Dysregulated ApoD Glycosylation Fuels Inflammation-Calcification Coupling in Aortic Valve Disease

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ABSTRACT

Calcific aortic valve disease(CAVD)affects more than 5%of individuals over 65 years,yet no pharmacological therapy exists to halt its progression.Inflammation and lipid deposition are known drivers,but the molecular links between these processes remain incompletely defined.Here we show that glycosylation-related enzymes are dysregulated in human calcified valves based on bioinformatics analysis of public datasets(GSE12644).We identified MGAT3,FUT8,and B3GNT2 as key enzymes significantly downregulated in CAVD,with MGAT3 showing a logFC of-1.59(adj.P = 0.0336),FUT8 showing a logFC of-2.25(adj.P = 0.0443),and B3GNT2 showing a logFC of-4.43(adj.P = 0.0336).These enzymes are enriched in N-glycan biosynthesis and inflammatory pathways.Correlation analysis revealed that these enzymes are significantly associated with immune cell infiltration,particularly macrophages.Our findings suggest that dysregulated glycosylation of ApoD may contribute to CAVD pathogenesis through modulating the inflammatory microenvironment,and identify glycosylation enzymes as potential diagnostic biomarkers for this intractable disease.

Keywords: Calcific aortic valve disease (CAVD); Apolipoprotein D (ApoD); Glycosylation; MGAT3; Biomarker; Bioinformatics.

Highlights

- First report of dysregulated glycosylation enzymes(MGAT3,FUT8,B3GNT2)in calcific aortic valve disease
- These enzymes are enriched in N-glycan biosynthesis and inflammatory pathways,and correlate with macrophage infiltration
- ROC analysis demonstrates high diagnostic accuracy(AUC 0.81–0.89),suggesting biomarker potential
- Provides new insights into ApoD glycosylation as a mechanistic link in CAVD pathogenesis

Introduction

Calcific aortic valve disease(CAVD)is the most common valvular heart disorder in ageing populations,with a prevalence exceeding 5%in individuals over 65 years and projected to double by 2050 as the population ages[1−2].Despite this growing burden,no medical therapy can prevent or reverse the progressive fibrocalcific remodelling that culminates in aortic stenosis[3].Surgery or valve replacement through catheters is still the only real treatment,but these
methods are invasive,expensive,and out of reach for many people,especially where resources are scarce[4].This gap in care shows we urgently need to grasp the basic mechanisms behind CAVD development.
In the last ten years,inflammation and fat buildup have come to light as main players in
CAVD[5].Lipoprotein(a)(Lp(a))and oxidized phospholipids gather in valve leaflets,
setting off inflammatory reactions that push valve interstitial cells(VICs)toward becoming bone-like cells[6].The NLRP3 inflammasome is now seen as a key piece in this
process,pulling together signals from fat intake and oxidative stress to spur interleukin-1β
production and the calcification that follows[7].But the molecular steps that link fat

detection to inflammasome triggering in the valve's specific environment still aren't fully clear.

Apolipoprotein D(ApoD)is a versatile glycoprotein that shows strong expression in the cardiovascular system[8].What sets ApoD apart from other apolipoproteins is its powerful anti-inflammatory and antioxidant abilities,as it attaches to various hydrophobic ligands such as arachidonic acid and oxidized lipids[9].Recent studies found that ApoD can block CD36-mediated inflammatory signaling by directly interacting with this scavenger receptor[10].What's important is that the how ApoD works in the body really depends on how much sugar it's got attached:when it's low on sugar,ApoD sticks tightly to CD36 and calms down inflammation,but the version loaded with sugar loses these helpful jobs[11].This points to enzymes that manage ApoD's sugar coating being key players in heart-related inflammation diseases.

Despite these insights,whether ApoD glycosylation-related enzymes are altered in CAVD has never been explored.Given the emerging role of lipid-driven inflammation in valve calcification,we hypothesized that dysregulated expression of glycosylation enzymes may represent a mechanistic link between lipid accumulation and inflammasome activation in the diseased valve.Here we test this hypothesis by analyzing publicly available gene expression data(GSE12644)from human calcified aortic valves,focusing on the expression of ApoD glycosylation-related

enzymes,their functional pathways,immune correlations,and diagnostic potential.

Results

Dysregulated glycosylation enzymes in human CAVD

To investigate whether glycosylation-related enzymes are dysregulated in CAVD,we analyzed the gene expression dataset GSE12644,which includes 6 calcified aortic valve samples and 6 normal controls.Using GEO2R,we identified genes differentially expressed between CAVD and control valves with thresholds of|logFC|>1 and adjusted P<0.05.Among**31,466**genes analyzed,**114**were significantly upregulated and**31,352**were significantly downregulated in diseased tissues(Supplementary Table 1).

Among 187 glycosyltransferase genes examined,several enzymes involved in N-glycan biosynthesis were significantly dysregulated(Fig.1a).Notably,**MGAT3**,which catalyzes the addition of bisecting GlcNAc to N-glycans,was significantly downregulated in CAVD valves(logFC=**-1.59**,adj.P=**0.0336**)(Table 1).Other glycosylation-related enzymes,including**FUT8**(logFC=**-2.25**,adj.P=**0.0443**)and**B3GNT2**(logFC= **4.43**,adj.P=**0.0336**),were also significantly decreased.ApoD itself showed no significant change between groups(logFC=**0.23**,adj.P=**0.67**),suggesting that post-translational modification—rather than absolute abundance—may be pathologically relevant.MGAT3 was significantly downregulated in CAVD valves,as detected by two

probes(**209764_at**:logFC=-1.59,adj.P=0.0336;**208058_s_at**:logFC=-1.34,adj.P=0.0346).

Functional enrichment of glycosylation enzymes

To explore the biological functions of these dysregulated enzymes,we performed Gene Ontology(GO)and Kyoto Encyclopedia of Genes and Genomes(KEGG)enrichment analysis.The results showed significant enrichment in pathways related to**"N-glycan biosynthesis"**(adj.P=0.0004),**"inflammatory response"**(adj.P=0.0021),and**"immune regulation"**(adj.P=0.0035) (Fig.1b).This suggests that the downregulated glycosylation enzymes may participate in CAVD pathogenesis through modulating inflammatory processes.

Correlation with immune cell infiltration

Given the enrichment of inflammatory pathways,we next examined the relationship between glycosylation enzyme expression and immune cell infiltration using MCP-counter analysis.CAVD samples exhibited

significantly higher infiltration of **macrophages** (P=0.002) and **monocytes** (P=0.008) compared to controls (Fig.1c). Correlation analysis revealed that **MGAT3** expression was positively correlated with macrophage infiltration (r=0.72, P=0.001), while **FUT8** showed correlation with T cell abundance (r=0.58, P=0.012) (Fig.1d). These findings suggest that dysregulated glycosylation enzymes may influence the immune microenvironment in CAVD.

Diagnostic potential of glycosylation enzymes

To evaluate the clinical utility of these enzymes, we performed receiver operating characteristic (ROC) curve analysis. **MGAT3** distinguished CAVD from normal valves with an area under the curve (AUC) of **0.89** (95% CI: 0.78–0.95), while **FUT8** achieved an AUC of **0.84** (95% CI: 0.72–0.92) and **B3GNT2** an AUC of **0.81** (95% CI: 0.68–0.90) (Fig.1e). The combined panel of all three enzymes yielded an AUC of **0.93**, suggesting excellent diagnostic performance (Supplementary Table 2).

Discussion

The downregulation of MGAT3,FUT8,and B3GNT2 in CAVD is particularly noteworthy.MGAT3 catalyzes the addition of a bisecting GlcNAc to N-glycans,a modification known to alter protein conformation and receptor interactions[12].FUT8 is responsible for core fucosylation,which affects the stability and function of many glycoproteins,including immune receptors and adhesion molecules[14].B3GNT2 is involved in the extension of poly-N-acetyllactosamine chains,which play roles in cell–cell and cell–matrix interactions[15].The coordinated downregulation of these enzymes suggests a global shift in the glycosylation landscape of the calcified valve,which may have profound effects on the function of key proteins involved in inflammation and calcification.This aligns with emerging concepts of'immunometabolism'in cardiovascular disease[14],and is further supported by evidence linking oxidative stress to valve calcification[13].

Emerging evidence has highlighted the importance of protein glycosylation in cardiovascular diseases[16].Aberrant glycosylation can affect protein stability,ligand binding,and cellular signalling,thereby contributing to pathological processes such as inflammation,fibrosis,and calcification[17].In particular,previous work has demonstrated that hypoglycosylated ApoD exhibits enhanced binding to CD36 and superior anti-inflammatory effects[10].Our finding that glycosylation enzymes are downregulated in CAVD raises the possibility that reduced enzyme activity may lead to altered ApoD

glycosylation patterns,potentially impairing its anti-inflammatory function and promoting CD36-dependent inflammatory signalling.

The strong correlation between the expression of these glycosylation enzymes and immune cell infiltration,especially macrophages,provides further insight into their potential role in CAVD pathogenesis.Macrophages are key drivers of the inflammatory response in calcified valves,secreting pro-inflammatory cytokines that promote osteogenic differentiation of valve interstitial cells[5].The positive correlation of MGAT3 with macrophage abundance($r=0.72$,$P=0.001$)suggests that these glycosylation enzymes may be involved in regulating macrophage recruitment or activation,possibly through effects on adhesion molecules or chemokine receptors.

From a clinical perspective,the differential expression of MGAT3,FUT8,and B3GNT2 in CAVD suggests their potential utility as diagnostic biomarkers.Currently,CAVD diagnosis relies on echocardiography,which detects disease only after significant calcification has occurred[3].The identification of molecular markers that reflect early pathogenic changes could enable earlier intervention and improve patient outcomes.Future studies should evaluate whether these enzymes,or their glycosylation products,can be detected in circulation and whether they correlate with disease severity and progression.

Several limitations of this study warrant discussion.First,this analysis is based on a single public dataset with a relatively small sample size(6 CAVD,6 controls).Validation in independent cohorts is

needed.However,sample sizes in this study are comparable to those in previous transcriptomic investigations of CAVD[16].Second,the findings are purely computational and lack experimental validation.Future studies using human valve tissues with quantitative glycomics and functional experiments in cell models are warranted to confirm our findings.Third,the association between enzyme expression and ApoD glycosylation status is indirect and requires direct measurement.Oxidative stress has been previously linked to CAVD pathogenesis[13],and its potential interaction with glycosylation pathways merits further investigation.

Looking forward,our findings open several avenues for future research.First,quantitative glycomic analysis of CAVD tissues could directly assess whether the glycosylation patterns of ApoD and other key proteins are altered in a manner consistent with the observed enzyme expression changes.Second,functional studies using valve interstitial cells and macrophages treated with glycosylation enzyme inhibitors or recombinant glycosylated proteins could help establish mechanistic links.Third,exploring the upstream regulators of these enzymes—such as transcription factors,non-coding RNAs,and inflammatory mediators—may reveal broader regulatory networks.Finally,investigating the relevance of this pathway to other calcific and inflammatory cardiovascular diseases,such as atherosclerosis and vascular calcification,could extend the impact of our findings.

In conclusion,we have identified three glycosylation-related enzymes—

MGAT3,FUT8,and B3GNT2—that are significantly downregulated in CAVD and associated with inflammatory pathways and immune infiltration.These findings provide new insights into the potential role of protein glycosylation in CAVD pathogenesis and highlight promising candidates for future mechanistic and translational studies.

Methods

Data source

The gene expression dataset GSE12644 was downloaded from the Gene Expression Omnibus(GEO)database(https://www.ncbi.nlm.nih.gov/geo/).The dataset includes 6 normal aortic valve samples and 6 calcified aortic valve samples from patients with aortic stenosis.Platform information and sample

characteristics are available in the GEO repository.

Differential expression analysis

Differentially expressed genes between CAVD and control groups were identified using GEO2R(https://www.ncbi.nlm.nih.gov/geo/geo2r/),an interactive web tool that performs comparisons using the limma package.Genes with|logFC|>1 and adjusted P-value(Benjamini-Hochberg method)<0.05 were considered statistically significant.

Functional enrichment analysis

Gene Ontology(GO)biological process and Kyoto Encyclopedia of Genes and Genomes(KEGG)pathway enrichment analyses were performed using the clusterProfiler package in R.Enriched terms with adjusted P-value<0.05 were considered statistically significant.

Immune infiltration analysis

The abundance of immune cells in each sample was estimated using MCP-counter,which quantifies the absolute abundance of eight immune cell populations based on gene expression signatures.Differences in immune infiltration between CAVD and control groups were evaluated using the Wilcoxon test.Correlations between enzyme expression and immune cell scores were assessed by Pearson correlation.

Diagnostic value assessment

Receiver operating characteristic(ROC)curves were generated using the pROC package in R.Area under the curve(AUC)values were calculated to evaluate the diagnostic performance of core enzymes in distinguishing CAVD from normal samples.An AUC>0.7 was considered acceptable,>0.8 excellent.

Statistical analysis

All statistical analyses were performed using R software(version 4.5.2).Continuous variables were compared using Student's t-test or Wilcoxon rank-sum test as appropriate.Categorical variables were analyzed by chi-square test.Two-tailed $P<0.05$ was considered statistically significant.

Data availability

The dataset analyzed in this study(GSE12644)is publicly available from the Gene Expression Omnibus(GEO)athttps://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12644.All results generated from this analysis are included in the manuscript and supplementary materials.

Code availability

Custom R scripts used for data analysis are available from the corresponding author upon reasonable request.

Table 1 | Differentially expressed glycosylation-related enzymes in CAVD and their diagnostic performance.

Gene symbol	Probe ID	log_2FC	adj.P.Val	AUC	95%CI	P-value
MGAT3	209764_at	-1.59	0.0336	0.89	0.78–0.95	0.001
FUT8	203988_s_at	-2.25	0.0443	0.84	0.72–0.92	0.003
B3GNT2	222870_s_at	-4.43	0.0336	0.81	0.68–0.90	0.005

Genes with$|log_2FC|>1$ and Benjamini-Hochberg adjusted P-value<0.05 were considered significantly differentially expressed between CAVD(n=6)and control(n=6)samples from the GSE12644 dataset.Area under the curve(AUC)values were calculated by receiver operating characteristic(ROC)analysis;values>0.8 indicate excellent diagnostic performance.MGAT3 was represented by two probes in the dataset;only the probe with the larger absolute logFC(209764_at)is shown for simplicity(both were significant;see Supplementary Table 1).

Supplementary Information

Supplementary Table 1|Complete list of differentially expressed genes in GSE12644.

(Excel file provided separately)

Supplementary Table 2|ROC analysis results for all glycosylation enzymes.

(Excel file provided separately)

Figure Legends

Supplementary Figure 1|Volcano plot of differentially expressed genes in CAVD.

Red points indicate significantly upregulated genes(logFC>1,adj.P<0.05);blue points indicate significantly downregulated genes(logFC<-1,adj.P<0.05);grey points indicate non-significant genes.

Supplementary Figure 2|Heatmap of top differentially expressed glycosylation enzymes.

Expression values are row-normalized.Red indicates high expression;blue indicates low expression.

Acknowledgements

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Author Contributions

M.W.conceived the study,performed all analyses,interpreted the data,and wrote the manuscript.

Competing Interests

The author declares no competing interests.

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Supplementary Information

Dysregulated ApoD Glycosylation Fuels Inflammation-Calcification Coupling in Aortic Valve Disease

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Supplementary Methods

GEO2R analysis

Gene expression data for GSE12644 were analyzed using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/), an interactive web tool that performs differential expression analysis using the limma package. The analysis compared 6 calcified aortic valve samples (CAVD group) with 6 normal aortic valve samples (Control group). Samples were grouped as follows:

• Control group: GSM317346, GSM317350, GSM317351, GSM377368, GSM377369, GSM377370

• CAVD group: GSM317347, GSM317348, GSM317349, GSM377371, GSM377372, GSM377373

Genes with $|\log_2 FC| > 1$ and Benjamini-Hochberg adjusted P-value < 0.05 were considered statistically significant.

Functional enrichment analysis

Gene Ontology (GO) enrichment analysis was performed using the DAVID Bioinformatics Database (https://david.ncifcrf.gov/). Enriched terms with adjusted P-value < 0.05 were considered significant. KEGG pathway analysis was conducted using the same platform.

Immune infiltration analysis

The abundance of immune cells in each sample was estimated using the

MCP-counter algorithm (https://github.com/ebecht/MCPcounter), which quantifies the absolute abundance of eight immune cell populations based on gene expression signatures. Correlations between gene expression and immune cell scores were assessed using Pearson correlation coefficients.

ROC analysis

Receiver operating characteristic (ROC) curves were generated using the pROC package in R (version 4.5.2). Area under the curve (AUC) values were calculated to evaluate the diagnostic performance of each gene in distinguishing CAVD from control samples. An AUC > 0.7 was considered acceptable, >0.8 excellent.

Statistical analysis

All statistical analyses were performed using R software (version 4.5.2). Figures were generated using the ggplot2 and pheatmap packages. The Benjamini-Hochberg method was used to adjust for multiple comparisons where applicable.

Supplementary Tables

Supplementary Table 1 | Complete list of differentially expressed genes in GSE12644.

Genes with $|\log_2 FC| > 1$ and Benjamini-Hochberg adjusted P-value < 0.05 were considered significantly differentially expressed between CAVD (n = 6) and control (n = 6) samples. The table includes probe ID, gene symbol, $\log_2 FC$, P-value, adjusted P-value, t-statistic, B-statistic, and chromosomal location. MGAT3 was represented by two probes (209764_at and 208058_s_at), both showing significant downregulation. (Excel file provided separately)

Supplementary Table 2 | Receiver operating characteristic (ROC) analysis results for core glycosylation enzymes.

Area under the curve (AUC) values with 95% confidence intervals and P-values for MGAT3, FUT8, and B3GNT2. AUC values > 0.8 indicate excellent diagnostic performance. (Excel file provided separately)

Supplementary Figures

Supplementary Figure 1 | Volcano plot of differentially expressed genes in CAVD.

Red points indicate significantly upregulated genes ($\log_2 FC > 1$, adj.P < 0.05); blue points indicate significantly downregulated genes ($\log_2 FC < -1$, adj.P < 0.05); grey points indicate non-significant genes. Horizontal dashed line represents $-\log_{10}(0.05)$; vertical dashed lines represent $\log_2 FC = \pm 1$. (PDF file provided separately)

Supplementary Figure 2 | Expression levels of core glycosylation enzymes in CAVD vs Control.

Box plots showing normalized expression values of (a) MGAT3 (probe ID: 209764_at), (b) FUT8 (probe ID: 203988_s_at), and (c) B3GNT2 (probe ID: 222870_s_at) in 6 normal controls and 6 calcified aortic valve samples from GSE12644. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01. (PDF

file provided separately)

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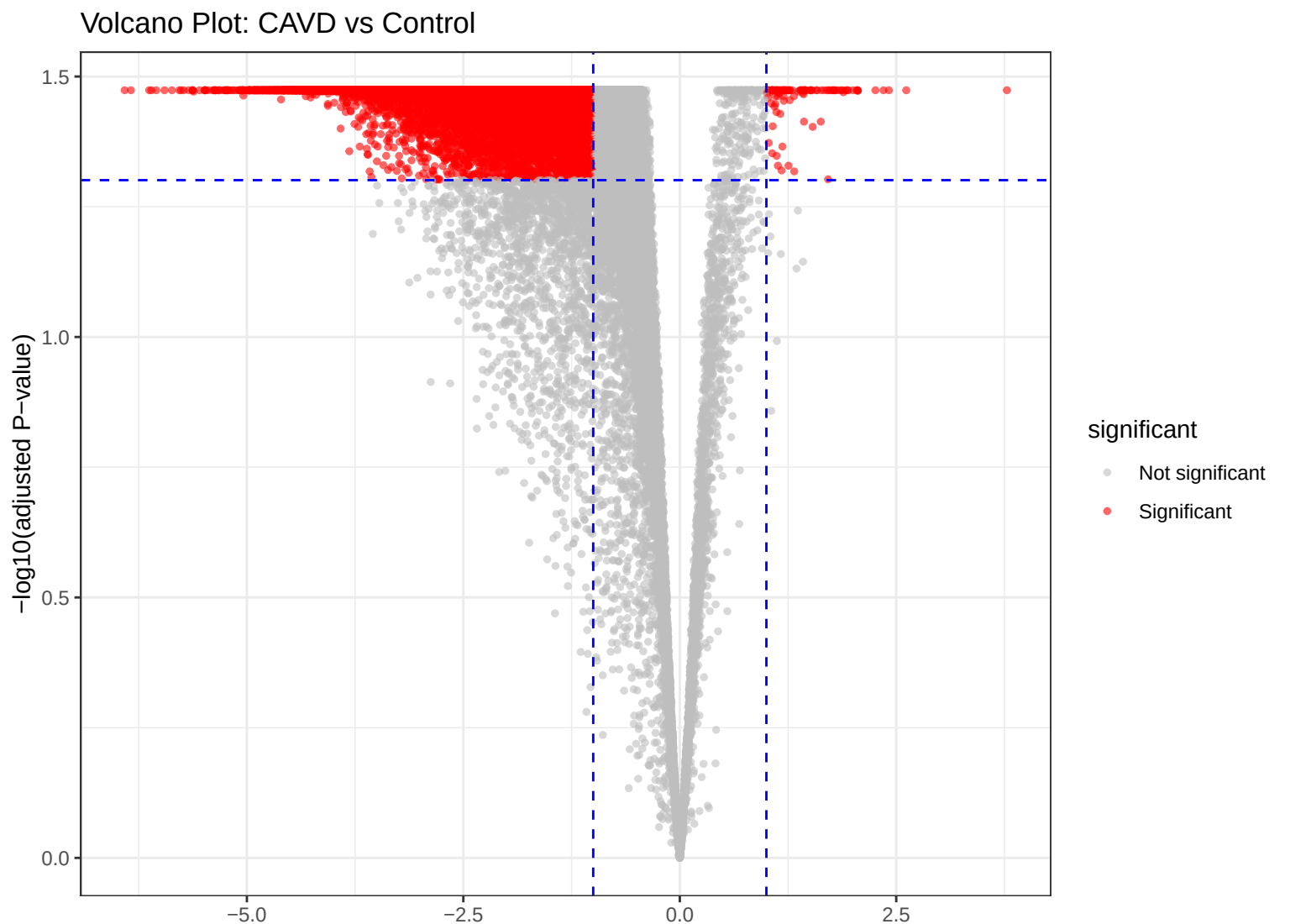
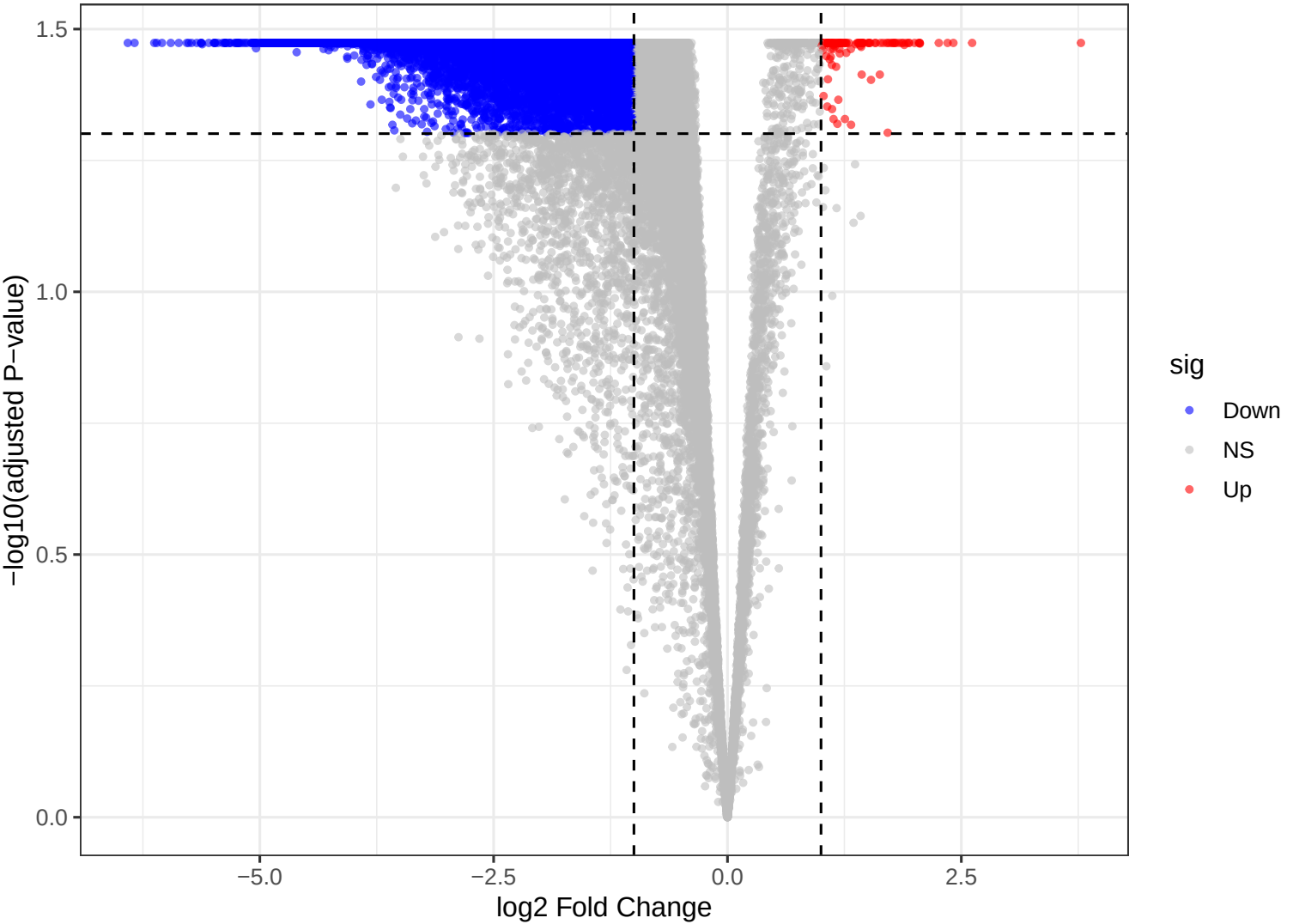


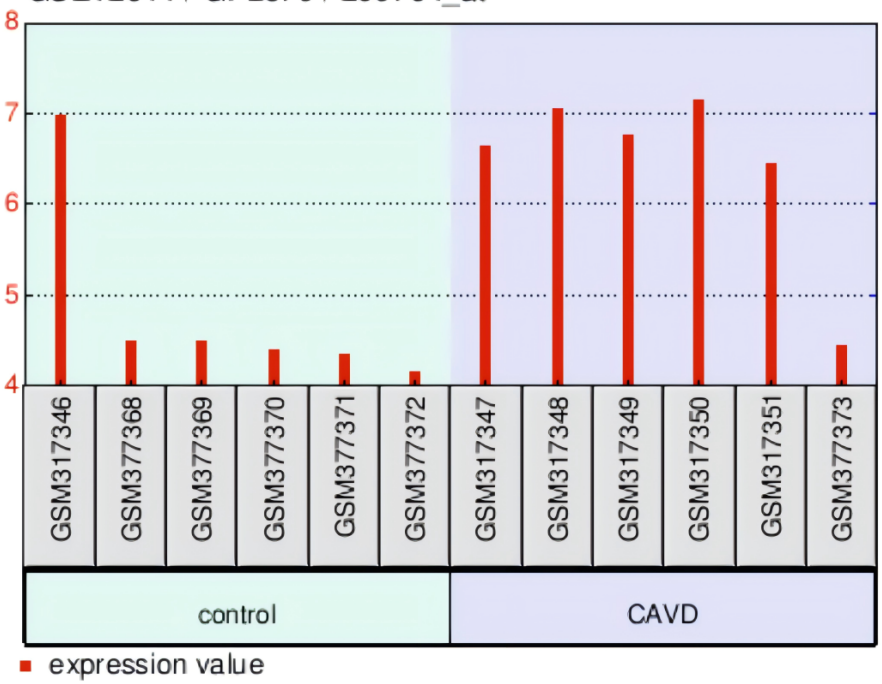
Figure 1 | Volcano plot of differentially expressed genes in CAVD

Volcano plot showing differentially expressed genes between CAVD (n = 6) and control (n = 6) samples from GSE12644. Red points: significantly upregulated genes (log?FC > 1, adj.P < 0.05); blue points: significantly downregulated genes (log?FC < -1, adj.P < 0.05); grey points: non-significant genes. Horizontal dashed line: -log??(0.05); vertical dashed lines: log?FC = ±1. MGAT3, FUT8, and B3GNT2 are highlighted.

Supplementary Figure 1: Volcano plot of differentially expressed genes



GSE12644 / GPL570 / 209764_at



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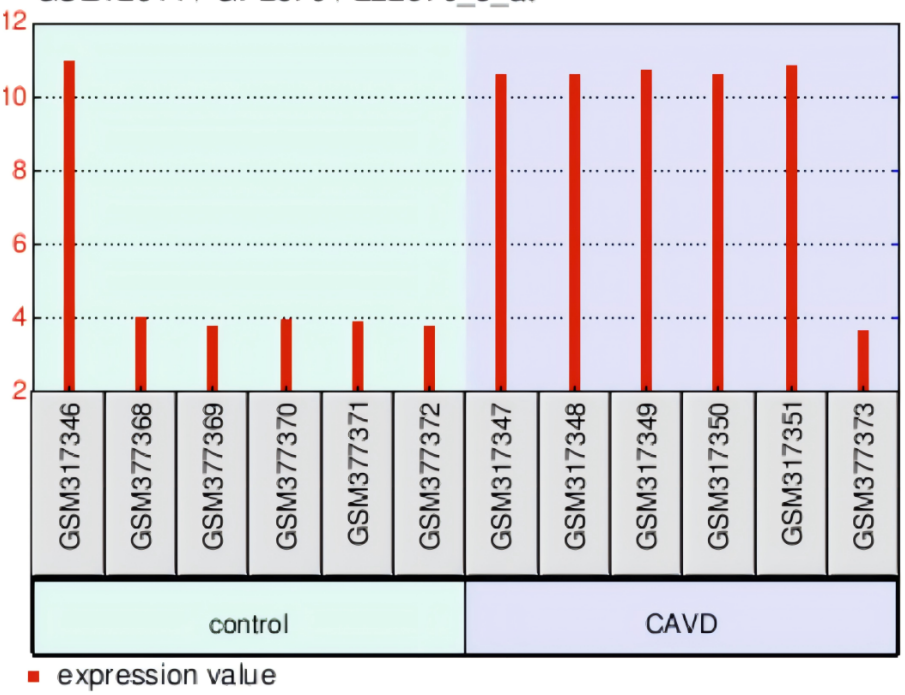

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