Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2024**; 29(2): 64
https://doi.org/10.31083/j.fbl2902064

Original Research

Immune Response Associated Gene Signatures in Aortic Dissection Compared to Aortic Aneurysm

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Academic Editors: Paramjit S. Tappia and Rajesh Katare
Submitted: 2 November 2023 Revised: 4 December 2023 Accepted: 25 December 2023 Published: 6 February 2024

Abstract

Background: Thoracic aortic dissections (TAD) are life-threatening events mostly requiring immediate surgical treatment. Although dissections mainly occur independently of thoracic aortic aneurysms (TAA), both share a high comorbidity. There are several indications for an involvement of the immune system in the development of TAD, just as in TAA. Nevertheless, specific disease-relevant genes, biomolecular processes, and immune-specific phenotypes remain unknown. **Methods**: RNA from isolated aortic smooth muscle cells from TAD (n = 4), TAA (n = 3), and control patients were analyzed using microarray-based technologies. Additionally, three publicly available bulk RNA-seq studies of TAD (n = 23) and controls (n = 17) and one single-cell RNA-seq study of TAA (n = 8) and controls (n = 3) were analyzed. Differentially expressed genes were identified and used to identify affected pathways in TAD. Five selected genes were validated by quantitative real-time polymerase chain reaction (PCR). **Results**: We identified 37 genes that were significantly dysregulated in at least three TAD studies—24 of them were not shown to be associated with TAD, yet. Gene ontology analysis showed that immune response was significantly affected. Five of the genes (*CCL2*, *RNASE2*, *HAVCR2*, *CXCL8*, and *IL6R*) were revealed as core genes that affect immune response in TAD. We compared the gene expression of those genes to TAA and found that *CXCL8*, *IL6R,* and potentially also *CCL2* were upregulated in TAD. **Conclusions**: The identified immune-related genes showed TAD-specificity, independent of possible pre-existing comorbidities like TAA. So, these genes represent potential biomarkers and therapeutic targets linked to the immune response in acute TAD. Additionally, we identified a set of differentially expressed genes that represents a resource for further studies.

Keywords: RNA-seq; immune response; aortic aneurysm; aortic dissection

1. Introduction

Thoracic aortic aneurysms (TAA) and thoracic aortic dissections (TAD) are diseases, whose origin is linked to an ongoing weakening of the aortic wall. The TAA subtype, being associated with a tricuspid (i.e., healthy) aortic valve (TAV) is characterized by a continuous enlargement of the aortic diameter and a progressive enzymatic degradation of the elastic fibers within the aortic wall. This is mainly caused by an increased expression of matrix-degrading enzymes like matrix metalloproteinases and a reduced expression of tissue inhibitors of matrix metalloproteinases (TIMPs). Additionally, the TAV-TAA is associated with a disturbance of cellular and metabolic processes, genetic damages, and atherosclerotic processes in the aortic wall [1–3]. A TAA is sometimes, but not necessarily, a pre-existing condition that leads to the development of TAD. When the aortic diameter exceeds 6 cm the risk for rupture or dissection increases by 6.9% per year [4]. As a preventive measure aortic replacement surgery is performed as it reduces the risk for rupture and dissection with a mortality rate of about 20% before reaching the hospital and about 30% before admission [5]. Apart from risk assessment based on the aortic diameter, there are no clinical parameters to predict the event of aortic dissection. Accordingly, the search for biomarkers indicative for TAAs and TADs in the sense of an early diagnosis remains a central task for research. Once diagnosed, disease-progression inhibitory pharmacological therapies are a gold standard, yet, given the fact that TAA and TAD disease pathogenesis remains unclear, target-oriented drugs cannot be designed.

Importantly, previous studies showed that immune system-related genes and immune cell infiltration may play key roles in the pathogenesis of TAD. Immune cell infiltration can lead to a destabilization of the aortic wall, enabling the tearing of the wall by the blood flow [6,7]. In contrast to the abdominal aortic aneurysm, in TAV-TAA the role of inflammation is still controversially discussed. Although recently some signs for the involvement of immune cells and biomolecular processes were identified, especially their possible role in the transformation from TAA into TAD is still unclear [8].

Table 1. Clinical information on TAD, TAA, and control patients.

				p-value		
Parameter	C (n = 3)	TAA (n = 3)	TAD (n = 4)	C vs. TAA	C vs. TAD	TAD vs. TAA
Age (Years)	64.3 ± 2.3	65.0 ± 3.6	63.8 ± 5.5	0.801	0.872	0.749
Sex (Male)	33%	66%	75%	0.721	0.352	0.846
Aortic diameter (mm)	nd	56.5 ± 3.5	52.7 ± 5.1	nd	nd	0.897
Current smoker	nd	0%	0%	nd	nd	1.000
Coronary heart disease	nd	0%	0%	nd	nd	0.846
Hypertension	33%	66%	75%	0.721	0.352	0.846
Hyperlipidemia	66%	33%	75%	0.721	0.846	0.352
Diabetes	66%	33%	50%	0.721	0.519	0.519
Aortic stenosis	nd	33%	25%	nd	nd	0.846
Aortic regurgitation	nd	33%	50%	nd	nd	0.203

Data are presented as mean ± standard deviation (SD). nd = not determined. Mean age (years) is shown for controls, TAD, and TAA. Differences that are statistically significant (p-values < 0.05) between the two conditions were calculated using a two-sided t-test. C, control; TAA, thoracic aortic aneurysms; TAD, thoracic aortic dissections.

Transcriptome analysis is an excellent tool to identify key genes and pathways involved in disease development. Measuring gene expression levels and identifying transcripts that show a differential expression between two conditions, such as diseased and healthy controls, enables the identification of deregulated molecular physiology [9]. Additionally, the differences that do exist between TAA and TAD patients particularly regarding their immunological phenotypes have not been studied yet. Therefore, we performed a previously established genome-wide expression analysis [10] to identify signature genes and biomolecular processes underlying TAD with pre-existing TAA, and compared the data to TAA. The present study aimed to identify a gene signature for TAD development that is independent from TAA. The data generated by these analyses form a solid theoretical foundation for further exploration and understanding of the immune response involved in TAA and TAD progression.

2. Materials and Methods

2.1 Samples and Study Population

The design of this study was approved by the Ethics Committee of the Johannes Kepler University Linz (EK 1111/2018). All patients included in this study gave their written informed consent, no minors were included in the study. This study confirms the Declaration of Helsinki.

Primary smooth muscle cells were isolated from tissue of the ascending thoracic aorta after a maximum of 1 hour postoperatively from patients with a thoracic aortic aneurysm, patients with an acute Stanford A dissection, and healthy controls (Table 1). All patients were caucasians and had a tricuspid aortic valve and were matched regarding sex and age; patients with genetic disorders affecting connective disorders were excluded from the study. All patients in the TAD group also had a pre-existing TAA (Table 1). Cell isolation and handling was described in more detail in previous studies [1,11]. To summarize, the tunica media was separated mechanically followed by an enzymatic collagenase-elastase treatment, which allowed the cells to settle into cell culture dishes. Before starting experiments all cell lines were tested for possible cellular contaminations like endothelial cells or fibroblasts by Western blot analyses.

2.2 Microarray Gene Expression

Total RNA was extracted from isolated aortic smooth muscle cells from TAD patients (n = 4), TAA patients (n = 3), and controls (n = 3) using TRIzol reagent (# 15596018, Lot: 19698001, Life Technologies, Carlsbad, CA, USA), followed by purification using the RNeasy mini kit from Qiagen (Valencia, CA, USA, Lot: 166038437), adhering to the guidelines provided by the manufacturers. To assess the RNA samples' quality and quantity, a UV–Vis spectrophotometer (Thermo, NanoDrop 2000, Waltham, MA, USA) was utilized, measuring absorbance at 260 nm and 280 nm. Transcription profiles of Long noncoding RNA (lncRNA) and messenger RNA (mRNA) in the samples were analyzed using Clariom D solutions, compatible with the Affymetrix Gene Chip (Santa Clara, CA, USA). The microarray data were processed and displayed through the Affymetrix Expression Console Software, version 1.2.1 (Thermo Fisher Scientific, Waltham, MA, USA). We normalized the primary data, specifically the CEL files, at the transcript level employing the robust multi-array average technique. Following the provided guidelines, we calculated the median summarization of the transcript expressions. For signal scanning and further analysis, equipment from Affymetrix (Affymetrix Gene Chip Operating Software) was utilized. The Affymetrix gene expression data that support the findings of this study was uploaded in GEO NCBI (PRJNA90739). The Affymetrix gene expression data comparing TAD and controls, TAA and controls, and TAA and TAD are further referred to as C-TAD 1, C-TAA

1, and TAA-TAD, respectively.

2.3 Acquisition and Analysis of Publicly Available Transcriptomic Data

Next, to reduce intra-laboratory bias and increase the power of the study by analyzing a larger number of biological samples, sequencing data from three additional RNA-seq studies were sourced from the Sequence Read Archive database (https://www.ncbi.nlm.nih.gov/sra). These were part of the following accessible gene expression projects (all analyzing aortic tissue of patients with aortic dissection and healthy controls): PRJNA229053 (TAD n = 7, controls n = 5)—a gene expression study of ascending aorta from patients with acute Stanford type A aortic dissection [12], PRJNA612822 (TAD n = 6, controls n = 6)—a study of the m6A methylome in the aorta of patients with acute aortic dissection and controls [13], and PRJNA642644 (TAD n = 10, controls n = 6)—a transcriptome analysis of ascending aortic tissue in Stanford type A aortic dissection [14], further referred to as C-TAD 2, C-TAD 3, and C-TAD 4, respectively.

Transcriptomic data analysis was performed according to our recently established workflow [10]. Dysregulation of genes was analyzed using DESeq2 (Version 3.17) [15]. In the study, genes were labeled as differentially expressed if their unadjusted Wald test *p*-value was below 0.05. We opted not to adjust these *p*-values for multiple hypothesis testing. This decision was made to reduce the likelihood of false negatives, a common issue in studies with limited sample sizes. As a result, the *p*-values in our analysis are primarily descriptive. Our methodology favored an exploratory strategy, utilizing uncorrected *p*-values to lower the risk of overlooking significant genes, a frequent challenge in studies with a small number of biological replicates. The methodology involves analyzing data from different laboratories and independent studies. Such a multicentered approach minimizes the bias that might arise from a single laboratory setting, thereby enhancing the reproducibility and reliability of our findings. Genes were referred to as differentially expressed within a study if the Wald test *p*-value was <0.05.

Data from PRJNA649846 (TAA n = 8, controls n = 3)—a single-cell RNA-sequencing (scRNA-seq) analysis of ascending aortic tissues in aneurysmal and in control aortic tissue [16] was also obtained from the Sequence Read Archive database (https://www.ncbi.nlm.nih.gov/sra) and every sample was analyzed individually with the Seurat workflow [17] in R (https://www.R-project.org/; version 4.2.0). The publicly available count matrices were loaded in the R software environment and then a Seurat object was created. Next, a quality control and a selection of cells for further analysis was performed. Cells that had unique feature counts over 2500 or less than 200 were filtered out. After applying the previously mentioned filters on the dataset, the data was normalized by LogNormalize with a default scale factor. Then a linear transformation of the data was performed, followed by a default principal component analysis (PCA). A heuristic ranking of PCAs based on the percentage of variance was terminated. Then the cells were clustered based on the euclidean distance in PCA space and the default Louvain algorithm was used to iteratively group cells together, a resolution of 0.5 was used as parameter to set granularity of downstream clustering. Then Uniform Manifold Approximation and Projection (UMAP) was used as a dimension reduction technique to visualize the datasets. The datasets of the individual samples were merged for visualization in **Supplementary Fig. 1.** In order to make the scRNA-seq data comparable to the other studies analyzed, the mean expression of the identified genes was analyzed in all clusters, and compared between all TAA samples (n = 8) and controls (n = 3). The study workflow is summarized in Fig. 1.

2.4 Data Analysis of Differentially Expressed Genes

The free software environment R (https://www.R-project.org; version 3.12.0.) was used for statistical analysis and graphic representation of the data. Heatmaps were created with the ggplot package [18], Venn diagrams were generated with the ggVennDiagram package [19]. Gene enrichment analysis was conducted with the clusterProfiler package [20] using differentially expressed genes (DEGs) that were dysregulated in at least three TAD studies. For the netplot analysis, we loaded the DEGs into the clusterProfiler package, performed gene set enrichment analysis, and created visual representations of enriched pathways using the netplot function. To compare two means in real-time quantitative PCR (RT-qPCR) experiments a two-sided *t*-test was performed. The data examined in the course of this study can be found in the supplemental information accompanying this publication.

2.5 RT-qPCR

RNA was extracted from isolated aortic cells of TAD patients, TAA patients, and healthy controls, (n = 6 per group) with the Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA, USA, # T2010S, Lot: 10144556). We prepared complementary DNA (cDNA) with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific #10680471) following the manufacturer's instructions. After cDNA preparation, we performed qPCR using 1× Luna Universal qPCR Master Mix (NEB #M3003), 0.5 µM final concentration for primers, and 10 ng of cDNA on a BIORAD CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA), using the following conditions: 2 min at 95 °C followed by 40 cycles with 15 sec at 95 °C, 30 sec at 63 °C, and 40 sec at 72 °C and after finishing hold at 4 °C. Statistical analysis was performed using a two-sided *t* test. Expression was normalized and analyzed with the Bio-Rad CFX Manager Software (Version 2.3, Bio-Rad Laboratories, Hercules, CA,

USA). Ubiquitin C (*UBC*) was used as a housekeeping gene because it was shown previously that UBC is stably expressed in aorta [21,22] and it did not show dysregulation in any of the included RNA-seq studies. The relative expression was calculated in comparison with the housekeeping gene. Primers were designed using Primer3Plus (Version 3.3.0, https://www.primer3plus.com/index.html) [23]. All primers have an exon-exon junction and sequences are listed in **Supplementary Table 1**. The study workflow is summarized in Fig. 1.

3. Results

3.1 Identification of DEGs in TAD

To identify genes dysregulated in the context of TAD, we analyzed not only gene expression data generated by our team (C-TAD 1; PRJNA90739), but also compared our data to three publicly available bulk RNA-seq studies: PRJNA229053, PRJNA612822, and PRJNA642644, further referred to as C-TAD 2, C-TAD 3, and C-TAD 4, respectively. Each study consists of a group of patients who had developed TAD as well as a control group without TAD. In each study, we initially identified a different number of genes exhibiting dysregulated expression: 1970, 4550, 5191, and 5245 DEGs in C-TAD 1, C-TAD 2, C-TAD 3, and C-TAD 4, respectively. This was followed by a comparative analysis across all studies to assess the number of DEGs In summary, we analyzed gene expression data of 26 TAD samples and 24 control samples from four different studies (**Supplementary Table 2**). For subsequent analyses, we compared the individual studies and only included DEGs observed in at least three studies (with a Wald test p-value < 0.05), which are consistently either up- or down-regulated. We obtained a total of 37 genes (Fig. 2A; **Supplementary Table 3**) dysregulated in at least three studies, four of them in all four studies: CC-Chemokin-Ligand-2 *(CCL2)*, Ribonuclease A Family Member 2 *(RNASE2)*, Hepatitis A Virus Cellular Receptor 2 *(HAVCR2)*, and Fibulin 1 *(FBLN1)*. 25 genes have not been shown to be associated with TAD before, according to our literature search. When clustering those genes on the basis of gene expression log2 fold change (log2FC), distinct intensity patterns of dysregulation were revealed (Fig. 2B).

3.2 Functional Enrichment Analysis of the DEGs

To analyze their relevance in the context of TAD, we next assessed the cellular function of the identified 37 DEGs with a gene set enrichment analysis. We identified 96 significantly affected pathways in TAD (Fig. 3A and **Supplementary Table 4**). A considerable number, 20 (20.83%), are immune-related pathways, and the majority of these (17 pathways) are in the top 30 of significantly affected pathways (Fig. 3A). By analyzing these pathways, we noticed that *FBLN1*, which is well known for TAD development, was not identified as critical in the significantly affected pathways.

We also found that C-X-C Motif Chemokine Ligand 8 (*CXCL8*) and Interleukin 6 Receptor (*IL6R*) have key roles in the dysregulation of pathways in TAD. Both genes are involved in the dysregulation of a majority of the dysregulated pathways in TAD. *CXCL8* is involved in 79 (82.29%) and *IL6R* in 54 (56.25%) of the 96 significantly affected pathways (**Supplementary Table 4**). Both genes are involved in all of the 20 identified significantly affected immune-related pathways in TAD.

Therefore, in addition to the previously mentioned genes dysregulated in all four studies found in immune-related pathways (*CCL2*, *RNASE2*, *HAVCR2*), we identified two more strong candidates as possible targets, *IL6R* and *CXCL8*—both genes were significantly upregulated in three out of four studies. In summary, we identified immune response and immune system related pathways as the most significant affected pathways in TAD; We identified five genes which are mainly involved in immune response processes (Fig. 3B).

3.3 The Role of CCL2, RNASE2, HAVCR2, CXCL8, and IL6R in TAA

In the next step we analyzed the specificity of the before identified genes for TAD by analyzing their expression in another TAD-linked condition, namely TAA. Therefore, we first analyzed gene expression differences between TAA and controls in our microarray experiment. In our study none of the five identified genes showed a significantly dysregulated expression in TAA compared to controls, and no increased log2FC above 1 (Fig. 4). In comparison, as described earlier, the TAD-specific genes are significantly increased and have an increased log2FC compared to controls. The TAD-specificity was also confirmed by comparing the gene expression between TAA and TAD. Except for *HAVCR2*, all genes showed a significant increase or a log2FC >1 in TAD.

Additionally we analyzed a publicly available scRNA-seq experiment and analyzed gene expression differences between TAA and controls. The scRNA-seq study was analyzed, because this is the only appropriate publicly available gene expression study that sequenced ascending aortic tissues in aneurysmal and in control aortic tissue. We identified 19 different clusters, representing 19 different cell types, among the 11 analyzed aortic tissue samples (**Supplementary Fig. 1**). The expression of the five target genes was analyzed in each individual sample. The mean expression of the target genes in each cluster did not show a significant upregulation of *CCL2*, *RNASE2*, *HAVCR2*, *CXCL8* and *IL6R*. However, there was a trend that showed an upregulation in *RNASE2* (**Supplementary Fig. 2**). Due to low expression or quality reasons, the target genes *CCL2*, *RNASE2*, *HAVCR2*, *CXCL8*, and *IL6R* could not be identified in every single cluster. Furthermore, in the individual clusters where these genes could be identified, we found

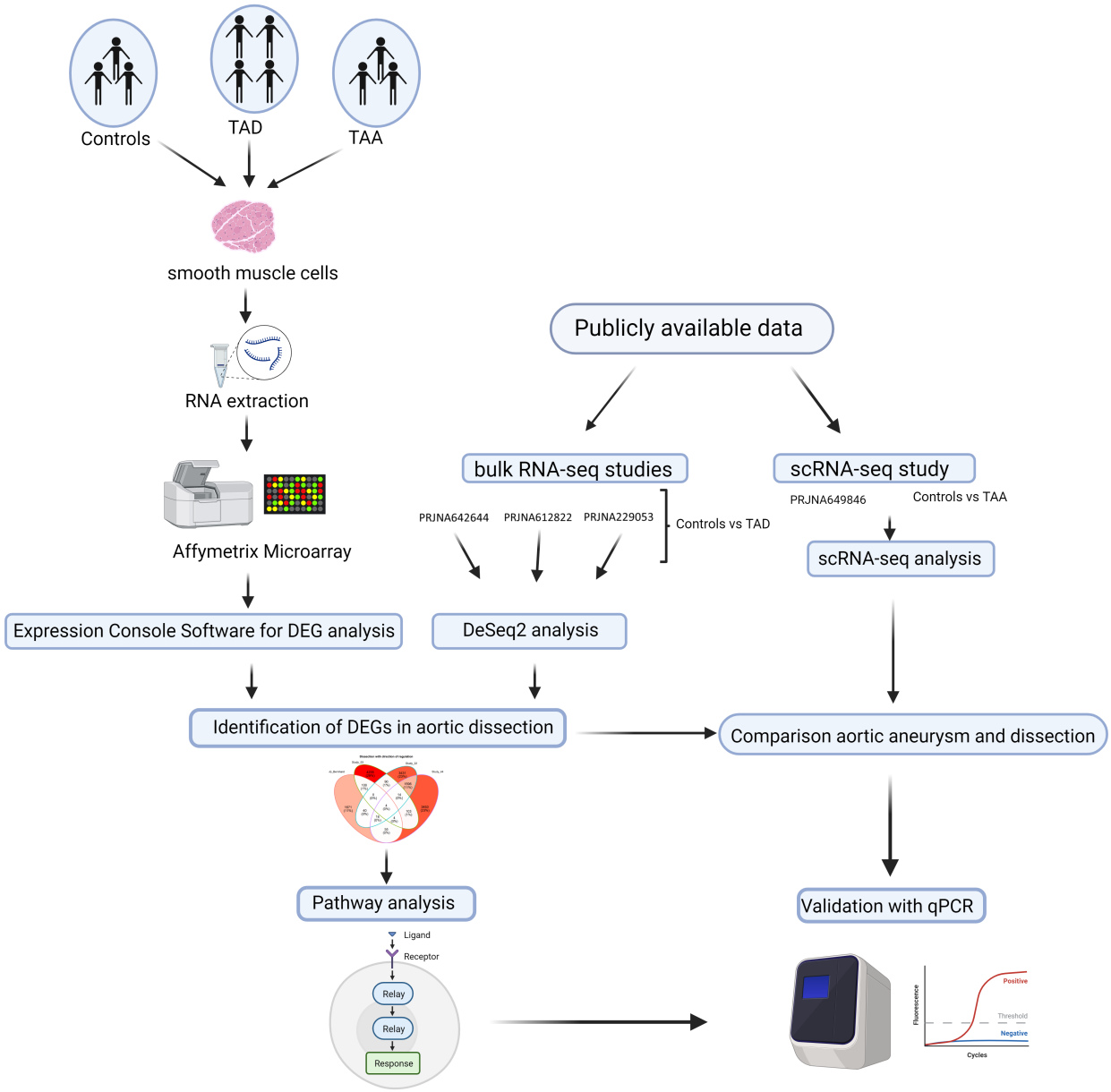

Fig. 1. Microarray and sequencing analysis workflow for TAD, TAA, and control samples. RNA was extracted from TAD (n = 4), TAA (n = 3), and control samples (n = 3). Samples were measured by Affymetrix Gene Chip and DEGs were analyzed by Affymetrix Gene Chip Operating Software. Parallel to microarray experiments, three publicly accessible bulk RNA-seq studies were analyzed with DeSeq2: PRJNA229053 (TAD n = 7, controls n = 5), PRJNA612822 (TAD n = 6, controls n = 6), and PRJNA642644 (TAD n = 10, controls n = 10). Common genes were identified by comparing the DEGs across the individual studies. A pathway analysis was performed and data was compared to Affymetrix Gene Chip Operating Software results (C vs TAA) and one publicly available scRNA-seq study PRJNA649846 (TAA n = 8, controls n = 3). Results were validated with RT-qPCR. TAD, thoracic aortic dissections; TAA, thoracic aortic aneurysms; DEGs, differentially expressed genes; C, control; scRNA-seq, single-cell RNA-sequencing; RT-qPCR, real-time quantitative polymerase chain reaction.

no significant differences in *CCL2*, *RNASE2*, *HAVCR2*, *CXCL8*, and *IL6R* between controls and TAA across any single cluster, thereby guiding our decision not to pursue further cluster-specific annotation.

3.4 Validation of Immune Related Genes with RT-qPCR

To validate the observed dysregulation of our fife selected candidate genes, we next performed RT-qPCR for *CCL2*, *RNASE2*, *HAVCR2*, *CXCL8*, and *IL6R* on primary aortic smooth muscle cells from TADs, TAAs, and controls.

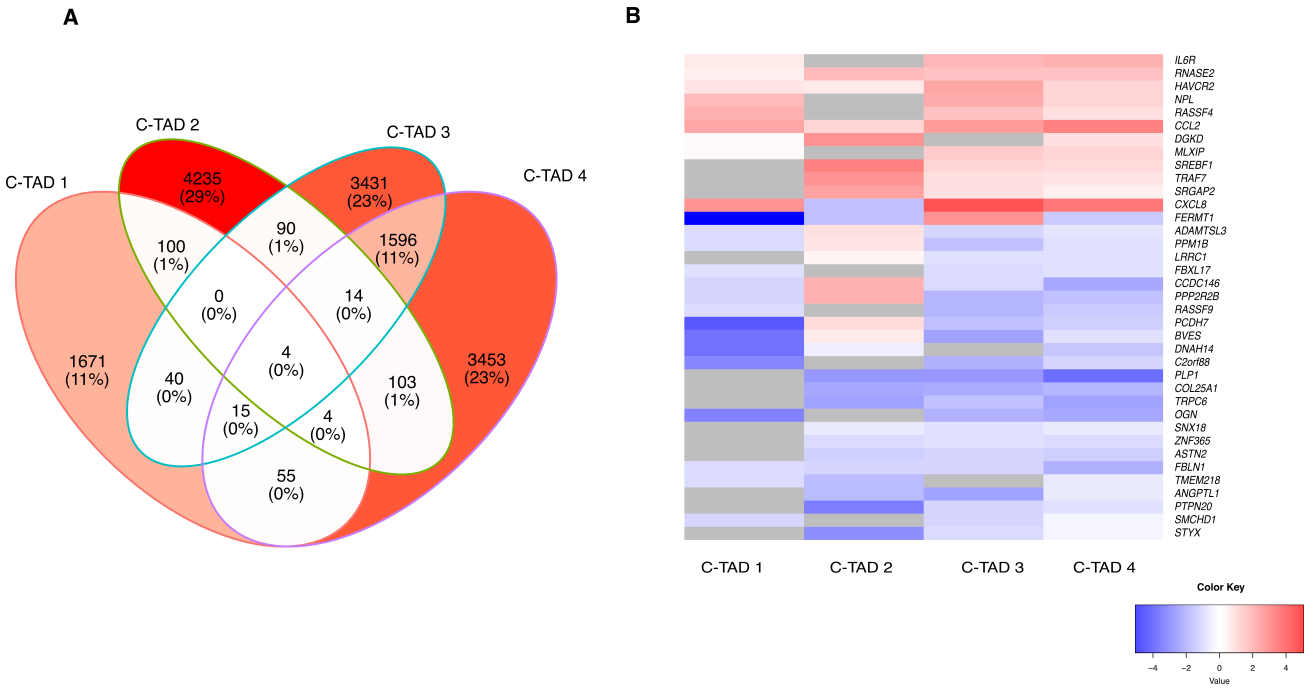

Fig. 2. Identification of common DEGs. (A) Venn Diagram showing the numbers of DEGs (p-value < 0.05) identified in the individual studies. (B) Heatmap of DEGs in TAD, showing 37 rows, each representing a DEG identified in at least three studies. The color intensity in the boxes indicates the log2 fold changes (log2FCs) in gene expression; with red signifying upregulated genes and blue denoting downregulated genes. Gray indicates not available data.

We found the same pattern for the genes in the RT-qPCR as in the RNA-Seq data. While all genes were higher expressed in TADs compared to controls, only *RNASE2* (p-value = 0.023), *CXCL8* (p-value = 0.030), and *IL6R* (p-value = 0.036) showed significant differences (Fig. 5). *CCL2* also showed a trend of higher expression, but not significant (p-value = 0.420)—potentially due to the low number of samples analyzed (n = 6 for all groups).

The comparison of TAA and controls showed similar gene expression between the groups for most genes; *RNASE2* was the only gene showing a significantly higher expression (p-value = 0.017) in TAA compared to controls.

Also, the comparison of TAD and TAA showed a similar pattern like the previous analysis, so the gene expression was increased in TAD samples, with a significant difference for *IL6R* (p-value = 0.047). Only *HAVCR2* showed a similar expression compared to controls. Despite missing significance (probably because of the small number of analyzed samples) the other genes showed an increased fold change in TAD compared to TAA (2.25× for *CCL2*, 1.89× for *RNASE2*, and 52.6× for *CXCL8*).

In addition, we also validated *FBLN1* (dysregulated in all four studies but not immune-related) by RT-qPCR, and could show a significant difference (p-value = 0.0007) between controls and TAD (**Supplementary Fig. 3**).

4. Discussion

In this study, we performed gene expression analyses of TAD not only on our own data, but also included publicly available data and compared the data to TAA. Using a previously developed strategy for efficiently reducing the number of relevant target genes in gene expression data [10] we could identify 37 target genes that were dysregulated in TAD. 24 of the genes have not been suggested to be associated with TAD before (**Supplementary Table 3**). With our study we could identify pathological signature genes and processes in TAD. Our results indicate that immune response and immune related pathways have the highest impact on TAD development, linked to the key genes *RNASE2*, *CXCL8*, *IL6R,* and potentially also *CCL2*. In general, we identified hallmark genes and biomolecular processes in TAD using high-throughput gene expression data that could provide a range of potentially new drug targets (genes) and therapeutics. We also identified known affected pathways involved in the function of extracellular matrix and metabolism, such as extracellular matrix structural constituent, collagen-containing extracellular matrix, positive regulation of cellular metabolic process and cellular metabolic process [24]. Furthermore, the detection of *FBLN1*, which is already well known for its causal interconnection in TAD development [24–26], in all of the studies highlights the good performance of this approach.

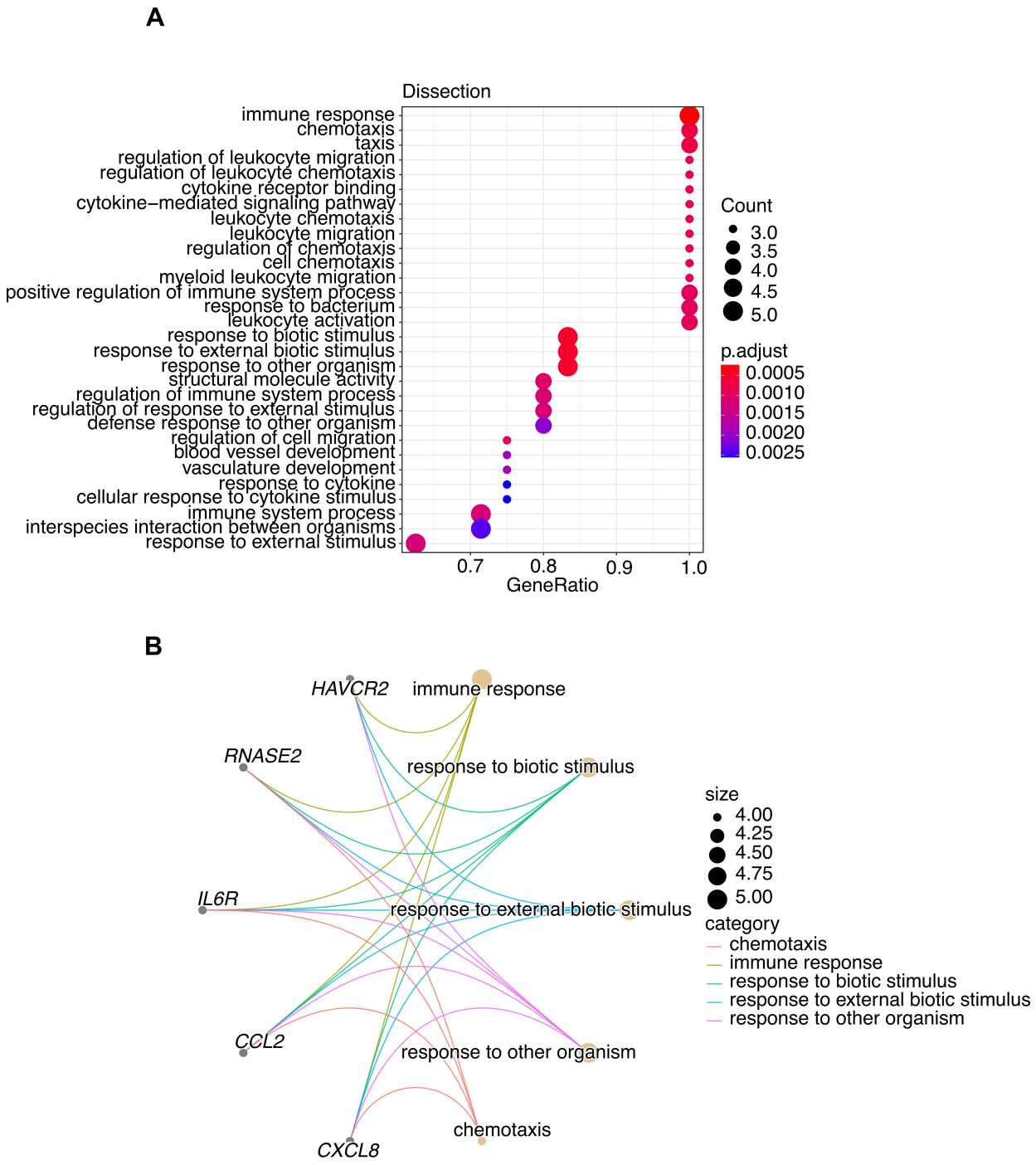

Fig. 3. Gene Ontology analysis argues for a central role of immune-related pathways in TAD pathogenesis. (A) Top 30 significantly affected biological processes, cellular components and molecular functions of 37 DEGs dysregulated in at least three TAD studies. The size of each dot reflects the quantity of impacted genes linked to the functional annotation (count). The Gene Ratio represents the ratio of affected gene counts to the size of the respective pathway. As for the dot color, it signifies the significance level: blue for lower significance and red for higher significance. (B) Netplot showing the molecular interaction/reaction network pathways of the five genes analyzed by gene ontology analysis. The dot size of the pathway indicates the number of the involved genes.

4.1 The Role of CCL2, RNASE2, HAVCR2, CXCL8, and IL6R in TAD and TAA

CCL2 is a well-known mediator of the innate response and inflammation [27] encodes for monocyte chemoattractant protein 1 (MCP1), which is a cytokine responsible for the recruitment of monocytes, memory T cells, and dendritic cells. Studies have already identified *CCL2* as a key player in acute TAD, as increased CCL2 serum protein levels and increased mRNA expression in mouse models have been detected [6,28,29]. The role of *CCL2* in aor-

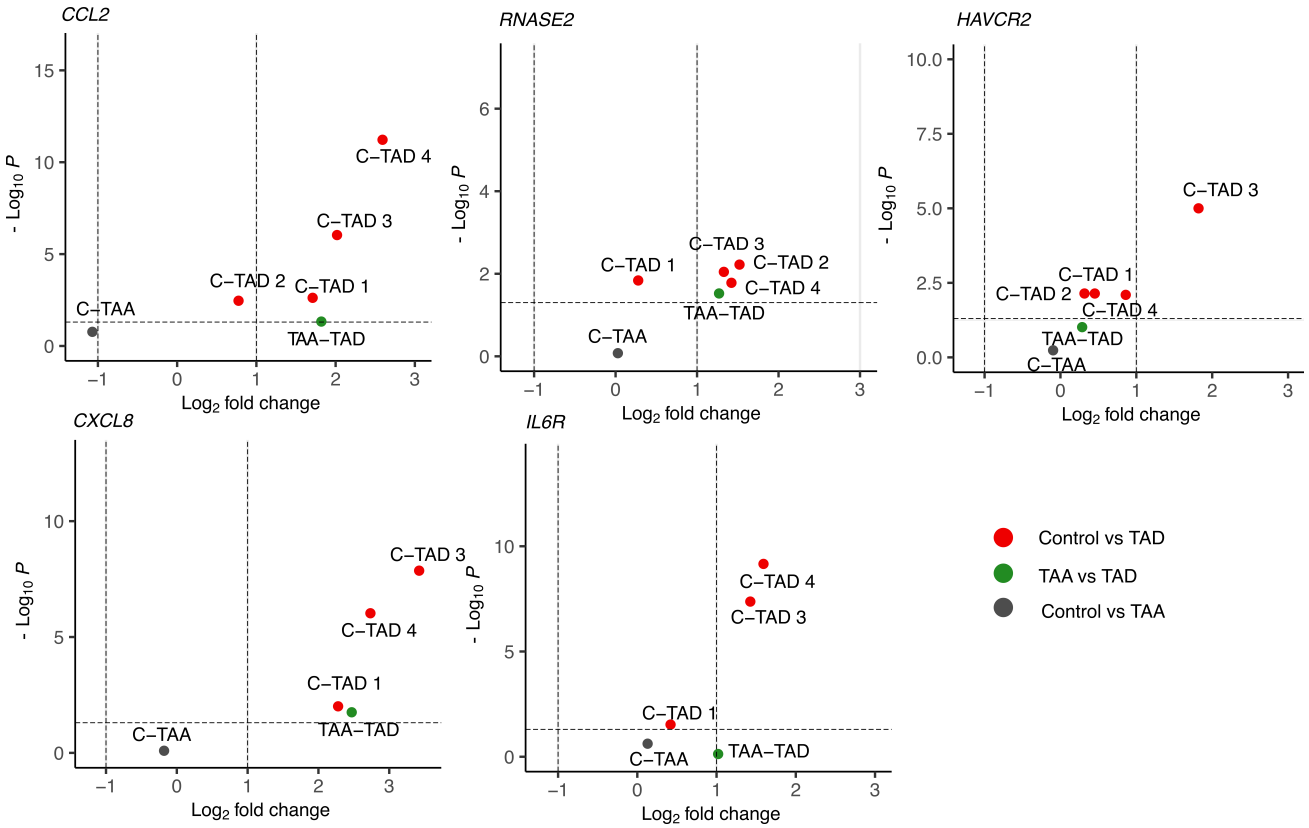

Fig. 4. TAD target genes show a greater degree of dysregulation than TAA. Figure shows the gene expression between TAA and controls (gray dots), TAD and controls (red dots), and TAD and TAA (green dots). The dotted horizontal line indicates a *p*-value of 0.05, the right vertical line a log2 fold change of 1.

tic aneurysm is still undefined. Increased levels of MCP-1 in the wall were observed in abdominal aortic aneurysm (AAA) patients [30]. The present study is the first one describing MCP-1 expression in TADs and TAAs. The analysis shows a significantly increased expression in TADs. However, probably due to low sample size only a trend could be shown in RT-qPCR.

RNASE2 encodes for an enzyme called Eosinophil-derived neurotoxin, an eosinophil degranulation product which also serves as an attractant for immune cells. The role of *RNASE2* in TAD and TAA development is completely unknown. Kan *et al.* [31] showed an increased gene expression of *RNASE2* in a human gene co-expression network study, but only in AAAs. In general, the recruitment of eosinophils seems to exert a protective role. In AAA it regulates macrophage polarization and blocks NF-κB activation in aortic inflammatory and vascular cells [32]. Also in TAD, low eosinophil counts are significantly associated with increased mortality rates. Qin *et al.* [33] stated recently, that peripheral blood eosinophil counts may be involved in thrombosis and could be an effective and efficient indicator for the diagnosis, evaluation, and prognosis monitoring of patients with TAD [33,34]. Our study is the first study that provides evidence for an interconnection of increased *RNASE2* gene expression and TAD and, according to the RT-qPCR also in TAA. It is speculated that during transition from TAA to TAD self-repair mechanisms are activated, leading to an upregulation of *RNASE2*, coupled with eosinophil recruitment.

HAVCR2 encodes for T cell immunoglobulin and mucin domain-3, which plays a role in immune regulation and inflammation. Beside macrophage activation *HAVCR2* is involved in activating $CD8^+$ T-cell exhaustion by secretion of cytokines like tumor necrosis factor-alpha (TNF-α) , interferon-gamma (IFN-γ) and Interleukin-2 (IL-2) [35,36]. Nothing is known about the role of *HAVCR2* in aortic aneurysm and TAD development so far. Importantly, the present study is the first study that provides data indicating a role of HAVCR2 in TAA and TAD; nevertheless RT-qPCR data could not support this hypothesis.

CXCL8 encodes for Interleukin-8 (IL-8), being a chemokine with an essential role in inflammatory immune responses. Largely produced by macrophages it is responsible for recruiting neutrophils. Also, for *CXCL8* its role in TAD has been suggested in several studies. Increased gene expression was observed in dissection tissue and blood [37,38]. Yet, the role of *CXCL8* in aortic aneurysm still remains unclear. Lindeman *et al.* [39] found increased IL-8 mRNA and protein levels associated with aortic growth in the aortic wall of AAA patients; data for a role of IL-8

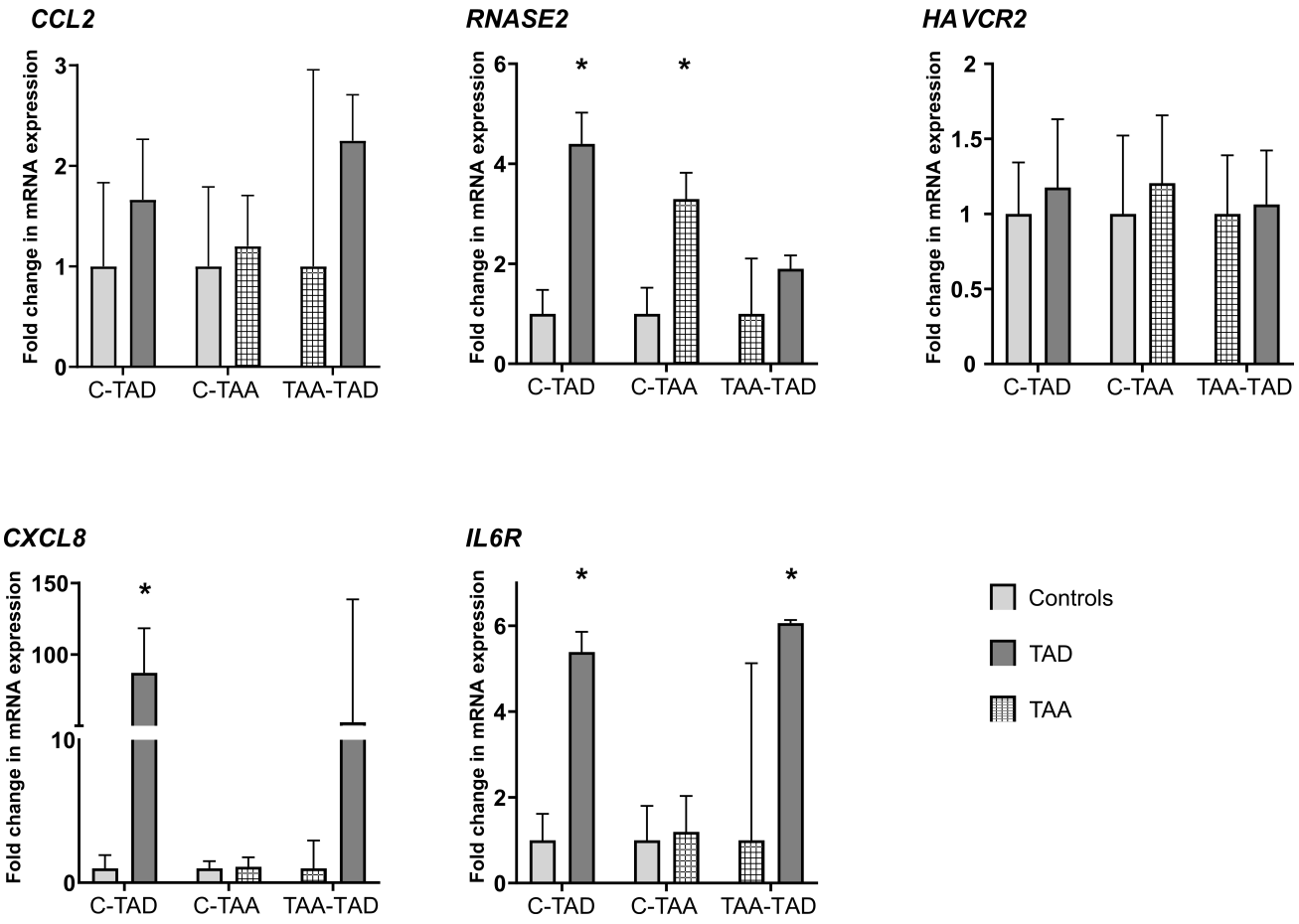

Fig. 5. Validation of transcriptomic data by RT-qPCR. Expression of *CCL2*, *RNASE2*, *HAVCR2*, *CXCL8* and *IL6R* in, TADs (n = 6), TAAs (n = 6), and controls (n = 6) were normalized to ubiquitin C (*UBC*) as a reference gene by the Bio-Rad CFX Manager software. The control group expression level is designated as 1, so values >1 indicate up-regulation. Each histogram represents the mean ± standard error (SE) of six individual cell lines and three replicates per individual. Significant differences are marked as * for $p < 0.05$ and were calculated using a two-sided *t*-test.

in TAA pathogenesis is still missing. Data from our study show that there is no increase in *IL-8* gene expression in TAA. These findings may make *CXCL8* an interesting clinical parameter to detect the transition of a TAA to a TAD.

IL-6R (encoded by *IL6R* gene) and its interaction partner IL-6 play a major role in inflammatory immune responses. IL-6 is a pro-inflammatory cytokine and an anti-inflammatory myokine secreted among others also from blood vessel smooth muscle cells [40]. In aortic aneurysm, as well as in TAD, a role of IL-6 has been suggested in multiple studies: Both, abdominal aortic aneurysms (AAA) as well as TAA show elevated mRNA and protein levels of IL-6 to be associated with increasing aortic diameter [39,41,42]. Increased IL-6 protein levels and increased IL-6 gene expression in blood and tissue have also been detected in Stanford A dissection [37,38]. Wu *et.al.* [43] further showed its high predictive value for a poor postoperative outcome after TAD surgery. In our study we could confirm these results and provide new data suggesting an important role of IL-6 in TAA and TAD.

The immune system has not only functions, such as innate and acquired immunity, but also plays important roles in hematopoiesis, inflammation, regeneration, and proliferation [44]. Our results indicate that the up-regulation of those functions is fundamentally important in TAD, but not in TAA. A potential reason is that disease-specific changes in gene expression could be greater the more severe the disease is [45].

4.2 Strengths and Limitations

A strength of the study is the definition of TAD specific gene dysregulations, allowing for a clear separation of TADs from TAAs. Further, this study is the first to separate transcriptional profiles of TAD and TAA using data from multiple studies. The procedure applied strengthens the overall precision and outcome by consequent removal of false positive information. Accordingly, our results are based on a high sample size of RNA-Seq data and a minimized laboratory bias.

A weakness of the study is that potential post-translational modifications cannot be determined by our approach. One of the method's limitations is its dependency on data from various studies, mostly conducted by distinct research groups. While data quality-based filtering was applied, it's not possible to account for potential errors in sample preparation and data collection. Within each study, genes were classified as differentially expressed if their Wald test *p*-value was less than 0.05. These *p*-values weren't adjusted for multiple hypothesis testing, as such correction could lead to a high rate of false negatives, especially in individual studies with small sample sizes (for instance, when correcting for multiple testing, no genes are identified as dysregulated in at least three studies). In this study, the number of false negative genes was reduced in subsequent comparisons of the individual studies. Used *p*-values are purely descriptive and therefore in need of validation with another method, such as RT-qPCR. Importantly, we have very little to no information on the use and effects of various drugs (for example, statins) that could potentially affect gene expression. Despite some weaknesses of the study, without doubt, using new data analysis tools, completely new and unexpected candidate genes could be identified. TAD-specific gene expression may lay a basis for new early diagnosis markers of TADs.

5. Conclusions

This study has provided valuable insights into the genetic and molecular factors associated with TAD. The identification of specific dysregulated genes and their connection to immune-related pathways highlights the potential for future research to develop targeted therapies and diagnostic tools for TAD. These findings hold promise for improving patient care and enhancing our understanding of this life-thratening cardiovascular condition. However, it's important to acknowledge the limitations of this research. One notable weakness is our methodology's inability to account for potential post-translational modifications, which could play a significant role in TAD pathogenesis. Moreover, our reliance on data from various studies, conducted by distinct research groups, introduces a degree of variability that might impact the consistency of our findings. This is particularly relevant as the Wald test *p*-values used to classify differentially expressed genes were not adjusted for multiple hypothesis testing. Such an approach, while beneficial in reducing the number of false negatives, necessitates cautious interpretation and further validation, for instance, through methods like RT-qPCR. In conclusion, while our study has uncovered unexpected candidate genes for TAD, underlining its potential in developing early diagnosis markers, further studies are needed to delve deeper into thee genetic pathways and to validate these findings in broader and more diverse datasets. Such research will be crucial in confirming the utility of these genes as biomarkers and therapeutic targets, ultimately contributing to improved outcomes for patients with TAD.

Availability of Data and Materials

All datasets on which the conclusions of a manuscript depend are already provided as part of the submitted article.

Author Contributions

CD and MR performed the research, analyzed the data and wrote the manuscript. BA und DB designed the research study and provided help and advice on data interpretation and writing the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The design of this study was approved by the Ethics Committee of the Johannes Kepler University Linz (EK 1111/2018). All patients included in this study gave their written informed consent, no minors were included in the study. This study confirms the Declaration of Helsinki.

Acknowledgment

Not applicable.

Funding

Supported by Johannes Kepler Open Access Publishing Fund.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2902064.

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