13.51)	12 (14.29)	1	19 (13.57)
Peripheral artery disease	NA	23 (27.71)	…	57 (59.38)
Other aneurysms	NA	25 (29.76)	…	12 (12.5)
Cerebrovascular disease	3 (8.11)	36 (43.9)	0.0003	21 (15)
Cardiovascular disease	1 (3.34)	16 (19.05)	0.0846	27 (19.29)
Chronic obstructive pulmonary disease	1 (2.7)	15 (17.86)	0.0481	19 (13.57)
Chronic kidney disease	2 (5.4)	17 (20.24)	0.0726	19 (13.57)
Antihypertensive	2 (5.4)	37 (44.05)	5.82E-05	20 (14.29)
Nonsteroidal anti-inflammatory drugs	0 (0)	15 (18.07)	0.01	20 (14.29)
Glucocorticoids	1 (2.7)	6 (7.23)	0.58	7 (5)
Statins	7 (18.92)	63 (75.9)	1.63E-08	20 (14.29)
Anticoagulants	3 (8.11)	46 (55.42)	3.06E-06	20 (14.29)

Continuous variables are presented as mean (range), and categorical variables are presented as n (%). Two-sample *t* tests and χ^2 tests were used to compare the means of continuous phenotypes and the distribution of categorical phenotypes, between AAA and control groups, respectively. Missing values were excluded from the calculations of each variable. Hypertension was defined on the basis of clinical history and the use of antihypertensive medication. Dyslipidemia was diagnosed through clinical history and the use of hypolipidemic medication. Diabetes was identified by clinical history and the use of insulin or oral hypoglycemic medications, without differentiation between type 1 or type 2. Peripheral artery disease was assessed on the basis of clinical symptoms and clinical history. Other aneurysms included thoracic and visceral aortic aneurysms, iliac artery aneurysms, and popliteal artery aneurysms, and were diagnosed using computed tomography or ultrasound. Cerebrovascular diseases were determined by a history of transient ischemic attack or stroke. Cardiovascular diseases were assessed by history of acute myocardial infarction or angina pectoris, or admission with clinical symptoms, ECG changes, or a positive enzymatic curve diagnosed by a cardiologist. Chronic obstructive pulmonary disease was identified on the basis of clinical history. Chronic kidney disease was assessed by clinical history. Medication data (antihypertensive, nonsteroidal anti-inflammatory drugs, glucocorticoids, statins, and anticoagulants) was extracted from the medical history. Medication use in the control group was not always recorded and may have been underestimated.

ensured that the sum of all TPMs in each sample was the same, facilitating the comparison between samples. All samples reached a minimum of 10 million reads aligned to the reference genome with STAR (Figure S1A and S1B).[16]

As part of the quality control, we also checked that the reported sex of the samples matched the biological sex of the sequenced data. To do this, we compared the expression levels of the *XIST* gene, which regulates the X chromosome inactivation mechanism in women and has null expression in men, with the expression of male-exclusive genes, calculating an average expression of Y chromosome genes. One sex mismatch sample was removed from the study (Figure S1C).

Differential Expression Analysis

Before conducting differential expression analyses, we normalized the TPM counts using quantile normalization and removed lowly expressed genes with <0.5 TPMs in >50% of the samples. For the comparison of AAAs against control samples, we kept 14 675 genes and removed 12 615 genes. For the comparison among AAAs of varying diameters using the AAA-only sequencing panel, we kept 14 779 genes and removed 12 511 genes.

To evaluate the impact of technical covariates on the results, we performed a principal component analysis on all samples and tested the correlation between the principal component analysis and all the technical covariates. Technical covariates that had significant correlations ($P<0.05$) with the first 4 principal components were included in the analyses as fixed-effect covariates (Table S1). Additionally, all comparisons were adjusted for age and sex. To preserve the regulatory effects that act through smoking, we did not include smoking as a covariate.

First, we calculated DEGs between AAA and controls using a linear regression, including age, sex, flow cell type, flow cell lane, GC mean content, RIN, percentage of RNA fragments >200, and Qubit as covariates.

Date of creation of the library was not included due to its high correlation with the status variable (Pearson's correlation=−0.74) (Figure S2A and S2B). Second, we calculated DEGs between aneurysms of varying diameter using a linear regression, including age, sex, date of creation of the library, batch number, GC mean content, RIN, percentage of RNA fragments >200, and Qubit. We corrected for multiple testing in both analyses using the Benjamini–Hochberg false discovery rate (FDR) method and considered significant DEGs those with an adjusted *P* value <0.05.[19] A sensitivity analysis was conducted to address the potential index event bias in the differential expression analysis by diameter. Further details are provided in Data S1.

Additionally, we performed a linear regression model using ischemic time information on artery aorta tissue samples from Genotype-Tissue Expression (GTEx) version 8 data[20] to identify genes whose expression could be altered by ischemic time and compared these to our DEG results. We corrected the linear regression model for age, sex, RIN, type 2 diabetes, body mass index, autolysis score, center, sequencing protocol, sequencing platform, and genotyping principal components. To determine the genes affected by ischemic time, we corrected for multiple testing and selected genes with an FDR-adjusted *P* value <0.05.[19] All analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria).

We considered novel DEG those genes that were differentially expressed in our analysis but had not been identified in earlier microarray studies between AAA samples and controls.[10–15]

Enrichment Analysis

After identifying DEGs between AAAs and control samples and between AAAs of varying diameters, we performed enrichment analyses using the R package 'clusterprofiler'[21,22] on the Gene Ontology (GO) databases for Biological Process, Cellular Component, and Molecular Function, as well as the Kyoto Encyclopedia of Genes and Genomes.[23] We corrected for multiple testing using the Benjamini–Hochberg FDR method and identified significantly enriched pathways with an adjusted *P* value <0.05.[24] We used "aPEAR"[25] to perform a cluster analysis of redundant pathways with a minimum cluster of size of 15 and hierarchical clustering. The enriched pathways and genes were visualized using "enrichplot."[26]

Study of the Inflammatory Infiltrate

We investigated the differences in the proportions of inflammatory infiltrates between AAAs and control samples using CIBERSORTx.[27] CIBERSORTx compares RNA sequencing data with a reference expression database of selected cell types, to estimate the proportion of each cell type. The residuals of our RNA sequencing data were calculated using a linear regression that included all covariates except the status variable. We used the residuals and the "lm22" signature matrix that contains expression data of 547 genes in 22 inflammatory cell types from microarray studies and can be used to distinguish inflammatory cell populations in RNA sequencing data.[27] We conducted a *t* test to compare the proportions of each cell type between AAAs and controls. A *P* value threshold corrected by multiple testing using Bonferroni for the number of cell types was set at $P<2.27\times10^{-3}$ (0.05/22).

Alternative Splicing

We used the SUPPA2 software to identify differences in splice events between AAA and control tissue samples. SUPPA2 can identify 7 different splice events including skipping exons, mutually exclusive exons, alternative 5′ or 3′ splice sites, retained introns, and alternative first or last exons. Since no external reference for splicing events was available in GTEx, we used our control samples as internal reference to characterize differential splicing events in AAAs. Based on gene annotation from GENCODE version 26,[18] we computed the proportion of splice inclusion for the TPM counts in each splice event by dividing the number of TPMs of 1 form of the event by the total number of TPMs. Finally, the magnitude of splicing change was calculated by subtracting the proportion of splice inclusions between AAAs and controls. Significant alternative splicing events were selected on the basis of a magnitude of splicing change >0.1 and FDR-corrected *P* <0.05, using the default parameters for calculation.

Allele-Specific Expression

Allele specific expression was investigated using PHASER[28] on 12 AAA samples with available genotype data. Allele-specific expression consists of the analysis of the differences in the expression levels of the different haplotypes present in a heterozygous individual. We quantified allele specific expression at the gene level using the GENCODE version 26 gene annotation.[18] To reduce the effect of the known mapping bias toward the reference allele[29], we performed an additional STAR mapping step with WASP filtering.[30]

We used the allele specific expression data obtained by the GTEx consortium to compare allele-specific expression between our AAA samples and GTEx control tissues.[31] To do this, we compared the proportional expression of each allele between our AAA samples and the GTEx controls, using a nonparametric Wilcoxon test.[32] Then, to identify genes with distinct allele-specific expression patterns, we corrected for multiple comparisons using FDR.[19] We performed

enrichment and cluster analyses in all genes showing FDR-adjusted *P* values <0.05.

RESULTS

Participant Characteristics

The Table shows the participant demographic and clinical data. Aortic tissue samples from 96 patients with AAA and 44 controls from the TABS cohort were used for RNA sequencing analysis.

We examined demographic and clinical variables between patients with AAA and controls to determine whether these might influence our expression-level differences. We found significant differences between patients with AAA and controls in sex (Figure S3A); age (Figure S3B); smoking status; the prevalence of hypertension, dyslipidemia, cerebrovascular disease, and chronic obstructive pulmonary disease; and medication use, including antihypertensives, nonsteroidal anti-inflammatory drugs, statins, and anticoagulants. In relation to smoking status (N=122), which is a known risk factor for AAA development and rupture, significant differences were found in never smokers and past smokers, with 70.27% and 8.11% in controls, and 17.86% and 50% in patients with AAA, respectively. No significant differences were found in current smokers (Table, Figure S3C).

Differential Expression Analyses Between AAAs and Controls

The analysis of differential expression between aortic samples from 96 patients with AAA and 44 controls revealed 7454 genes displaying significant differences in expression (adjusted *P*<0.05) (Figure S4A and Table S2). Using GO and Kyoto Encyclopedia of Genes and Genomes enrichment analyses, we found a total of 1152 and 89 enriched terms, respectively (Figure S5A and Figure S6A). The complete results of the enriched terms for GO and Kyoto Encyclopedia of Genes and Genomes are shown in Tables S3 and S4. To better characterize the biological processes associated with the DEGs in the enrichment analysis, we performed a cluster analysis of GO pathways. We found that most of the DEGs were associated with the immune system: regulation of mononuclear cell proliferation, leukocyte chemotaxis, regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains, mast cell degranulation, major histocompatibility complex (MHC) class II protein complex, positive regulation of T-cell activation, and cluster of differentiation (CD) 4–positive α-β T-cell differentiation (Figure 2A). Other represented metabolic pathways were related to sequestering of calcium ion, regulation of actin filament length, and ATP synthesis coupled electron transport (Figure 2A). All these analyses corroborated previous associations with inflammatory, actin filament, intracellular calcium, and ATP synthesis regulation processes. The results of the differential expression analysis after adjusting for significant confounders are provided in Data S1.

To account for differences in gene expression between AAA and control tissue that could be attributed to ischemic time (time between the donor's death and sample collection when blood flow is interrupted), we removed 10 737 DEGs associated with ischemic time in the GTEx aorta samples (N=387)[20] from the total DEGs found between AAAs and controls, leaving 3002 DEGs (Figure S4B and Table S5).

We then performed a new enrichment analysis and identified 424 enriched GO terms and 65 Kyoto Encyclopedia of Genes and Genomes pathways (Figure S5B and Figure S6B) (complete results are available in Tables S6 and S7, respectively), which represented removal of 728 and 24 pathways susceptible of being caused by ischemic time, respectively. Cluster analysis of GO enriched terms confirmed identified clusters related to the regulation of calcium ion retention, ATP synthesis coupled electron transport, and immune response centered on T-cell activation (MHC class II protein complex, positive regulation of T-cell activation). On the other hand, other clusters also associated with the immune system were no longer represented (regulation of mononuclear cell proliferation, leukocyte chemotaxis regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains, CD4-positive, α-β T-cell differentiation, mast cell degranulation) together with the regulation of actin filament length (Figure 2B). By accounting for genes whose expression was altered by ischemic time, we identified a set of genes that are less likely to be affected by the experimental limitations of these types of studies.

Vascular inflammation is a well-known driver of AAA development and progression. Even in our most stringent analysis, which removed pathways possibly caused by ischemic time, there was a notable enrichment of pathways associated with the immune response. Consequently, we decided to investigate the influence of the inflammatory infiltrate on AAAs by comparing the abundance of 22 immune cell types from gene expression profiles between our AAA and control samples. After correcting for multiple testing, we found significant differences on the abundances of CD8 T cells, natural killer (NK) resting cells, and dendritic activated cells (Figure 2C) between AAA and control samples. AAA samples had a higher proportion of CD8 T cells, while controls had more NK resting cells and dendritic activated cells (complete results of the study of the inflammatory infiltrate in AAA and control samples are available at Table S8 and Figure S7).

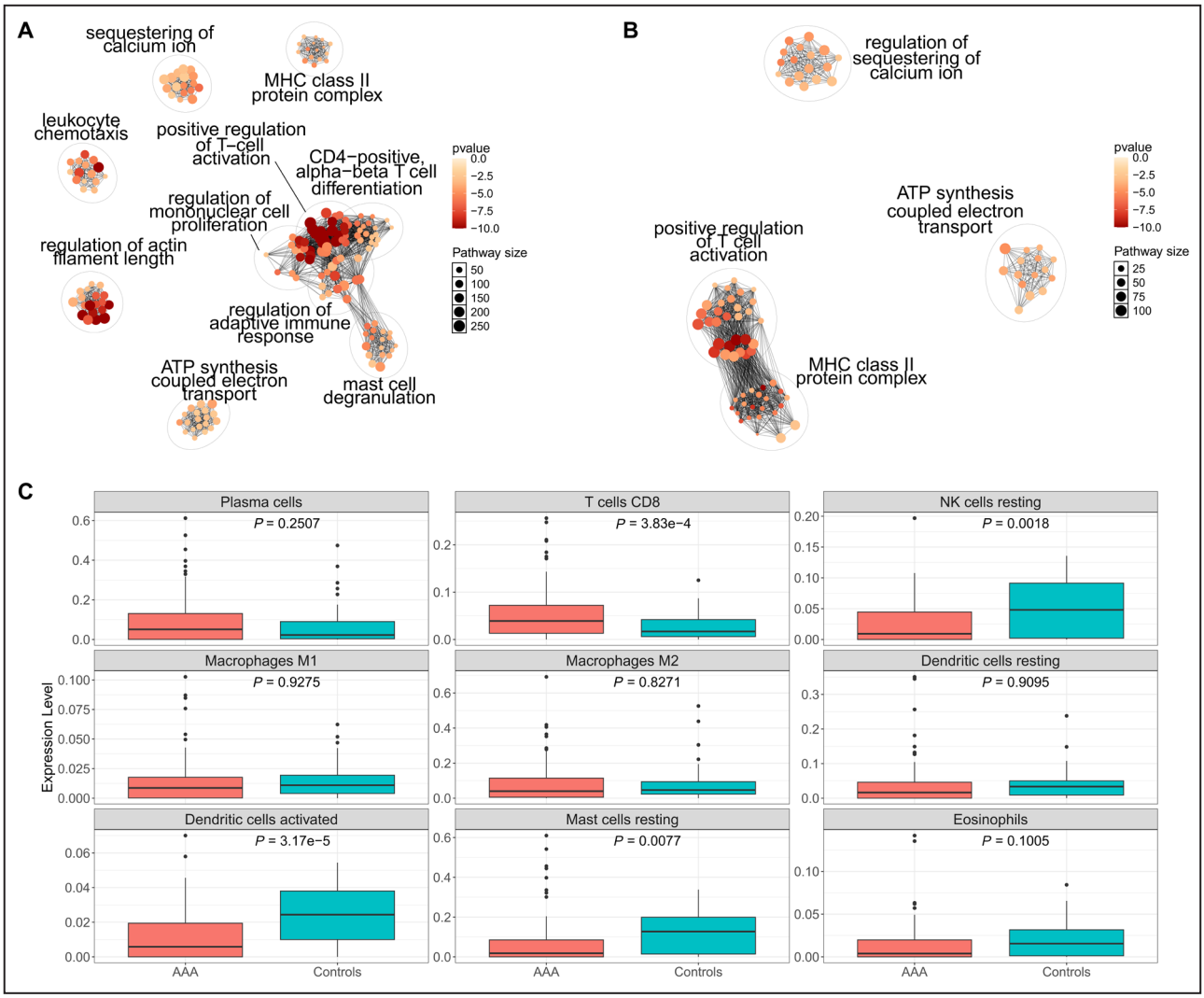

Figure 2. Differential gene expression and immune cells analyses in AAA.
Hierarchical clustering analysis results with all the DEGs between AAA and control samples (**A**) and after removing DEGs by ischemic time (**B**). The cluster name "Regulation of mononuclear cell proliferation" in 2A is an abbreviation for "Regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains." **C**, Comparison of the proportion of inflammatory cells between AAA and control samples, using CIBERSORTx. *P* values correspond to a *t* test comparing the proportions' means of each cell type between AAAs and controls. AAA indicates abdominal aortic aneurysm; CD, cluster of differentiation; DEGs, differentially expressed genes; MHC, major histocompatibility complex; and NK, natural killer.

Study of Alternative Splicing

We investigated alternative splicing patterns between the AAA and control groups to identify specific splicing patterns associated with AAA. We identified 15 significant alternative splicing events on 11 unique genes (*FHL1*, *GNAS*, *ASAH1*, *SPP1*, *ARL1*, *MORF4L2*, *CYCS*, *HMGB1*, *HMGN1*, *SELENOP*, *RNASE4*) between AAAs and controls. The analysis revealed that the number of alternative first exon events was more represented than any other splice event among AAA and control samples (Figure 3A; complete results are available in Table S9). A functional enrichment analysis was conducted on the 11 genes with significant alternative splicing events, but no significantly enriched metabolic pathways were found. Interestingly, 7 of the 11 genes were also differentially expressed between AAAs and controls, suggesting that the expression of specific splicing variants could be altered in AAAs (Figure 3B).

Differential Expression Analysis by Aortic Diameter

The diameter of AAAs is a significant risk factor for rupture. We analyzed the DEGs by diameter to identify alterations in gene expression throughout the progression of the disease. We observed a total of

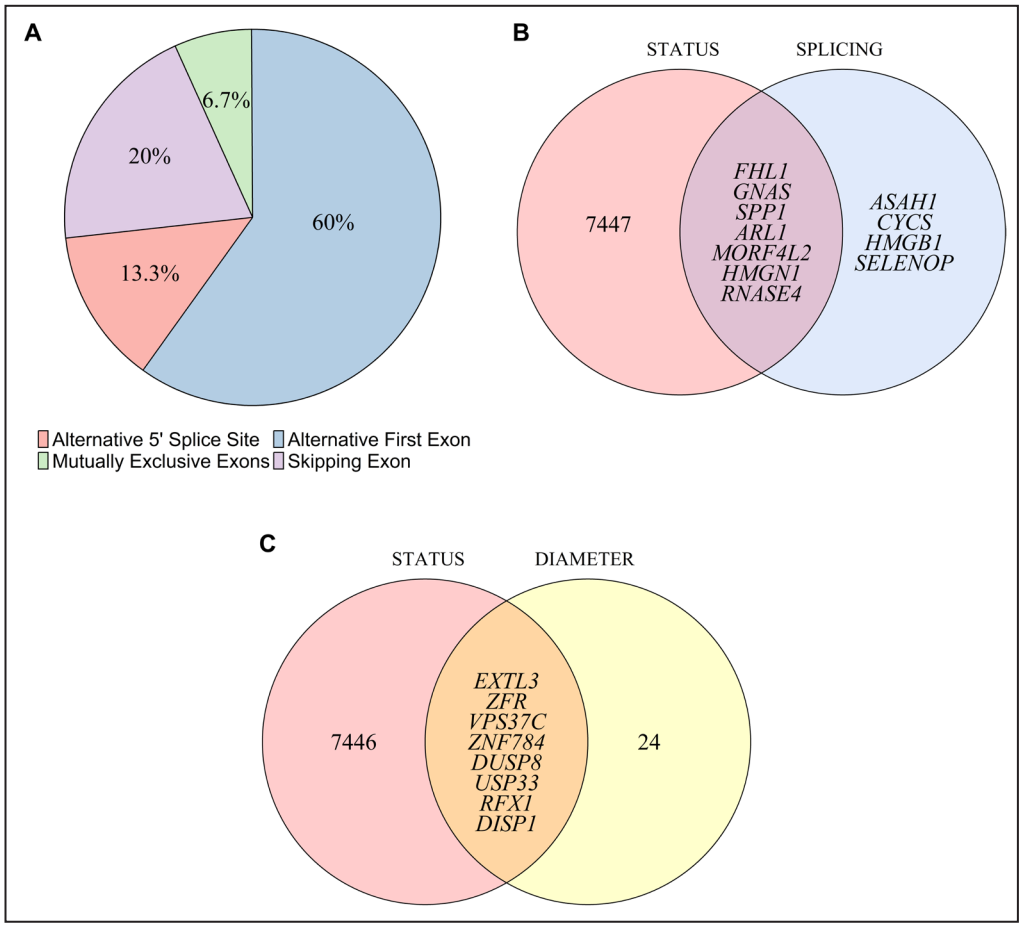

Figure 3. Alternative splicing and differential gene expression analyses results.
A, Significant alternative splicing types identified between AAA and control samples. **B**, Venn diagram showing the overlap between DEGs in AAA and control samples, and genes with differential alternative splicing patterns in AAA and control samples. **C**, Venn diagram showing the overlap between DEGs in AAA and control samples, and DEGs by AAA diameter. DIAMTER, DEGs by AAA diameter; SPLICING, genes with differential alternative splicing patterns in AAA and control samples; and STATUS, DEGs between AAA and control samples. AAA indicates abdominal aortic aneurysm; DEGs, differentially expressed genes; and MHC, major histocompatibility complex.

32 DEGs among aneurysms of varying diameters (N=84), although no enriched pathways were identified (Figure S4C and Table S10). Of the 32 DEGs by diameter, 8 (*EXTL3*, *ZFR*, *DUSP8*, *DISP1*, *USP33*, *VPS37C*, *ZNF784*, *RFX1*) were also DEGs between AAAs and controls (Figure 3C), suggesting that these 8 genes are altered in disease formation and also during disease progression. We performed a sensitivity analysis adjusting for smoking status, a known risk factor associated with both AAA onset and progression, to control for a potential index bias. The analysis revealed that among the 32 genes associated with diameter, 19 remained associated after adjusting for smoking. Additionally, among the 8 genes that were differentially expressed in the AAA–control analysis and also among different diameters, 4 (*EXTL3*, *ZFR*, *DUSP8*, *RFX1*) remained associated after the adjustment for smoking.

Allele-Specific Expression

We investigated the potential effect of AAA-associated genetic variants on gene expression in diseased tissue by studying allele-specific expression in 12 AAA samples with available genetic data. On average, we identified 529 genes with significant allele-specific expression (adjusted $P<0.05$) in the 12 AAA samples. Among these genes, 90 exhibited significant allele-specific expression in >5 of the 12 AAA samples. Additionally, to determine whether these associations were related to AAA or were characteristic of the aortic tissue, we compared allele-specific expression patterns between our AAA samples and 387 GTEx aortic samples, used as controls. The comparison between AAA and control samples identified 1815 genes with significant differences in the allele-specific expression patterns (adjusted $P<0.05$; Figure 4A). An enrichment

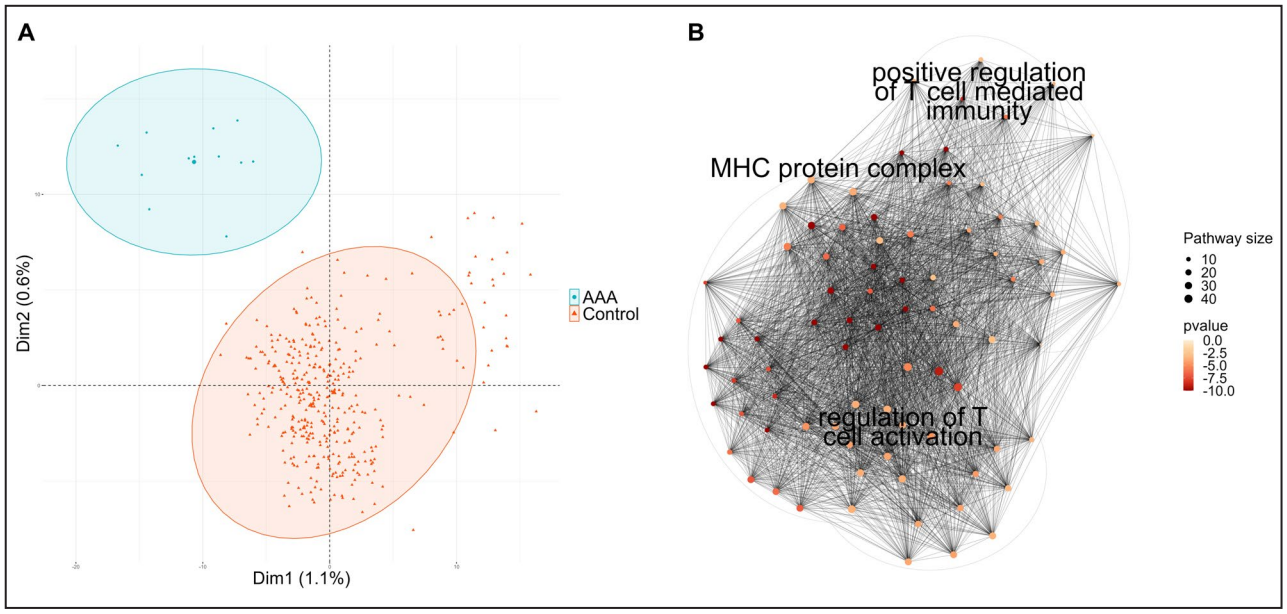

Figure 4. Allelic specific expression results.
A, Results of principal component analysis clustering based on allele-specific patterns between AAA and control samples. **B**, Hierarchical clustering analysis results with genes with different allele-specific patterns between AAA and control samples. AAA indicates abdominal aortic aneurysm; Dim1, dimension 1; Dim2, dimension 2; and MHC, major histocompatibility complex.

analysis on GO terms for these 1815 genes revealed 91 enriched pathways. The posterior cluster analysis revealed 3 clusters strongly related to the immune system: MHC protein complex, positive regulation of T-cell–mediated cytotoxicity, and regulation of T-cell activation (Figure 4B). The association between the immune system and AAAs was also observed in the differential expression analysis between AAA samples and controls, validating the robustness of these results.

Finally, among the 90 genes that exhibited significant allele-specific expression in >5 AAA samples, we selected those that also showed significant differential allele-specific expression between AAA and GTEx control samples, and those present in loci identified in the largest genome-wide association studies (GWASs) on AAA risk to date,[7] to identify haplotypes associated with AAA risk. Among the selected genes, *SNURF* was the only gene that also presented differential allele-specific expression patterns between AAA and control tissues, and *SPP1* and *THBS2* were prioritized on the basis of their presence in a locus identified in the previous GWAS on AAA. This allowed us to hypothesize that the presence of particular genetic haplotypes in these 3 genes determined their differential expression associated with risk of AAA, providing supporting evidence of their putative causal effect on disease risk.

DISCUSSION

This study analyzed differential expression between AAA aortic tissue samples and control aortic samples using whole transcriptome data obtained through RNA sequencing and identified 7454 DEGs. In addition, we studied the effect of ischemic time on gene expression to obtain a more credible list of genes associated with AAA development, which resulted in 3002 DEGs, of which 1452 were new. Using our RNA sequencing data, which allows alternative splicing analysis compared with microarrays,[33] we conducted a novel exploration of alternative splicing between AAA and control samples, to identify the potential underlying mechanism of the observed differential expression. Furthermore, we analyzed the differential expression between AAA of different diameters to identify genes altered during disease progression. Finally, we analyzed allele-specific expression to gain insights into how genetic variants impact expression in the diseased tissue and provide evidence for causality.

Study of Ischemic Time-Independent Pathways Involved in AAA Development

Clustering analysis with the enriched pathways after accounting for the ischemic time effect revealed a strong association with MHC class II protein complex, positive regulation of T-cells, intracellular calcium ion regulation, and the regulation of the ATP synthesis clusters of pathways. The results of our clustering analysis confirm the previously observed associations with AAAs while also suggesting that these associations are independent of ischemic time, which is a significant confounder factor in most studies using donor

samples. A detailed analysis of the signaling pathways that comprise the ATP regulation cluster revealed a large presence of genes that code for subunits of complexes I (nicotinamide adenine dinucleotide ubiquinone oxidoreductase), III (ubiquinol-cytochrome c reductase), and IV (cytochrome c oxidase) of the electron transport chain. Our results indicate that 88% (22/25) of the DEGs coding for the subunits of the complexes that form the electron transport chain are expressed to a lower extent in AAAs, suggesting a lower synthesis of ATP in AAA tissue. This is consistent with 2 previous microarray studies in which the oxidative phosphorylation pathway was downregulated among AAA samples compared with controls.[10,12] Moreover, mitochondrial dysfunction has previously been studied in the development of AAAs[34,35] and other cardiovascular diseases[36] due to its key role in cellular alterations characteristic of cardiovascular diseases, including excessive production of reactive oxygen species, energy depletion, endoplasmic reticulum stress, and activation of apoptosis.

Several genes from the intracellular calcium regulation cluster have already been investigated for their role in AAAs.[37–42] Our findings suggest that intracellular calcium levels in smooth muscle cells were altered in AAA samples, which is consistent with the loss of vascular contractility in the dilated aorta.[43]

The association between the immune system and inflammatory response and AAA have widely been validated in previous similar studies on the basis of microarrays.[10–13,15] Further, there is a widely studied inflammatory component in AAA development involving both adaptative and innate immune responses.[44,45] The presence of inflammatory infiltrates in AAA tissue have been widely demonstrated, which play a key role in the development of the disease.[44,45] The results of our cluster analysis also corroborated the association with the immune system after accounting for ischemic time, highlighting the key role of T cells on AAA development.[46,47]

To better understand the effects of the inflammatory infiltrate in AAA development, we compared the proportions of inflammatory infiltrates between AAA and control samples. Some previous studies have also analyzed the inflammatory infiltrates in AAA tissue through gene expression.[15,39] In one study,[15] immune cell proportions were estimated in AAA tissue layers (media and adventitia) (N=76), without comparing with controls. Their results, suggesting that plasma B cells and M2 macrophages were the 2 most represented cell populations in both layers, and M1 macrophages, eosinophils, and activated dendritic cells were among the least represented cells in both layers are consistent with our findings using whole aortic tissue. Consistent with our results, another study that combined 2 whole-tissue data sets on AAA (N=69) and control tissues (N=10),[39] found that resting mast cells were among the most frequent cell groups in AAA samples. In addition, our results align with this study in detecting higher proportions of CD8 T cells and lower proportions of resting NK cells in AAA samples.

We found a significantly lower proportion of activated dendritic cells in AAA tissue samples compared with control samples, which has not been detected in previous studies. Previously, dendritic cells had been suggested as AAA inducers through the promotion of infiltration and activation of neutrophiles and lymphocytes.[44,45] Interestingly, the whole-tissue study that combined 2 existing data sets, observed a significant increase in the proportion of resting dendritic cells within AAA samples compared with controls.[39] Future studies are needed to explore a putative role of dendritic cells in AAAs.

To rule out postmortem effects on expression as potential cause of variability among different studies analyzing the inflammatory infiltrate, we used a reference work evaluating changes in postmortem blood samples,[48] which found higher levels of resting NK cells and CD8 T-cells in postmortem samples. These findings suggested that the observed increase in CD8 T-cell levels in AAA tissue could be underestimated in our results, and that the greater presence of NK resting cells in control samples could be due, in part, to their origin from organ donors. Consistent with this hypothesis, previous studies have shown that levels of NK cells are higher in the peripheral blood of patients with AAA compared with controls,[49] and that these cells play a role in the development of the disease.[44,45] These results also suggest the existence of a highly cytotoxic environment led by CD8 T cells in AAA tissue.

Finally, we compared DEGs between AAA and control samples, with the candidate genes identified in a recent large GWAS on AAA risk[7] and found that in 84 of the 121 (69.42%) loci, 1 of the candidate genes was differentially expressed in the present study. This suggests that differential expression in these genes could be causing the association with disease and providing additional evidence to pinpoint the true causal gene in each associated locus.

Study of Alternative Splicing Between AAAs and Controls

The relevance of alternative splicing in the development of diseases, such as cancer and neurological and cardiovascular diseases, has been well established for years.[50] However, capturing the complexity of alternative splicing has been challenging. With the recent improvements in sequencing techniques, it is now possible to study alternative splicing in more depth.[50] This is the first study to elucidate the role of splicing in AAA development. We compared splice

events between AAAs and controls and identified 11 genes (*SPP1*, *FHL1*, *GNAS*, *MORF4L2*, *HMGN1*, *ARL1*, *RNASE4*, *ASAH1*, *CYCS*, *HMGB1*, *SELENOP*) with differentially represented splicing variants. We compared the splicing event types identified in our comparison between AAA and control samples with the presence of splicing events in the whole genome,[51] and the observed proportions were comparable to the expected values genome-wide. The most frequent splicing types were alternative first exons (60%) and skipping exons (20%), while the least frequent were alternative 5′ splice-site (13.3%) and mutually exclusive exons (6.67%). On the other hand, it was surprising that our results did not include alternative last exons or alternative 3′ splice-site and retained introns, despite their considerable genome-wide frequencies (10.72%, 9.2%, and 3.54%, respectively). This may be due to the limited sample size, as only 15 events were identified.

We observed 7 genes that showed differential expression between AAA and control samples and had a splicing variant significantly more represented in AAAs or controls (*SPP1*, *FHL1*, *GNAS*, *MORF4L2*, *HMGN1*, *ARL1*, *RNASE4*), indicating that splicing differences could be explaining the observed differential expression. Among these genes, *SPP1* and *FHL1* have been previously characterized in relation to AAAs,[52,53] whereas *GNAS* is a new DEG identified between AAA and control tissue in this study. The evidence of differential splicing events validates *GNAS* as a new robust DEG between AAA and control tissue and suggests that alternative splicing in this gene explains the differential expression and its implication to AAAs. Finally, for *MORF4L2*, *HMGN1*, *ARL1*, and *RNASE4*, although they have been identified in previous differential expression studies in relation to AAAs,[10–15] their specific role in AAAs has not been studied. For these genes, our results contribute to understanding the molecular mechanism leading to differential expression in AAA tissue.

SPP1 codes for osteopontin, a regulator of inflammation involved in cardiovascular diseases.[54] *SPP1* is more expressed in AAA tissue than controls, both in animal models and in humans, and it participates in AAA-associated extracellular matrix degradation.[55,56] Consistent with previous data, our results found increased expression in AAA tissue, and identified for the first time that skipping of exon 3 on the *SPP1* gene is more frequent in AAAs than in controls, suggesting that this form of alternative splicing may be associated with AAA development.

FHL1 codes for a protein that is highly expressed in skeletal and cardiac muscle. *FHL1* has been shown to be a promising blood biomarker for human ascending thoracic aortic aneurysm as a modulator of metalloproteases.[53] Our findings, and those obtained in previous microarray studies,[10–13,15] indicate that *FHL1* levels are lower in AAA tissue than in control tissue. We have detected for the first time that an alternative 5′ splicing site form in this gene occurs more frequently in controls, suggesting that AAA tissue would have reduced expression of this alternative isoform and reduced levels of *FHL1*.

Although not previously found in transcriptomic studies, mutations in the *GNAS* gene have been studied in mice for their effect promoting AAAs. *GNAS* codes for the α subunit of the heterotrimeric G stimulatory protein.[57] Heterotrimeric G stimulatory protein may play its protective role in AAA development through regulation of vascular muscle tissue and is considered a potential therapeutic target for AAAs.[57] Consistent with this protective role, our results confirmed lower *GNAS* expression in AAAs. Moreover, our results add a mechanistic insight by revealing an alternative first exon splicing variant that occurs more frequently in controls, suggesting that it could increase expression levels of the final protein and protect against AAAs.

MORF4L2 and *HMGN1* are DNA repair–related genes, which had significantly lower and higher expression levels in AAA tissue compared with control tissue, respectively. *MORF4L2* has been associated with atheroma plaque progression in atherosclerosis.[58] Both genes present alternative splicing events that are less frequent in AAA tissue. Further work to elucidate the specific role of these genes in the risk of AAA development is warranted.

Genes Associated With AAA Onset and Progression

We identified 8 genes (*EXTL3*, *ZFR*, *DUSP8*, *DISP1*, *USP33*, *VPS37C*, *ZNF784*, *RFX1*) that showed differential expression between AAA and control tissues, and also differential expression in AAA tissues of different diameters. Among these, *EXTL3*, *ZFR*, *DUSP8*, and *DISP1* have been previously identified as DEGs between AAAs and controls, but their association with AAA progression is novel,[10–15] and *USP33*, *VPS37C*, *ZNF784*, and *RFX1* are novel DEGs. The potential contribution of these genes to the development of AAAs needs to be thoroughly investigated to elucidate a putative role on disease onset and progression.

Among the genes with higher expression at larger diameters, *USP33* encodes a deubiquitinating enzyme that has been associated with the development of hypertension,[59] a known risk factor for AAAs.[60] Proteins with deubiquitinating functions have been proposed as potential candidate genes for treating AAAs and other cardiovascular diseases.[61] Additionally, *USP33* promotes the stabilization of β_2-adrenergic receptors, promoting vasodilation in smooth muscle.[62] We observed increased *USP33* expression with increased AAA diameter, which is consistent with the functionality of β_2-adrenergic receptors. It

is important to note that *USP33* lost significance in the sensitivity analysis adjusting for smoking status, indicating that its effect may have been overestimated due to index event bias. Nevertheless, the biological plausibility of *USP33*'s involvement in AAA progression remains strong, and further investigation is warranted.

Among the genes showing decreased expression levels with larger diameters, we highlight *DUSP8* and *RFX1*. *DUSP8* is a phosphatase downregulated in mouse models of aortic dilatation[63] that negatively regulates the mitogen-activated protein kinase pathway and controls the execution of immune responses.[64] Our findings align with those obtained in the animal model of aortic dilatation, suggesting that *DUSP8* may be a potential candidate gene for treating AAAs by regulating both aortic dilatation and the immune system.

RFX1 encodes a transcription factor that regulates genes involved in MHC class II,[65] and previous research has shown that a decrease in *RFX1* expression leads to activation of $CD14^+$ monocytes in patients with coronary artery disease.[66] Furthermore, CD14 protein plays a crucial role in recruiting macrophages during the early stages of AAAs, and knockout mice for *CD14* gene have been shown to resist the formation of AAAs in 2 different models.[67] Finally, our expression results indicate that *CD14* is upregulated in patients with AAA compared with controls (FDR P=0.002). Overall, these results suggest that low expression levels of the transcription factor *RFX1* may lead to increased *CD14* expression, which plays a crucial role in the development and progression of AAAs.

Two previous studies have considered the diameter of AAAs in differential expression analyses with aortic tissues. The first study[10] compared gene expression between small (n=20) and large AAAs (n=29) with controls, but did not compare AAAs of varying diameters. The second study[15] performed a correlation analysis on each gene between the diameter growth rate and gene expression in individuals (n=24) with 2 aortic measurements, distinguishing between the media and adventitia aortic layers, but did not identify any significant genes after multiple testing correction. The larger sample size in our study has enabled the identification of DEGs associated with aneurysm diameter for the first time. Further functional validation and the establishment of causality between these genes and AAAs could pave the way for the development of novel therapeutic targets aimed at halting aneurysm progression. This could have a significant impact on clinical practice by providing new opportunities for intervention in patients with AAA.

Identification of Haplotypes Associated With AAA Risk

We performed a clustering analysis of enriched biological pathways with the 1815 genes with significant differences in the allele-specific expression patterns between AAAs and controls. The cluster analysis revealed a strong association with immune system pathways, particularly those associated with T cells. These results are consistent with the clustering analysis of DEGs between AAAs and controls and demonstrate how specific haplotypes determine the differential expression of genes associated with AAA in the diseased tissue. By combining allele-specific expression information on AAA samples with the allele-specific expression results between AAA and controls, along with information from a previously published GWAS,[7] we explored the associations between genetic variants associated with AAA risk and gene expression. This approach aimed to unravel the association between genetic haplotypes and gene expression as determinants of AAA risk.

SNURF is the only gene that showed significant allele-specific expression in >5 of the 12 studied individuals with AAA and differential allele-specific expression patterns between AAA and control tissues, suggesting the existence of a specific haplotype associated with AAA risk. *SNURF* codes for an open reading frame of the *SNRPN* gene, with which it forms a bicistronic gene.[68] Both genes exhibit significantly lower expression in AAA than in controls in our data. Moreover, we observed that the rs705 single nucleotide polymorphism (minor allele frequency [MAF] [C]=0.45) determined *SNURF* allelic expression in 8 of 12 AAA samples, with the T allele being expressed twice as often as the C allele. Additionally, rs705 is an expression quantitative trait locus in blood of the *SNRPN* gene. Although causality cannot be derived with certainty from these results, they suggest a possible effect of the haplotype containing the rs705 T allele on *SNURF* expression and risk of AAA.

Among the genes with significant allele-specific expression in the 12 analyzed AAA samples that did not exhibit significantly different allele-specific expression patterns between AAA and control groups in our analyses, we selected those that were within a locus associated with AAA in the most recent GWAS on AAA risk.[7] *THBS2* encodes for thrombospondin 2, a protein that regulates cell–cell and cell-extracellular matrix interactions and has been studied in relation to multiple cardiovascular diseases.[69] *THBS1*, a member of the same family, has been identified as a regulator of AAA in animal models.[70] Two common genetic variants (rs58023137 [MAF] [T]=0.22) and rs9505895 (MAF [A]=0.2) were identified in previous GWASs[7] and in our allele-specific expression results. For both variants, the GWAS risk allele was associated with higher expression gene levels in 1 individual with AAA. Additionally, these 2 variants were also expression quantitative trait loci in aortic tissue regulating *THBS2* gene expression, supporting the known association between

thrombospondin and AAA,[7] and demonstrating the presence of risk haplotypes associated to increased expression and risk of AAA.

Regarding the *SPP1* gene, we found 6 genetic variants (rs35893069 [MAF] [T]=0.1), *rs6839524 (MAF [G]=0.12),* rs4754 (MAF [C]=0.28), rs1126616 (MAF [T]=0.27), rs1126772 (MAF [G]=0.21), rs9138 (MAF [C]=0.22) with allele-specific expression among the 12 AAA samples. *SPP1*, encoding for osteopontin, has been associated with AAA risk due to the role of this protein in the degradation of the extracellular matrix, 1 of the hallmarks of AAA.[55,56] However, none of the 6 genetic variants were found to be significant in the previous GWAS,[7] and therefore no association with allelic expression could be established.

Strengths and Limitations

This is one of the largest studies of differential expression between AAA tissue samples and controls, and we see this as a strength. However, due to the difficulty in obtaining aortic tissue samples, the sample size is still limited compared with transcriptomic studies in more accessible tissues, which may have limited our power.

Previous studies comparing transcriptomics between AAA and control samples have been limited to the use of expression microarrays.[10–15] In this study, we used RNA sequencing to obtain transcriptomic information. RNA sequencing provides greater sensitivity and a wider range of detection of both high and low expression genes and is not limited to microarray probes,[9,71] which allowed us to detect more DEGs. Additionally, RNA sequencing technology allowed for a more precise study of alternative splicing compared with microarrays, which is also a strength of this study. A further significant distinction between our study and previous research on AAA tissue differential expression is the consideration of genes known to be associated with ischemic time. The impact of ischemic time in previous studies analyzing differential expression with control samples obtained from donors has been a limitation in the field. By excluding genes affected by ischemic time, we anticipate more robust results.

In addition, our results should be interpreted within the context of the limitations of this study.

First, it is important to note that, while this study contributes to a more comprehensive understanding of the AAA pathophysiology and to the identification of potential new targets for further investigation, this does not necessarily imply the existence of a causal association between the identified genes and the disease. Some of the pathways identified in our study may be reactive responses to aortic dilatation rather than drivers of AAA progression. Further investigation is necessary to distinguish between pathways that are primary contributors to AAA progression and those that are secondary consequences of the disease. Similarly, identified DEGs by diameter indicate genes associated with advanced stages of AAA, but their specific roles in disease progression need to be further elucidated.

Second, this study was performed analyzing bulk RNA from tissue material. Alternatively, 2 previous single-cell RNA sequencing studies have been performed on 4 AAA human samples.[72,73] Single-cell RNA sequencing studies are useful for detecting transcriptomic differences in specific cell types and determining the altered pathways in each cell population.[74] However, the use of this technology comes with significantly higher costs compared with bulk RNA sequencing, which would imply the use of significantly smaller sample sizes and, therefore, a reduction in the statistical power. Additionally, gene expression might be heterogeneous between different samples of the same AAA, due in part by the coexistence of different stages of AAA development and disease progression in the same tissue. Because only 1 tissue sample was analyzed per individual, localized differences within the AAA tissue could have influenced the differential expression results in a way that did not necessarily reflect the whole AAA expression or status.

Third, while ischemic time data were not available for our control samples, ischemic time is known to influence gene expression in control samples obtained from deceased multiorgan donors. Therefore, by accounting for ischemic time, we were able to more accurately identify biological processes associated with AAA, while minimizing differences in sample origins between AAAs and controls. At the same time, despite ensuring more robust findings, the elimination of these genes could have been too rigorous and imply the loss of some biological pathways associated with AAA that, at the same time, were affected by ischemic time.

Fourth, the significant differences in age and sex distributions between individuals recruited as AAAs and controls are a limitation of the study. In addition, to minimize the impact of confounding variables on gene expression, we corrected for relevant technical variables, in addition to age and sex, that were available for all individuals. However, we chose not to adjust for all significant confounding factors, described in the Table, in our main analyses, as this would have considerably reduced the sample size and statistical power due to missing data. Specifically, adjustment for these confounders would have resulted in the loss of 15% of the individuals with AAA and 30% of the control samples. Despite acknowledging that this may be a limitation of the study, the fully adjusted results present high concordance with the main differential expression analysis.

Fifth, the analyses of the allele-specific expression are limited by the small sample size (n=12) and the

requirement for the individuals to be heterozygous to study the genetic variants. Therefore, further validation is necessary. However, this is the first study analyzing allele-specific expression in AAAs, and our results emphasize the value of this new data set combining genetic and expression data for the study of AAAs.

Sixth, the conclusions presented in this article are based on the results obtained from a cohort of European-ancestry individuals only. Further analyses in different ancestries will determine the generalization of these results.

Finally, given that our study is primarily based on RNA sequencing data, future functional validation studies in animal models are warranted to confirm the roles of the identified genes in the development and progression of AAAs.

Conclusions

Our analysis of the whole transcriptome from human aortic samples allowed us to identify numerous novel genes associated with AAAs, 26.6% more than in previous microarray studies.[10–15] Moreover, our efforts to account for the impact of ischemic time have provided more robust biological pathways associated with AAA development, represented by 3002 DEGs, of which 1452 were new. Additionally, the study of differential splicing processes between AAAs and controls have revealed novel molecular processes involving already known genes in relation to AAA, such as *SPP1*, *FHL1*, or *GNAS*.

The study also analyzed the differential expression of genes in individuals with AAAs of different diameters, providing novel genes associated with AAA progression. Further research on these genes and their potential causal relation to AAA may lead to potential treatments against aneurysm progression, which increases the risk of rupture.

Finally, the analysis of differential allele-specific expression in 12 AAA samples permitted the identification of haplotypes associated with expression of particular genes and AAA risk, providing evidence for their possible involvement in disease and shedding light into the molecular mechanism.

Overall, this study provides a comprehensive exploration of AAA expression patterns, revealing key insights into the pathophysiology of AAA initiation and progression. RNA sequencing was used to conduct one of the largest differential expression analyses to date, uncovering novel genes associated with AAA. Our consideration of ischemic time and AAA diameter improved the precision in identifying biological processes associated with AAA onset and progression. Furthermore, our analysis of differential allele-specific expression identified genetic haplotypes that influence gene expression on AAA tissue, which advances our understanding of AAA genetic background. These findings contribute to future research and potential advances in precision medicine to reduce AAA progression and mortality risk.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Data S1
Tables S1–S10
Figures S1–S7

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