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ORIGINAL RESEARCH

Exploration and Validation of Immune and Therapeutic-Related Hub Genes in Aortic Valve Calcification and Carotid Atherosclerosis

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Background: Cardiovascular diseases, such as aortic valve calcification (AVC) and carotid atherosclerosis (CAS), impose substantial health challenges on a global scale. Both disorders have overlapping risk factors, which might trigger similar immune-inflammatory reactions in both diseases.

Methods: Shared differentially expressed genes (DEGs) were identified in the AVC and CAS datasets from the Gene Expression Omnibus (GEO). Candidate hub genes associated with immunity were identified using LASSO and immune cell infiltration analysis, and single gene set enrichment analysis (GSEA) was performed on the datasets. Subsequently, the hub genes were confirmed by qRT–PCR validation in tissue samples.

Results: A total of 140 upregulated and 65 downregulated common genes were screened. Enrichment analyses highlighted immune system processes, response to stress, and cytokine pathways among the identified CEGs. LASSO identified candidate hub genes, including ANGPTL1, CX3CR1, and CCL4. Immune cell infiltration analysis emphasized the participation of immune cells, including macrophages, γδ T cells, and resting NK cells. The three hub genes were validated by qRT–PCR analysis.

Conclusion: Our study explored immunological processes, including immune-related genes and cells, involved in the development of AVC and CAS. In particular, the identified hub genes ANGPTL1, CX3CR1, and CCL4 play crucial roles in mediating immune-inflammatory responses, which are central to the pathogenesis of these cardiovascular diseases, and the involvement of these genes in key immune pathways suggests that they could serve as potential biomarkers for early diagnosis or as targets for therapeutic strategies.

Keywords: aortic valve calcification, carotid atherosclerosis, immune-related genes

Introduction

Aortic valve calcification (AVC) and carotid atherosclerosis (CAS) are prevalent cardiovascular diseases that pose significant challenges to public health systems worldwide.[1–3] Recent studies estimate that AVC affects approximately 25% of the elderly population aged 65 and over, leading to substantial morbidity and mortality due to aortic stenosis and subsequent heart failure.[4] CAS, similarly, is a major contributor to ischemic stroke, affecting an estimated 10% of adults aged 70–79, which underscores its critical impact on healthcare systems and patient quality of life.[5]

These conditions not only share risk factors such as age, hypertension, dyslipidemia, and smoking but also exhibit similar pathophysiological processes, notably chronic immune-mediated inflammation.[6,7] Research has increasingly highlighted the role of specific immune cells and cytokines in AVC and CAS.[8] For instance, monocytes and T cells have been implicated in the inflammation leading to calcification in AVC,[9] while macrophages and foam cells are central to the atheromatous plaque development in CAS.[10] The cytokine pathways, including those mediated by interleukins and

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tumor necrosis factors, are critical in both conditions, suggesting overlapping molecular pathways that could be targeted therapeutically.

Several studies have provided a foundation for this research, identifying various immune-related genes and their roles in cardiovascular diseases. However, these studies often lack a comprehensive analysis combining multiple datasets to robustly define key molecular players across both AVC and CAS. This study aims to bridge this gap by leveraging advanced bioinformatics tools to analyze shared genetic pathways, thus providing a more integrative understanding of these diseases' pathogenesis.[11]

In pursuit of this goal, we utilized high-throughput sequencing data from the Gene Expression Omnibus (GEO), specifically datasets related to AVC and CAS. The selection of datasets was guided by their relevance to the immuno-logical profiles and clinical manifestations of these conditions. Bioinformatics tools such as LASSO regression and Gene Set Enrichment Analysis (GSEA) were employed to identify and evaluate candidate genes and pathways, offering insights into potential diagnostic markers or therapeutic targets. This approach not only aligns with the current computational methodologies in genomic research but also sets the stage for subsequent validation studies and clinical applications.

Materials and Methods
Microarray Dataset Collection and Processing
Microarray profiles associated with AVC and CAS were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), which contains high-throughput gene expression profile information submitted by research institutions. The GSE153555 series included 10 patients with aortic valve calcification and 10 controls; the FPKM was subsequently converted to the TPM, after which the TPM was log2 transformed for subsequent analysis according to Bo Li et al.[12] Gene expression data from 29 CAS patients and 12 healthy controls were extracted from GSE100927, and 13 early-stage and 16 advanced-stage carotid atherosclerotic plaque expression data were extracted from GSE28829 for validation. Detailed descriptive information on the datasets is shown in Table 1. The flowchart of this study is summarized in Figure 1.

### Differentially Expressed Gene (DEG) Analysis
Background correction, normalization and gene symbol conversion were performed on the AVC and CAS datasets (GSE153555 and GSE100927). Later, DEGs in the AVC and CAS datasets were identified using the "Limma" (version 3.40.6) package[13] in R software. Then, DEGs in the AVC and CAS datasets were screened at the thresholds of adjusted $p \leq 0.05$ and $|\log2 \text{ (fold change)}| \geq 2$. The expression patterns of the DEGs were subsequently visualized as volcano plots and heatmaps using the "ggplot2" and "pheatmap" packages in R software, respectively.

Coexpression Genes (CEGs) and Common Gene Sets (CGSs)
The coexpressed genes (CEGs) were obtained from the genes related to the crosstalk between upregulated and down-regulated genes between the two datasets by a Venn diagram, and the 1793 immune-related genes (IRGs) were obtained from the IMMPORT database (http://www.immport.org/). The common gene set (CGS) was the intersection of IRGs and CEGs.

Table 1 Descriptive Statistics of the GEO Datasets

GSE Series	Type	Sample Size		Platform	Purpose
		Control	AVC/CAS		
GSE153555	mRNA	10	10	GPL16791	Identification
GSE100927	mRNA	12	29	GPL17077	Identification
GSE28829	mRNA	13	16	GPL570	Validation
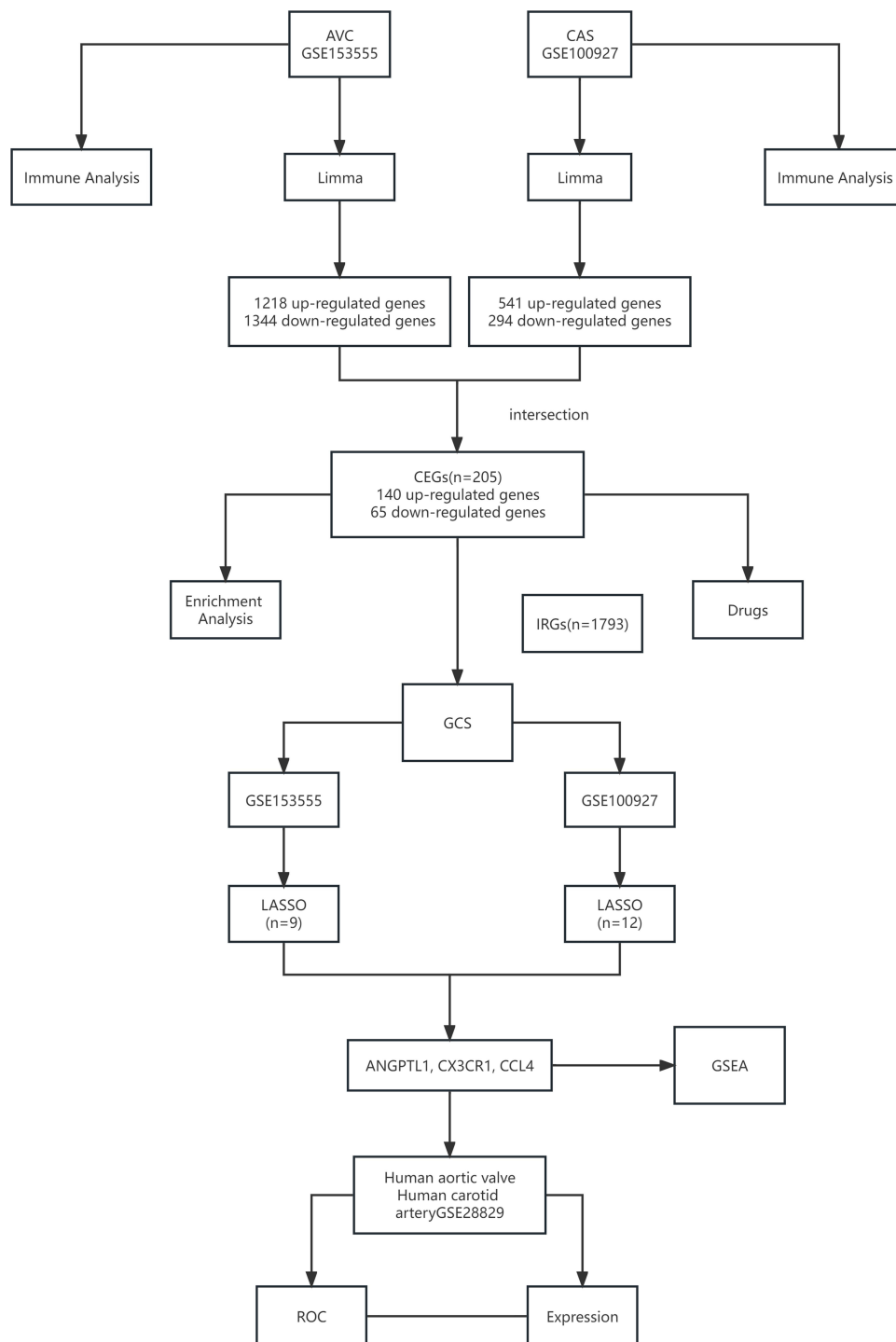
Figure 1 Flowchart of this study.

Identification of Candidate Drugs

The CMap database (https://clue.io/) is a repository of data containing thousands of gene transcription profiles obtained from cultured mammalian cells exposed to active small molecule drugs. The identification of small-molecule therapeutic candidates with AVC and CAS gene signatures was performed. CEGs were classified into two groups: upregulated and downregulated. Similarity was quantified using enrichment scores ranging from −1 to +1. A positive enrichment score

(close to +1) indicates that a small compound can induce the expression of the AVC gene, while a negative enrichment score (close to −1) indicates that a compound can mimic the normal state.

Functional Enrichment of the CEGs

The Gene Ontology (GO) system provides structured, computable information about the functions of genes and gene products.[14] The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a widely used database for the systematic study of gene functions.[15] Functional enrichment analysis was performed using the R package clusterProfiler,[16] where GO and KEGG analyses were performed based on the coexpressed genes (CEGs) shared by AVC and CAS.

Identification of Candidate Hub Genes via Machine Learning

LASSO is a regression method for selecting a variable to improve predictive accuracy and is also a regression technique for variable selection and regularization to improve the predictive accuracy and tractability of a statistical model. The R package "glmnet" was used to perform the LASSO regression by ten cross-validations. The intersecting genes between AVC and CA were considered candidate hub genes.

Gene–Gene Interaction Network Construction

GeneMANIA (http://www.genemania.org) is a resource-rich website that contains gene information, analyses gene lists and prioritizes genes for functional assays using a highly accurate prediction algorithm.

The Infiltration of Immune Cells

Immune cell infiltration analysis was performed using the CIBERSORT R package. A bar plot was generated to visualize the proportion of each type of immune cell in the different samples.

Gene Set Enrichment Analysis (GSEA)

To explore the potential role of key genes, GSEA (version 3.0) was performed for a single gene. The "c2.cp.kegg.v7.4. symbols.gmt" sequence from the MsigDB was selected as the reference gene set. $|NES| > 1$, NOM value of $p < 0.05$, and FDR q-val < 0.25 were set as the thresholds for enrichment significance.

Validation of Hub Genes

We collected 6 AVC tissue samples from patients who underwent aortic valve replacement surgery and 6 normal aortic valve tissue samples at our hospital. We collected 6 CAS tissue samples from patients who underwent carotid endarterectomy and 6 control carotid artery tissue samples free of atherosclerotic lesions. The study was approved by the ethics committee of our hospital, and all participants provided informed consent. The expression of hub genes was quantified by qRT–PCR according to the manufacturer's instructions. We used the 2-ΔΔCt method, with GAPDH serving as an internal control. In addition, we used GSE28829 to verify the mRNA expression of the three hub genes in CAS. Comparisons between patients and controls were performed using the *t*-test. p values less than 0.05 indicated statistical significance.

RNA Extraction and Real-Time PCR

Total RNA was extracted from aortic valve tissue using TRIzol. Reverse transcriptase (Thermo) was used to synthesize cDNA strands according to the manufacturer's protocol. qRT–PCR was performed using SYBR Green Real Time PCR Master Mix (CW0957) on an LC480 fluorescence quantitative PCR instrument (Roche, Switzerland). GAPDH was used as an internal control, and four duplicate wells were used for each subject. The relative expression levels of the genes were analyzed by the 2-ΔΔCT method. The primers used were as follows: ANGPTL1: F, 5′- AGTGGACACTGGACATTGCAG-3′; R 5′- GCT TCCTCTTTACCATCTGTGG-3′; CCL4, F: 5′- CTGTGCTGATCCCAGTGAATC-3′, R: 5′-TCAGTTCAGTT CCAGGTCATACA-3′; CX3CR1, F: 5′- ACTTTGAGTACGATGATTTGGCT-3′, R: 5′-GGTAAATGTCGGTGACAC TCTT-3′; and GAPDH: F, 5′-ATCCCATCACCATCTTCC-3′, R: 5′-GAGTCCTTCCACGATACCA-3′.

Statistical Analysis

The data were statistically analyzed using R 4.2.2 or GraphPad Prism 9.0. An unpaired *t*-test was used to compare two groups. Correlation analysis was performed by Spearman correlation. $p < 0.05$ was considered to indicate statistical significance.

Results

Identification of Differentially Expressed Genes (DEGs) in AVC and CAS

The red dots represent upregulated genes, the green dots represent downregulated genes, and the black dots represent nonsignificant genes according to the volcano plots (Figure 2A and B). Each row shows the DEGs, and each column refers to one of the samples from patients or controls, where red and blue represent DEGs with up- and downregulated gene expression, respectively (Figure 2C and D). According to the criteria (Padj <0.05 and |logFC| > 2), a total of 2562 DEGs were identified from the GSE153555 dataset, including 1218 upregulated and 1344 downregulated genes (Figure 2A). In the GSE100927 dataset, 835 DEGs (541 upregulated and 294 downregulated genes) were screened (Figure 2B).
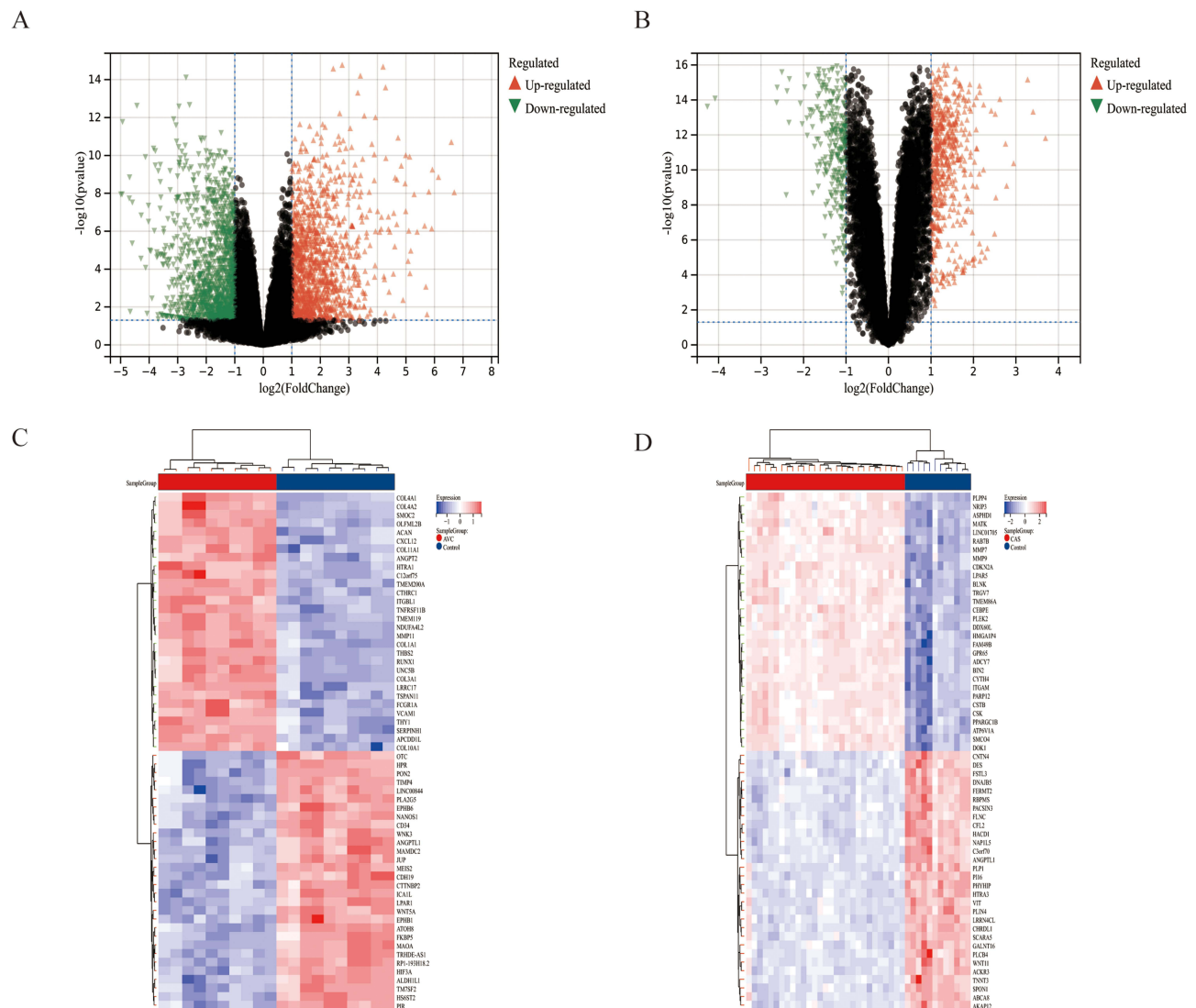
Figure 2 Volcano plots and heatmaps of DEGs identified from the AVC and CAS datasets. (**A** and **B**) Red and green plots represent DEGs with up-regulated and down-regulated gene expression in AVC-related GSE153555 dataset and CAS-related GSE100927 dataset respectively. (**C** and **D**) The heatmaps display the top 30 upregulated and downregulated DEGs identified from the two datasets above.

## Coexpression Genes (CEGs) Shared Between AVC and CAS

The genes that exhibited crosstalk between the two datasets were intersected by Venn diagrams, and 140 upregulated and 65 downregulated genes (a total of 205 CEGs) were screened (Figure 3A and B), suggesting that the pathogenesis of AVC and CAS may be similar. Then, we downloaded 1793 immune-related genes (IRGs) from the IMMPORT database and obtained a common gene set (CGS) of IRGs and CEGs, which included 59 genes (Figure 3C).

Identification of Candidate Drugs

Therefore, we uploaded 140 upregulated and 65 downregulated genes into the CMap database, and many potential therapeutic SMAs were identified. The SMAs with the highest absolute enrichment values were selected (Table 2) and included linsitinib, thalidomide, gefitinib, palonosetron, ascorbyl palmitate, altretamine, and several glucocorticoid receptor agonists, suggesting their potential therapeutic effects on AVC and CAS.

Functional Enrichment of the CEGs

To further elucidate the functional categories of the CEGs, GO enrichment and KEGG pathway analyses were performed. As shown in Figure 4A, for the biological process (BP) category, the CEGs were enriched mainly in the immune system process, stress response and immune response. Among the cellular component (CC) terms, most of the genes were enriched mainly in the extracellular region, vesicle and plasma membrane (Figure 4B). CEGs were involved mainly in signaling receptor activity, antigen binding, endopeptidase activity, and cytokine activity (molecular functions [MFs]) (Figure 4C). KEGG enrichment results showed that cytokine–cytokine receptor interactions and several immune disease
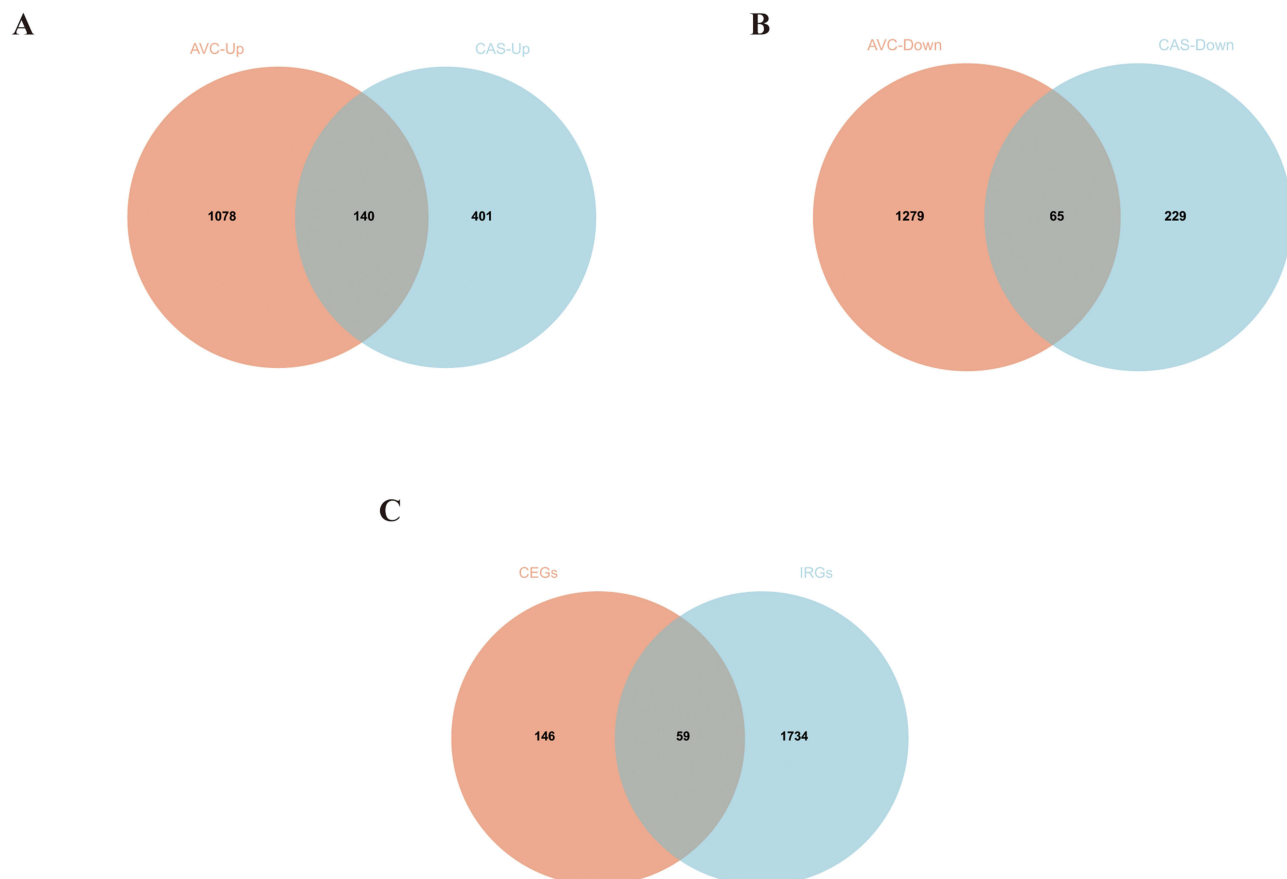
Figure 3 The CEGs and CGS shared between AVC and CAS. (**A**)Venn diagram displays the 140 up-regulated genes intersected by 1218 up-regulated genes in the GSE153555 dataset and 541 up-regulated genes in the GSE100927 dataset. (**B**) Venn diagram of 65 common down-regulated genes between the two datasets. (**C**) The common gene set (CGS) of IRGs and CEGs shows an intersection of 59 genes.

Table 2 Small Molecules Predicted with the Common Shared Hub Genes

Rank	Score	Name	Description
1.00	−96.99	Linsitinib	IGF-1 inhibitor
2.00	−94.62	Thalidomide	TNF production inhibitor
3.00	−92.37	Fluticasone	Glucocorticoid receptor agonist
4.00	−92.02	Fluocinolone	Glucocorticoid receptor agonist
5.00	−90.89	Dexamethasone	Glucocorticoid receptor agonist
6.00	−90.55	Memantine	Glutamate receptor antagonist
7.00	−88.20	Gefitinib	EGFR inhibitor
8.00	−86.96	Palonosetron	Serotonin receptor antagonist
9.00	−86.94	Ascorbyl-palmitate	Antioxidant
10.00	−86.90	Halometasone	Glucocorticoid receptor agonist

pathways were significantly enriched (Figure 4D). These results strongly suggested that cytokine pathways and the immune response play critical roles in the progression of AVC and CAS.

Identification of Candidate Hub Genes via Machine Learning

LASSO regression of the machine learning algorithm was applied to screen candidate genes in GSE153555 and GSE100927. The LASSO regression algorithm identified nine potential candidate biomarkers in AVC patients (Figure 5A) and twelve in CAS patients (Figure 5B). The intersection of the nine genes from GSE153555 and twelve genes from GSE100927 was visualized by a Venn diagram (Figure 5C), and three genes (ANGPTL1, CX3CR1, and CCL4) were identified for final validation.

Gene–Gene Interaction Network Construction

In addition, a gene–gene interaction (GGI) network was constructed using GeneMANIA to explore the interactions among the three genes. We found that CX3CR1 and CCL4 could interact through intermediary molecules, while ANGPTL1 exhibited a different pattern and could be associated with TEK, NR2F6, FMO2, CXCR5 and FCN1 (Figure 6).

Immune Cell Infiltration Analysis

Immune cell infiltration analysis was performed to better elucidate the immunoregulatory effects of AVC and CAS. The proportions of 22 types of immune cells in each sample are shown for the AVC, CAS and control groups, which showed that both AVC and CAS patients had higher levels of gamma delta T cells and lower levels of resting NK cells; they are also associated with macrophages (Figure 7). In general, different types of immune cells were differentially expressed between AVC and CA patients and could serve as potential regulatory points for AVC and CAS treatment.

GSEA Identifies Key Genes Associated with Signaling Pathways

To further clarify the role of key genes in AVC and CAS, we performed gene set enrichment analysis (GSEA). Using the data from the GSE153555 and GSE100927 cohorts, the correlations between the three key genes and KEGG pathways were analyzed (Figure 8). The enrichment results indicated that ANGPTL1, CCL4, and CX3CR1 might be related to cell adhesion, cytokines, and immune cells (NK cells and T cells).

Validation of the Three Hub Genes

We found that ANGPTL1 was significantly downregulated and that CX3CR1 and CCL4 were significantly upregulated in both AVC and CAS patients (Figure 9A and B). The AVC data were obtained from human aortic valves, and the CAS data were obtained from human carotid arteries. The CAS-related dataset GSE28829 includes early and advanced carotid

A

B

C

D
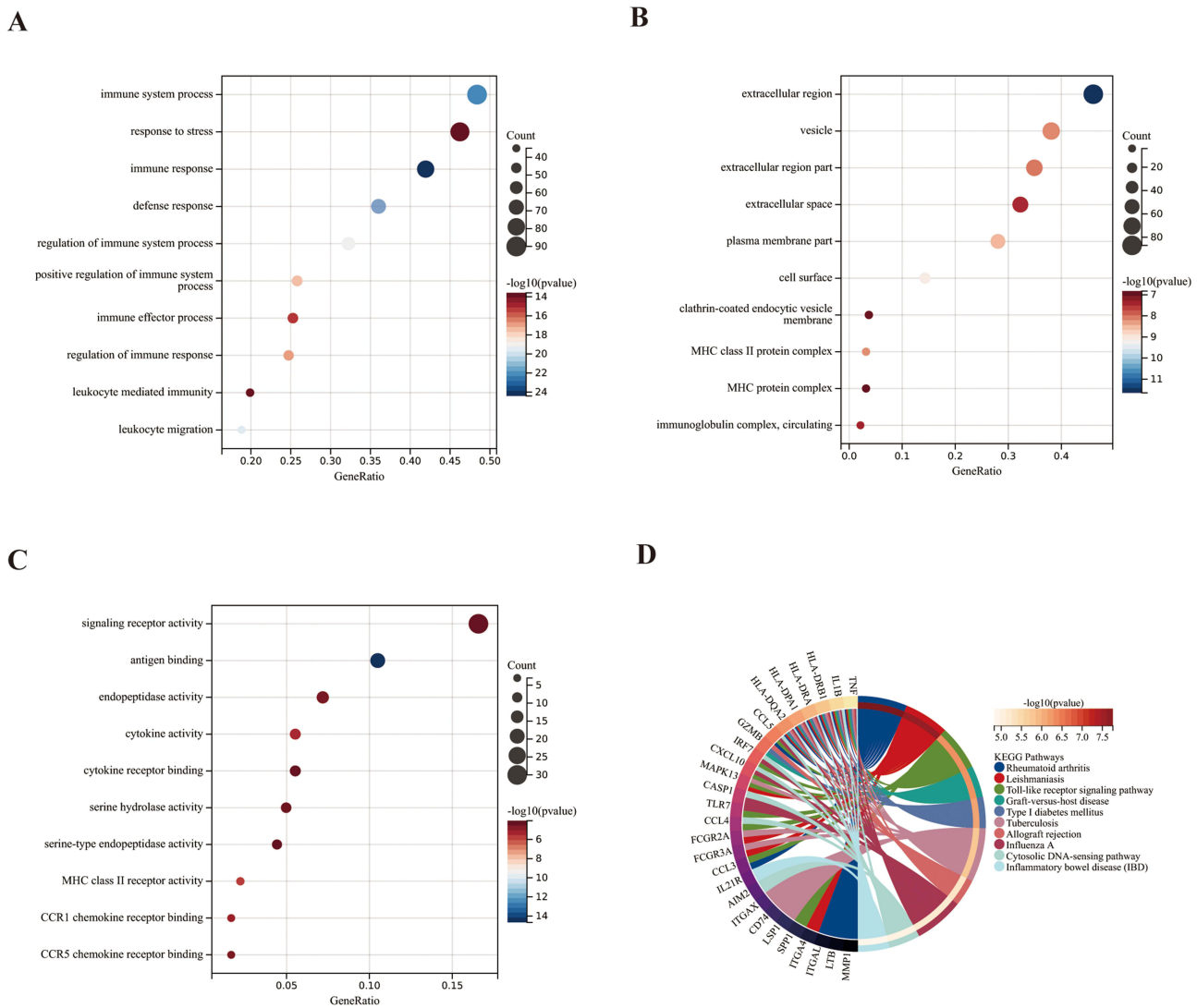
Figure 4 Gene ontology and pathway enrichment analysis of CEGs. (**A–C**) GO analysis of the CEGs, including biological process (BP), cellular component (CC), and molecular function (MF), respectively. The y-axis represents different GO terms, the x-axis represents gene ratio enriched in relative GO terms, the circle size refers to gene numbers, and the color represents p-value. (**D**) KEGG pathway analysis of the CEGs. Different colors represent various significant pathways and related genes.

artery plaque data, in which ANGPTL1 was downregulated and CX3CR1 and CCL4 were similarly upregulated in advanced carotid plaques (Figure 9C).

Discussion

Aortic valve calcification (AVC) and carotid atherosclerosis (CAS) are distinct cardiovascular conditions characterized by the accumulation of calcium in various areas. AVC specifically refers to the gradual buildup of calcium on the leaflets of the aortic valve, which can eventually result in aortic stenosis and the probable development of heart failure.[17,18] CAS, however, arises from the buildup of atherosclerotic plaques in the carotid arteries, which increases the likelihood of stroke.[19,20] Both AVC and CAS contribute significantly to the global burden of cardiovascular diseases.[6] With the aging population and persistent risk factors, the prevalence of these disorders is expected to increase, leading to significant health care expenses and diminished quality of life for those impacted.[21]

AVC and CAS have common risk factors, including hypertension, high cholesterol levels, smoking, and aging, and can potentially induce similar immune-related inflammatory reactions under both conditions.[22–25] We screened three immune-related genes via machine learning algorithms. Based on these findings, CCL4 and CX3CR1 exhibit
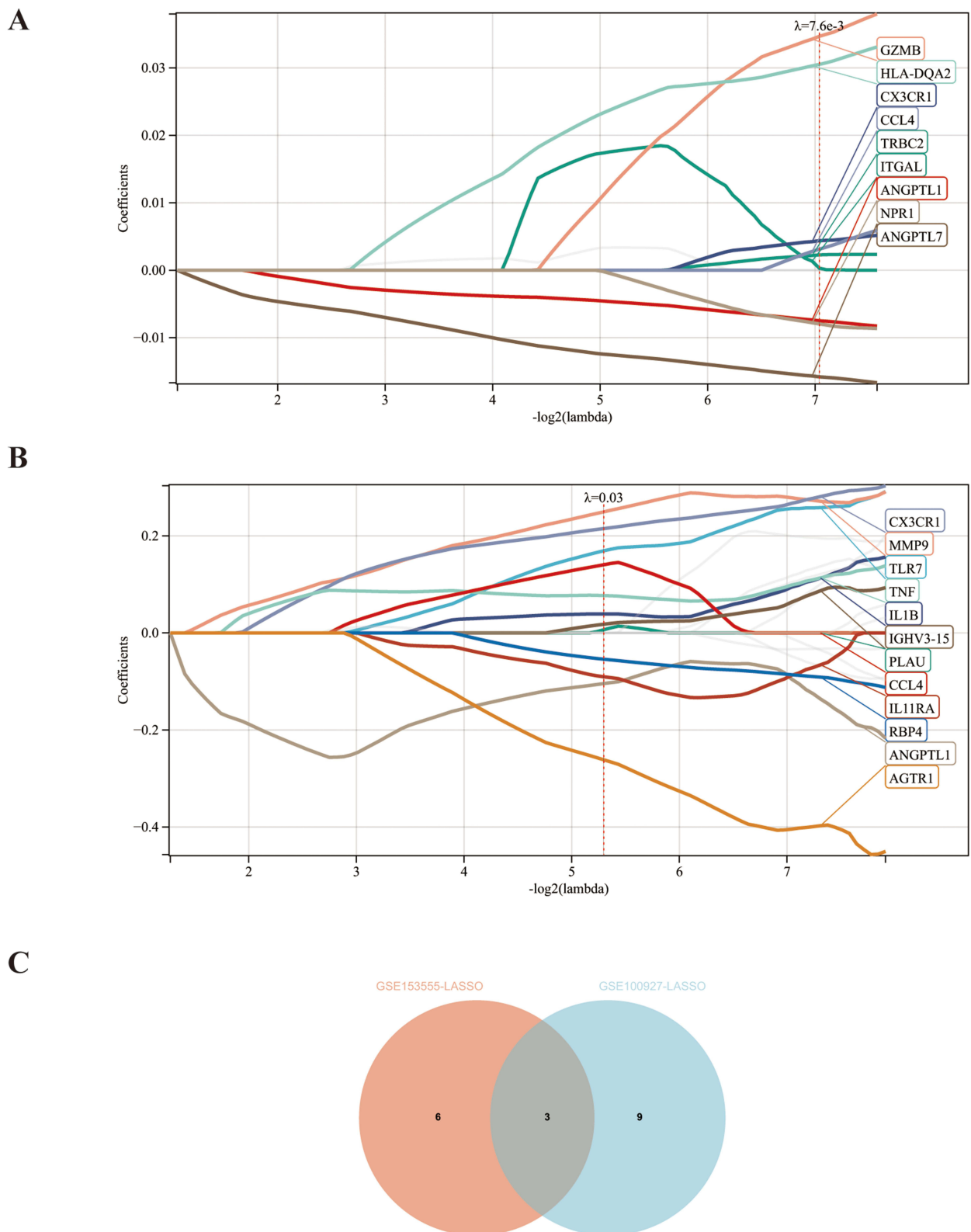
Figure 5 Machine learning in screening candidate hub genes for AVC and CAS. (**A**) Hub genes screening in the Lasso model for AVC. The number of genes (n =9) corresponding to the lowest point of the curve is the most suitable for AVC. (**B**) Hub genes screening in the Lasso model for CAS. The number of genes (n = 12) corresponding to the lowest point of the curve is the most suitable for CAS. (**C**) Venn diagram shows that three candidate hub genes are identified via the LASSO algorithms respectively.
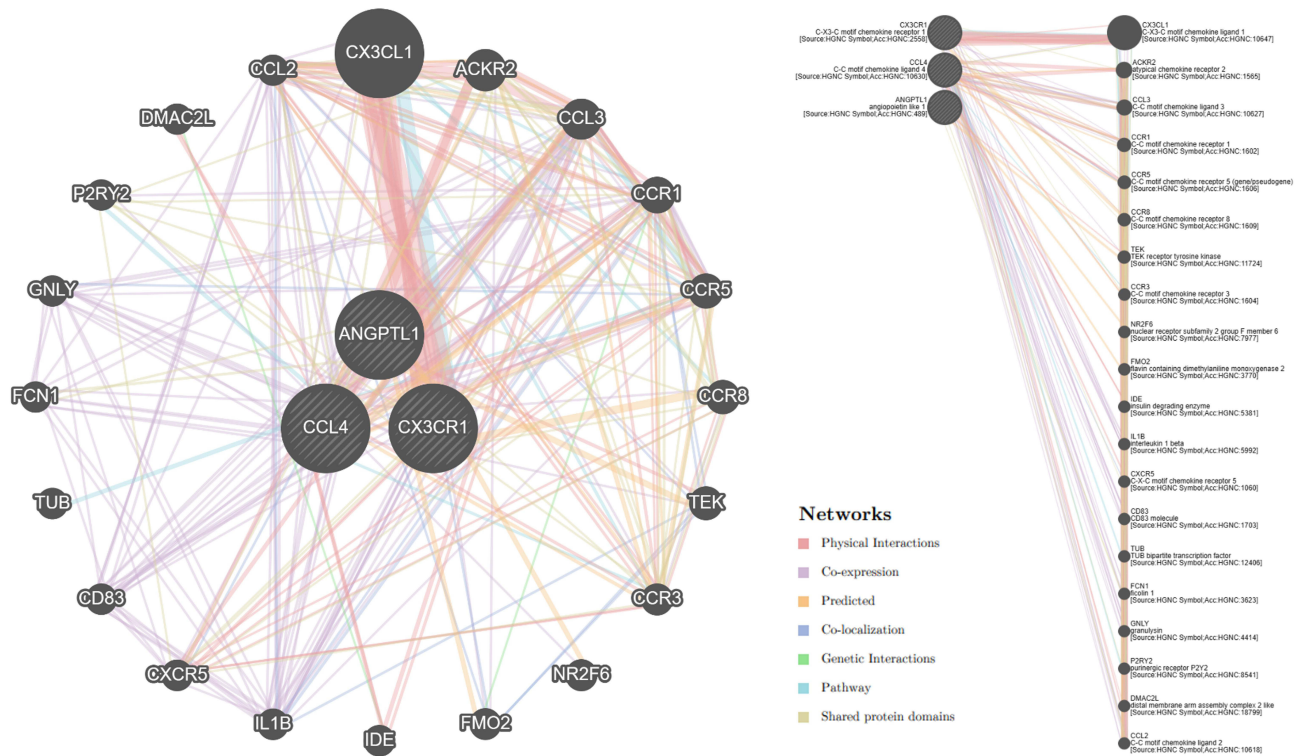
Figure 6 Gene-gene interaction network of ANGPTL1, CX3CR1 and CCL4 in GeneMANIA database.

a substantial increase in expression, while ANGPTL1 shows a decrease in expression among individuals with aortic valve calcification and carotid atherosclerosis. These findings indicate that these factors likely have a significant impact on the progression of these two diseases. Interestingly, compared to that in early carotid atherosclerotic plaques, the expression of the above three genes in advanced carotid atherosclerotic plaques exhibited a similar trend according to the GSE28829 data. These findings imply that three factors may have a significant impact on the development of these diseases.

CCL4 is a chemokine that can recruit monocytes and T lymphocytes and promote the infiltration of immune cells.[26,27] CX3CR1 is a receptor that is typically expressed on the surface of immune cells and can be involved in the migration and activation of immune cells, further exacerbating the local inflammatory response.[28,29] By regulating the activity of CCL4 and CX3CR1, it is possible to mitigate inflammation and thereby decelerate the progression of cardiovascular diseases.[30,31] Subsequent investigations can further elucidate the specific mechanism by which CCL4 and CX3CR1 contribute to the development of AVC and CAS and determine their potential as therapeutic targets.

ANGPTL1, also known as angiopoietin-like protein 1, was first identified in 1999. ANGPTL1 mostly exerts antiangiogenic effects and plays a significant role in protecting blood vessels; additionally, it acts as a tumor suppressor.[32] However, ANGPTL1 has not been reported to be involved in atherosclerosis, diabetes, hypertension or metabolic syndrome or to play an inflammatory role.[33] The downregulation of ANGPTL1 in both AVC and CAS patients indicates a potential protective function in these diseases; in its reduced state, it may not be possible to effectively counteract the inflammatory processes that contribute to AVC and CAS. The associations of ANGPTL1 with cell adhesion, cytokine activity, and immune cell regulation highlights its significance in immune-related pathways linked to AVC and CAS. ANGPTL1 potentially influences the regulation of immunological responses and suppresses the production of inflammatory cytokines. The therapeutic implications of ANGPTL1 in cardiovascular diseases are highly promising.[34] Elevating ANGPTL1 levels or harnessing its anti-inflammatory properties could be a promising strategy to mitigate the progression of AVC and CAS. However, further research is needed to determine the therapeutic potential of ANGPTL1 due to the intricate nature of these disorders and its diverse functions.
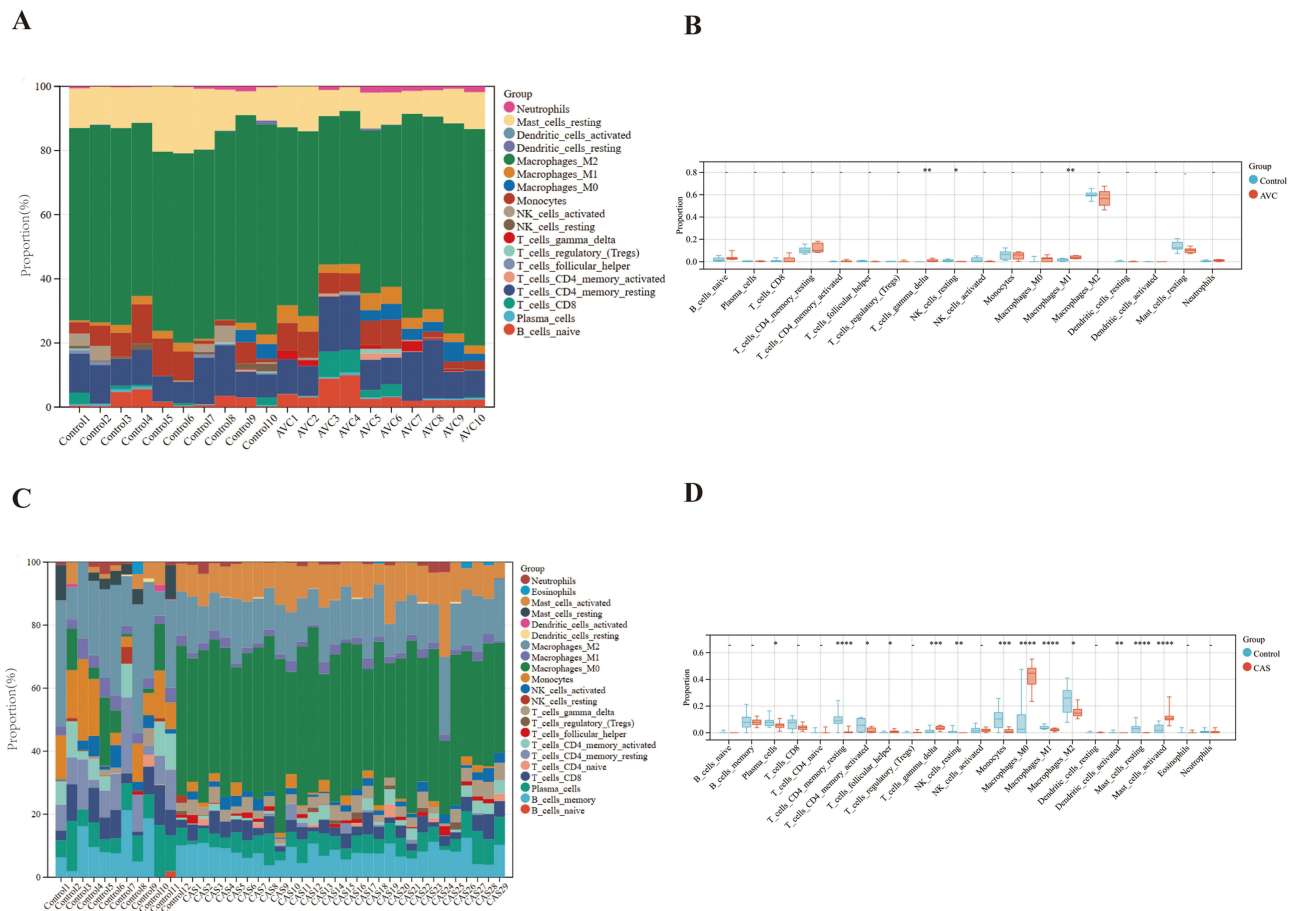
Figure 7 Immune cell infiltration analysis in GSE153555 and GSE100927 datasets. (**A**) The proportion of 22 kinds of immune cells in different samples visualized from the barplot in GSE153555. (**B**) The proportion of 22 kinds of immune cells in different samples visualized from the barplot in GSE100927. (**C**) Comparison regarding the proportion of 22 kinds of immune cells between AVC and control groups visualized by the vioplot. (**D**) Comparison regarding the proportion of 22 kinds of immune cells between CAS and control groups visualized by the vioplot.

Enrichment analysis revealed that the differentially expressed genes (DEGs) are linked to immunological factors, specifically involving immune cells and cytokines. TNF-α, also known as tumor necrosis factor (TNF-α), is a significant inflammatory mediator that has a crucial function in the pathogenesis of AVC and CAS.[10,35] Tumor necrosis factor (TNF) release can initiate an inflammatory reaction that prompts monocytes and other immune cells to move toward the aortic valve leaflet and carotid artery wall. This process ultimately results in the development and advancement of aortic valve calcification and carotid plaque formation.[36] Through the identification of drug candidates, thalidomide (a TNF inhibitor) has been screened as a potential therapeutic option for reducing the inflammatory response in patients with aortic valve calcification and carotid atherosclerosis.

The participation of immune cells in the development of AVC and CAS is an intricate and crucial element of these cardiovascular diseases.[8] When activated, macrophages generate a range of inflammatory mediators, such as cytokines and chemokines, which stimulate inflammation and harm tissues.[37,38] Furthermore, T cells play a crucial role in the adaptive immune system by identifying antigens produced by antigen-presenting cells (APCs) in many organs. Antigen recognition can initiate immunological responses and result in persistent inflammation, as observed in AVC and CAS.[9,39] Natural killer (NK) cells are components of the innate immune system, but their involvement in AVC and CAS has not been fully elucidated.[40,41] They have the potential to engage in interactions with other immune cells, such as macrophages and T cells, to influence the overall immunological response. Our results suggest that AVC and CAS patients have an increased proportion of γδ T cells and a decreased proportion of resting NK cells, which may be potential targets for immunomodulatory therapy aimed at preventing AVC and CAS.
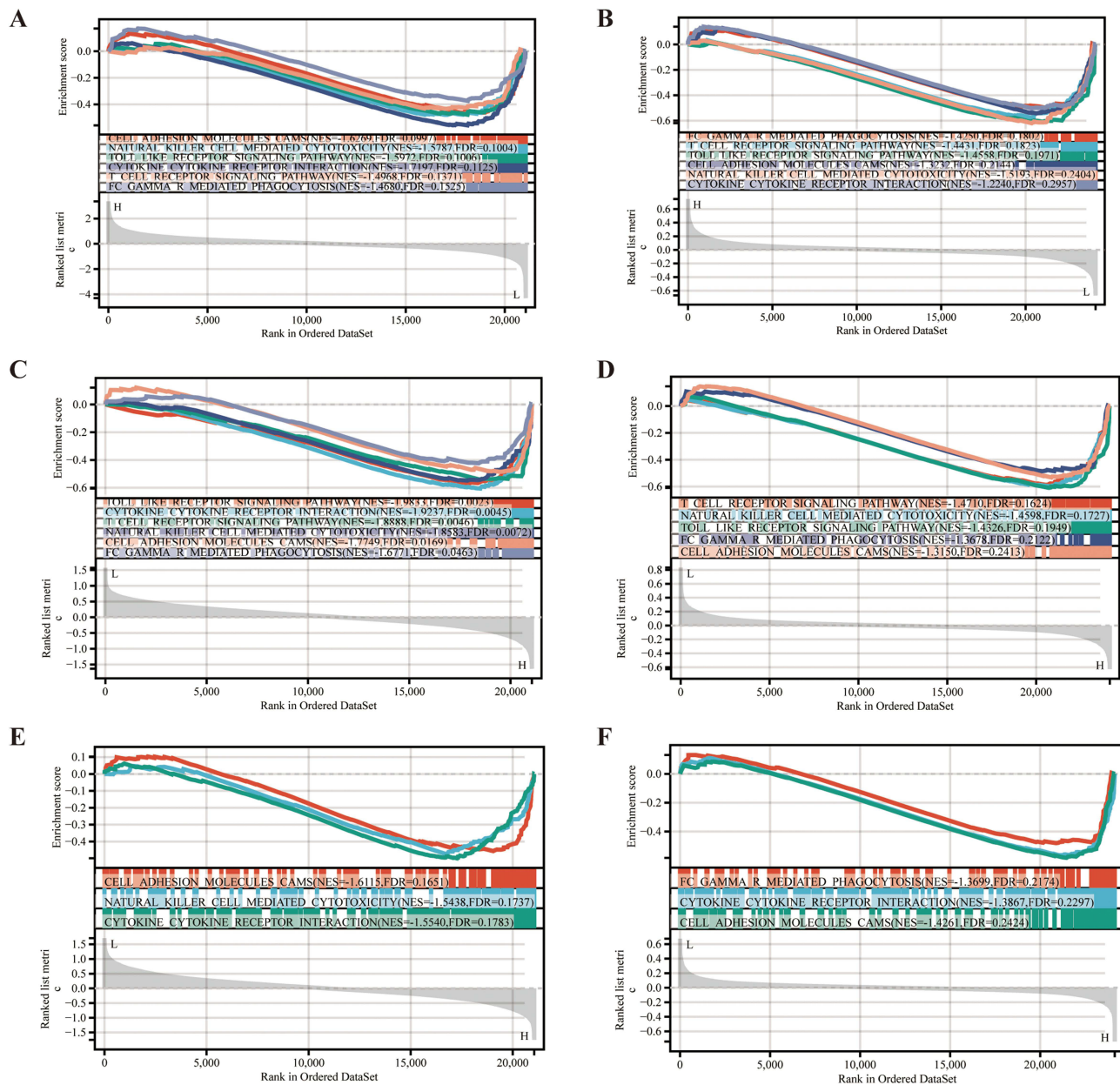
Figure 8 GSEA analysis focusing on the differential enrichment of KEGG pathways. (**A**) KEGG pathways of ANGPTL1 in GSE153555 datasets. (**B**) KEGG pathways of ANGPTL1 in GSE100927 datasets. (**C**) KEGG pathways of CX3CR1 in GSE153555 datasets. (**D**) KEGG pathways of CX3CR1 in GSE100927 datasets. (**E**) KEGG pathways of CCL4 in GSE153555 datasets. (**F**) KEGG pathways of CCL4 in GSE100927 datasets. (**A, C** and **E**) KEGG pathways of ANGPTL1, CX3CR1, CCL4 in GSE153555 datasets. (**B, D** and **F**) KEGG pathways of ANGPTL1, CX3CR1, CCL4 in GSE100927 datasets.

However, the transition from these findings to clinical applications requires further investigation. We recommend conducting in vivo studies to test the efficacy of these therapeutic strategies and to better understand the molecular dynamics regulated by ANGPTL1, CCL4, and CX3CR1 in the context of AVC and CAS. Additionally, longitudinal studies tracking the expression of these genes in patients over time could provide deeper insights into their roles at various stages of disease progression and response to treatment.

In conclusion, our study not only reinforces the understanding of key immunological genes in AVC and CAS but also opens up new avenues for targeted therapies aimed at modulating the immune response in these debilitating conditions. Continued research into these genes' specific functions and interactions will be crucial for developing effective therapeutic strategies that can be translated into clinical practice.
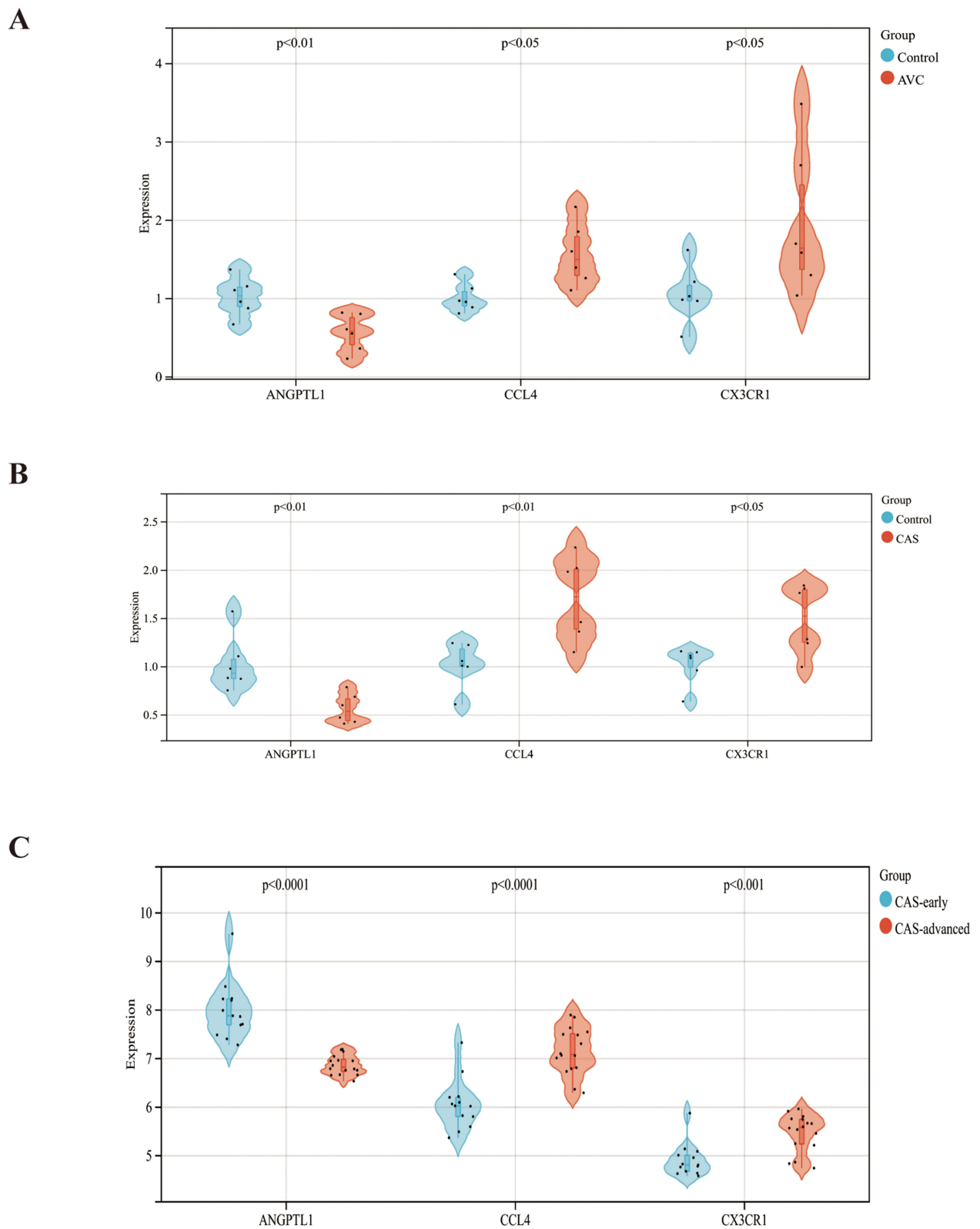
Figure 9 The expression of the three immune-related hub genes in Collected human aortic valve, carotid artery specimens and GSE28829. (**A**) The expression levels of ANGPTL1, CX3CR1, CCL4 in human aortic valve specimens. (**B**) The expression levels of ANGPTL1, CX3CR1, CCL4 in human carotid artery specimens. (**C**) The expression levels of ANGPTL1, CX3CR1, CCL4 in early and advanced atherosclerotic plaque from human carotid in GSE28829.

Limitations

The current study has several limitations. Initially, the sample size in this study was not very large. Therefore, it is imperative to validate our findings by reproducing them in additional datasets that include larger sample sizes when available. Additionally, the study is based on bioinformatics analysis. However, further experimentation is necessary to investigate the specific underlying mechanisms involved. Despite these limitations, this work offers insights into the same pathophysiological process between AVC and CAS and may help identify possible targets for treatment.

Conclusion

In this study, we effectively combined bioinformatics analysis with experimental confirmation to reveal shared immune-related genes linked to both aortic valve calcification and carotid artery atherosclerosis. These findings establish a framework for future research focused on comprehending the complex immunological foundation of various cardio-vascular illnesses and investigating innovative treatment approaches that target shared molecular pathways.

Data Sharing Statement

The datasets presented in this article can be found in online repositories. The names of the repositories and their accession numbers can be found in this article. The original data supporting the conclusions of this study have been provided by the authors without undue restrictions. The datasets generated and analyzed in this study are available in the GEO repository (https://www.ncbi.nlm.nih.gov/geo/). The datasets generated and analyzed in the current study are available from the corresponding author upon reasonable request.

Human Research and Ethics

Aortic valve samples for the validation experiment were obtained from patients undergoing aortic valve replacement, which is admitted to the hospital with aortic stenosis and aortic dissection. This study was approved by the Research Ethics Committee of Qilu Hospital (reference number: KYLL-202308-037) in accordance with the Declaration of Helsinki and the Code of Ethics of the World Medical Association. Written informed consent was obtained from all patients to participate in the study.

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Author Contributions

All authors conceived and designed the study. KW and YC contributed equally to this work. KW and YC performed the data collection and analysis and wrote the original draft. XG revised the article critically. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that this study was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

References

1. Virani SS, Alonso A, Benjamin EJ, et al. Heart disease and stroke statistics-2020 update: A report from the American heart association. *Circul*. 2020;141(9):e139–e596. doi:10.1161/CIR.0000000000000757

2. Yuan J, Usman A, Das T, Patterson AJ, Gillard JH, Graves MJ. Imaging carotid atherosclerosis plaque ulceration: Comparison of advanced imaging modalities and recent developments. *AJNR Am J Neuroradiol*. 2017;38(4):664–671. doi:10.3174/ajnr.A5026

3. Santangelo G, Bursi F, Faggiano A, et al. The global burden of valvular heart disease: From clinical epidemiology to management. *J Clin Med*. 2023;12(6):2178. doi:10.3390/jcm12062178

4. Whelton SP, Jha K, Dardari Z, et al. Prevalence of aortic valve calcium and the long-term risk of incident severe aortic stenosis. *JACC Cardiovasc Imag*. 2023. doi:10.1016/j.jcmg.2023.02.018

5. Alexandratou M, Papachristodoulou A, Li X, et al. Advances in noninvasive carotid wall imaging with ultrasound: A narrative review. *J Clin Med*. 2022;11(20):6196. doi:10.3390/jcm11206196

6. Blaser MC, Buffolo F, Halu A, et al. Multiomics of tissue extracellular vesicles identifies unique modulators of atherosclerosis and calcific aortic valve stenosis. *Circul*. 2023;148(8):661–678. doi:10.1161/CIRCULATIONAHA.122.063402

7. Marrero N, Razavi AC, Boakye E, et al. Association of inflammation and lipoprotein (a) with aortic valve calcification. *JACC Cardiovasc Imag*. 2023;16(9):1230–1232. doi:10.1016/j.jcmg.2023.02.013

8. Broeders W, Bekkering S, El Messaoudi S, Joosten LAB, van Royen N, Riksen NP. Innate immune cells in the pathophysiology of calcific aortic valve disease: Lessons to be learned from atherosclerotic cardiovascular disease? *Basic Res Cardiol*. 2022;117(1):28. doi:10.1007/s00395-022-00935-6

9. Álvarez-Heredia P, Domínguez-Del-Castillo JJ, Reina-Alfonso I, et al. A straightforward cytometry-based protocol for the comprehensive analysis of the inflammatory valve infiltrate in aortic stenosis. *Int J Mol Sci*. 2023;24(3):2194. doi:10.3390/ijms24032194

10. Goikuria H, Vandenbroeck K, Alloza I. Inflammation in human carotid atheroma plaques. *Cytokine Growth Factor Rev*. 2018;39:62–70. doi:10.1016/j.cytogfr.2018.01.006

11. Shendure J, Ji H. Next-generation DNA sequencing. *Nat Biotechnol*. 2008;26(10):1135–1145. doi:10.1038/nbt1486

12. Li B, Ruotti V, Stewart RM, Thomson JA, Dewey CN. RNA-Seq gene expression estimation with read mapping uncertainty. *Bioinfo*. 2010;26(4):493–500. doi:10.1093/bioinformatics/btp692

13. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47. doi:10.1093/nar/gkv007

14. Gene Ontology Consortium. The gene ontology resource: 20 years and still GOing strong. *Nucleic Acids Res*. 2019;47(D1):D330–D338. doi:10.1093/nar/gky1055

15. Kanehisa M, Goto S. KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res*. 2000;28(1):27–30. doi:10.1093/nar/28.1.27

16. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: An R package for comparing biological themes among gene clusters. *OMICS*. 2012;16(5):284–287. doi:10.1089/omi.2011.0118

17. Moncla L-HM, Briend M, Bossé Y, Mathieu P. Calcific aortic valve disease: Mechanisms, prevention and treatment. *Nat Rev Cardiol*. 2023;20(8):546–559. doi:10.1038/s41569-023-00845-7

18. Yang C, Xu H, Jia R, Jin Z, Zhang C, Yuan J. Global burden and improvement gap of non-rheumatic calcific aortic valve disease: 1990–2019 findings from global burden of disease study 2019. *J Clin Med*. 2022;11(22):6733. doi:10.3390/jcm11226733

19. Wang C, Fang X, Tang Z, et al. Frailty in relation to the risk of carotid atherosclerosis and cardiovascular events in Chinese community-dwelling older adults: A five-year prospective cohort study. *Exp Gerontol*. 2023;180:112266. doi:10.1016/j.exger.2023.112266

20. Khan F, Gonçalves I, Shore AC, et al. Plaque characteristics and biomarkers predicting regression and progression of carotid atherosclerosis. *Cell Rep Med*. 2022;3(7):100676. doi:10.1016/j.xcrm.2022.100676

21. Kronenberg F, Mora S, Stroes ESG, et al. Lipoprotein (a) in atherosclerotic cardiovascular disease and aortic stenosis: A European atherosclerosis society consensus statement. *Eur Heart J*. 2022;43(39):3925–3946. doi:10.1093/eurheartj/ehac361

22. Takx RAP, Zanen P, Leiner T, van der Graaf Y, de Jong PA. The interdependence between cardiovascular calcifications in different arterial beds and vascular risk factors in patients at high cardiovascular risk. *Atherosclerosis*. 2015;238(1):140–146. doi:10.1016/j.atherosclerosis.2014.11.024

23. Abdul-Rahman T, Lizano-Jubert I, Garg N, et al. The common pathobiology between coronary artery disease and calcific aortic stenosis: Evidence and clinical implications. *Prog Cardiovasc Dis*. 2023;79:89–99. doi:10.1016/j.pcad.2023.06.002

24. Hjortnaes J, Butcher J, Figueiredo J-L, et al. Arterial and aortic valve calcification inversely correlates with osteoporotic bone remodelling: A role for inflammation. *Eur Heart J*. 2010;31(16):1975–1984. doi:10.1093/eurheartj/ehq237

25. Bortnick AE, Buzkova P, Otvos JD, et al. High-density lipoprotein and long-term incidence and progression of aortic valve calcification: The multi-ethnic study of atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2022;42(10):1272–1282. doi:10.1161/ATVBAHA.122.318004

26. Ohukainen P, Syväranta S, Näpänkangas J, et al. MicroRNA-125b and chemokine CCL4 expression are associated with calcific aortic valve disease. *Ann Med*. 2015;47(5):423–429. doi:10.3109/07853890.2015.1059955

27. Chang -T-T, Yang H-Y, Chen C, Chen J-W. CCL4 inhibition in atherosclerosis: Effects on plaque stability, endothelial cell adhesiveness, and macrophages activation. *Int J Mol Sci*. 2020;21(18):6567. doi:10.3390/ijms21186567

28. Martínez-Hervás S, Vinué A, Núñez L, et al. Insulin resistance aggravates atherosclerosis by reducing vascular smooth muscle cell survival and increasing CX3CL1/CX3CR1 axis. *Cardiovasc Res*. 2014;103(2):324–336. doi:10.1093/cvr/cvu115

29. Lu M, Zhao W, Han S, et al. Activation of the human chemokine receptor CX3CR1 regulated by cholesterol. *Sci Adv*. 2022;8(26):eabn8048. doi:10.1126/sciadv.abn8048

30. Chang TT, Chen J-W. Emerging role of chemokine CC motif ligand 4 related mechanisms in diabetes mellitus and cardiovascular disease: Friends or foes? *Cardiovasc Diabetol*. 2016;15(1):117. doi:10.1186/s12933-016-0439-9

31. Bonacina F, Martini E, Svecla M, et al. Adoptive transfer of CX3CR1 transduced-T regulatory cells improves homing to the atherosclerotic plaques and dampens atherosclerosis progression. *Cardiovasc Res*. 2021;117(9):2069–2082. doi:10.1093/cvr/cvaa264

32. Santulli G. Angiopoietin-like proteins: A comprehensive look. *Front Endocrinol*. 2014;5:4. doi:10.3389/fendo.2014.00004

33. Thorin E, Labbé P, Lambert M, et al. Angiopoietin-like proteins: cardiovascular biology and therapeutic targeting for the prevention of cardiovascular diseases. *Can J Cardiol*. 2023;39(12):1736–1756. doi:10.1016/j.cjca.2023.06.002

34. Kadomatsu T, Tabata M, Oike Y. Angiopoietin-like proteins: Emerging targets for treatment of obesity and related metabolic diseases. *FEBS J.* 2011;278(4):559–564. doi:10.1111/j.1742-4658.2010.07979.x

35. Galeone A, Brunetti G, Oranger A, et al. Aortic valvular interstitial cells apoptosis and calcification are mediated by TNF-related apoptosis-inducing ligand. *Int J Cardiol.* 2013;169(4):296–304. doi:10.1016/j.ijcard.2013.09.012

36. Grim JC, Aguado BA, Vogt BJ, et al. Secreted factors from proinflammatory macrophages promote an osteoblast-like phenotype in valvular interstitial cells. *Arterioscler Thromb Vasc Biol.* 2020;40(11):e296–e308. doi:10.1161/ATVBAHA.120.315261

37. Neels JG, Gollentz C, Chinetti G. Macrophage death in atherosclerosis: Potential role in calcification. *Front Immunol.* 2023;14:1215612. doi:10.3389/fimmu.2023.1215612

38. Tagzirt M, Rosa M, Corseaux D, et al. Modulation of inflammatory M1-macrophages phenotype by valvular interstitial cells. *J Thorac Cardiovasc Surg.* 2022;166(5):e377–e389. doi:10.1016/j.jtcvs.2022.08.027

39. Ma X, Zhuo Y, Huang Y, et al. Reduced diversities and clonally expanded sequences of T-cell receptors in patients with essential hypertension and subclinical carotid atherosclerosis. *Hypertension.* 2023;80(11):2318–2329. doi:10.1161/HYPERTENSIONAHA.123.21112

40. Bonaccorsi I, Spinelli D, Cantoni C, et al. Symptomatic carotid atherosclerotic plaques are associated with increased infiltration of natural killer (NK) cells and higher serum levels of nk activating receptor ligands. *Front Immunol.* 2019;10:1503. doi:10.3389/fimmu.2019.01503

41. Raddatz MA, Madhur MS, Merryman WD. Adaptive immune cells in calcific aortic valve disease. *Am J Physiol Heart Circ Physiol.* 2019;317(1):H141–H155. doi:10.1152/ajpheart.00100.2019