Review

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Network-Guided Multiomic Mapping of Aortic Valve Calcification

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ABSTRACT: Despite devastating clinical sequelae of calcific aortic valve disease that range from left ventricular remodeling to arrhythmias, heart failure, and early death, the molecular insights into disease initiation and progression are limited and pharmacotherapies remain unavailable. The pathobiology of calcific aortic valve disease is complex and comprehensive studies are challenging valvular calcification is heterogeneous and occurs preferentially on the aortic surface, along a fibrocalcific spectrum. Here, we review efforts to study (epi-)genomic, transcriptomic, proteomic, and metabolomic aspects of aortic valve calcification in combination with network medicine-/systems biology-based strategies to integrate multilayered omics datasets and prioritize druggable targets for experimental validation studies. Ultimately, such holistic approach efforts may open therapeutic avenues that go beyond invasive and costly valve replacement therapy.

Key Words: aortic valve ■ aortic valve stenosis ■ endothelial cell ■ genome-wide association study ■ transcatheter aortic valve replacement

CALCIFIC AORTIC VALVE DISEASE: A DEADLY CONDITION LACKING MEDICAL THERAPY

Calcific aortic valve disease (CAVD) is a progressive and cell-mediated disorder that affects up to 25% of those aged >65 years. Without treatment, diagnosis of symptomatic aortic stenosis (AS) is associated with a mean survival time of <2 years.[1] Treatments are strikingly limited to invasive surgical valve replacement or transcatheter valve replacement (TAVR). Despite shared risk factors with atherosclerosis, statins or inhibitors of bone metabolism and other atherosclerotic remedies have consistently failed to slow AS progression and pharmacotherapies remain unavailable.[2]

Aortic valve anatomy is complex and highly anisotropic: composed of a tri-layered architecture, the disease-prone and collagen-rich fibrosa sits nearest to the ascending aorta, the disease-protected elastin-rich ventricularis faces into the left ventricle and proteoglycans in the spongiosa act as an intermediate layer. In healthy aortic valves, quiescent fibroblast-like valvular interstitial cells (VICs) reside throughout all layers. During disease development, heterogeneous VICs[3] undergo myofibrogenic/osteogenic differentiation and contribute directly to ECM (extracellular matrix) disruption, collagen accumulation, and calcific nodule formation, collectively resulting in leaflet thickening/stiffening, left ventricular pressure overload, heart failure, and premature death.[4] The aortic- and ventricle-facing layers are lined with valvular endothelial cells (VECs), a cell population that facilitates valvular mechano-sensation of stretch, compression, and shear stress and which may undergo endothelial-to-mesenchymal transition in response to microenvironmental cues.[5] Putative disease drivers are highly multifactorial, with myofibrogenesis, osteogenesis, oxidized lipid accumulation, inflammation, ECM disarray, dysbalanced calcium/phosphate metabolism, cellular senescence, and mechanobiology all being implicated to varying and complementary degrees.[4,6] This complexity (and our resultant lack of detailed mechanistic understanding of molecular pathways modulating valvular homeostasis and disease initiation/progression) contributes to the thus far unsuccessful search for a medical

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Nonstandard Abbreviations and Acronyms

AS	aortic stenosis
AV	aortic valve
BAV	bicuspid aortic valve
CAVD	calcific aortic valve disease
ECM	extracellular matrix
GWAS	genome-wide association study
Lp(a)	lipoprotein(a)
scRNA-seq	single-cell RNA-sequencing
TAVR	transcatheter aortic valve replacement
VEC	valve endothelial cell
VIC	valve interstitial cell

therapy, the need for which is particularly dire as CAVD is headed towards a demographic cliff: coincident epidemics of aging, obesity, and diabetes will conspire to increase its global prevalence by 240% over the next 2 decades.[7] In addition, human tissue resources for research purposes are declining due to growing rates of TAVR procedures. Here, we discuss applications of multiomics and systems biology approaches toward a more holistic understanding of CAVD pathobiology (Figure). Having been widely embraced throughout industrial and academic drug discovery, these state-of-the-art methodologies are no longer fishing expeditions and promise to potentiate the identification of novel drug targets to delay or even halt disease progression.

(EPI-)GENOME: A CRUCIAL DETERMINANT OF AORTIC VALVE CALCIFICATION ACROSS VALVE MORPHOLOGIES

The incidence of familial clustering and heritability of bicuspid aortic valves (BAV), a congenital valve abnormality that represents a potential risk factor for the premature development of CAVD and accelerated disease progression, is high.[8] Heritability of CAVD affects all AV morphotypes, as individuals whose siblings have a history of clinical AS are at ≈3.5-fold increased risk of AS even after exclusion of cases with congenital valvulopathy.[9] This favors the involvement of genetic contributors across the broad spectrum of CAVD. Genome-wide association studies (GWAS) have linked single-nucleotide polymorphisms (ie, single-nucleotide substitutions at a specific position in the genome) to heightened susceptibility of valvular calcification and incident AS, with instrumental variable approaches (ie, Mendelian Randomization) ushering in a new era for the study of causative CAVD drivers. This is exemplified by the groundbreaking work of Thanassoulis et al.[10] unveiling an *LPA* variant (rs10455872) that, mediated by high Lp(a) (lipoprotein[a]), associates with AV calcification and incident AS. A subsequent GWAS in 2457 AS cases and 349 342 controls confirmed this gene-trait linkage, while 2 new variants near *PALMD* and in *TEX41* reached genome-wide significance (rs7543130 and rs1830321, respectively).[11] A meta-analysis in 5115 European cases and 354 072 controls confirmed *PALMD* and *LPA*-associated variants and identified 2 new loci in interleukin-6 (*IL6*) and alkaline phosphatase (*ALPL*),[12] key regulators of VIC calcification.

The genetic basis of CAVD is complex, as exemplified by the gene-phenotype linkage of BAV, a congenital valve abnormality with strikingly variable genetic etiologies, ranging from intricate inheritance patterns to sporadic cases. Beyond certain syndromes and congenital malformations involving the left ventricular outflow tract, the heritability of BAV appears to be largely polygenic,[13] with an up to 10-fold increased prevalence in first-degree relatives as compared to the general population. In a recent GWAS involving 466 BAV cases and 4660 controls, a noncoding variant near *GATA4* (rs6601627) reached genome-wide significance with a protein-altering variant in this cardiac-specific transcription factor (rs3729856) showing near-significant association.[14] In human iPSC-derived cells, *GATA4* disruption led to impaired endothelial-to-mesenchymal transition, a process essential for proper valve development which is rekindled as CAVD evolves.[15] Beyond their association with incident AS, the afore-noted risk loci near *PALMD* (rs7543130) and *TEX41* (rs1830321) also associate with BAV.[11] In a subsequent GWAS with the largest sample of patients with BAV thus far, totaling 2236 BAV cases and 11 604 controls, the previously identified loci near *PALMD*, *TEX41*, and *GATA4* were confirmed, while a new missense variant (rs2550262) in *MUC4* was identified.[13] Loss of *Muc4* leads to delayed AV development in zebrafish, while *MUC4* overexpression in human cancer cells accelerates their transition toward a mesenchymal phenotype, suggesting that *MUC4* might be essential for endothelial-to-mesenchymal transition and thus AV formation.

Heritable phenotypes may also result from epigenetic modifications, typically involving DNA methylation, histone modification, or RNA-mediated processes. Multidimensional genomic profiling of calcified human AVs unveiled hypomethylation-induced upregulation of the long noncoding RNA *H19*, coinciding with amplified AV calcification and accelerated CAVD progression, with in vitro studies pointing toward Notch-signaling as a major *H19*-modulated pathway determining VIC's osteogenic fate.[16] Notably, the study by Mkannez et al[17] was the first to connect the epigenome to pro-osteogenic effects mediated by Lp(a)-derived metabolites: while the expression and activity of lysophosphatidic acid (ie, bioactive derivate of Lp(a)-bound oxidized phospholipids) degrading membrane-associated PLPP3 (phospholipid

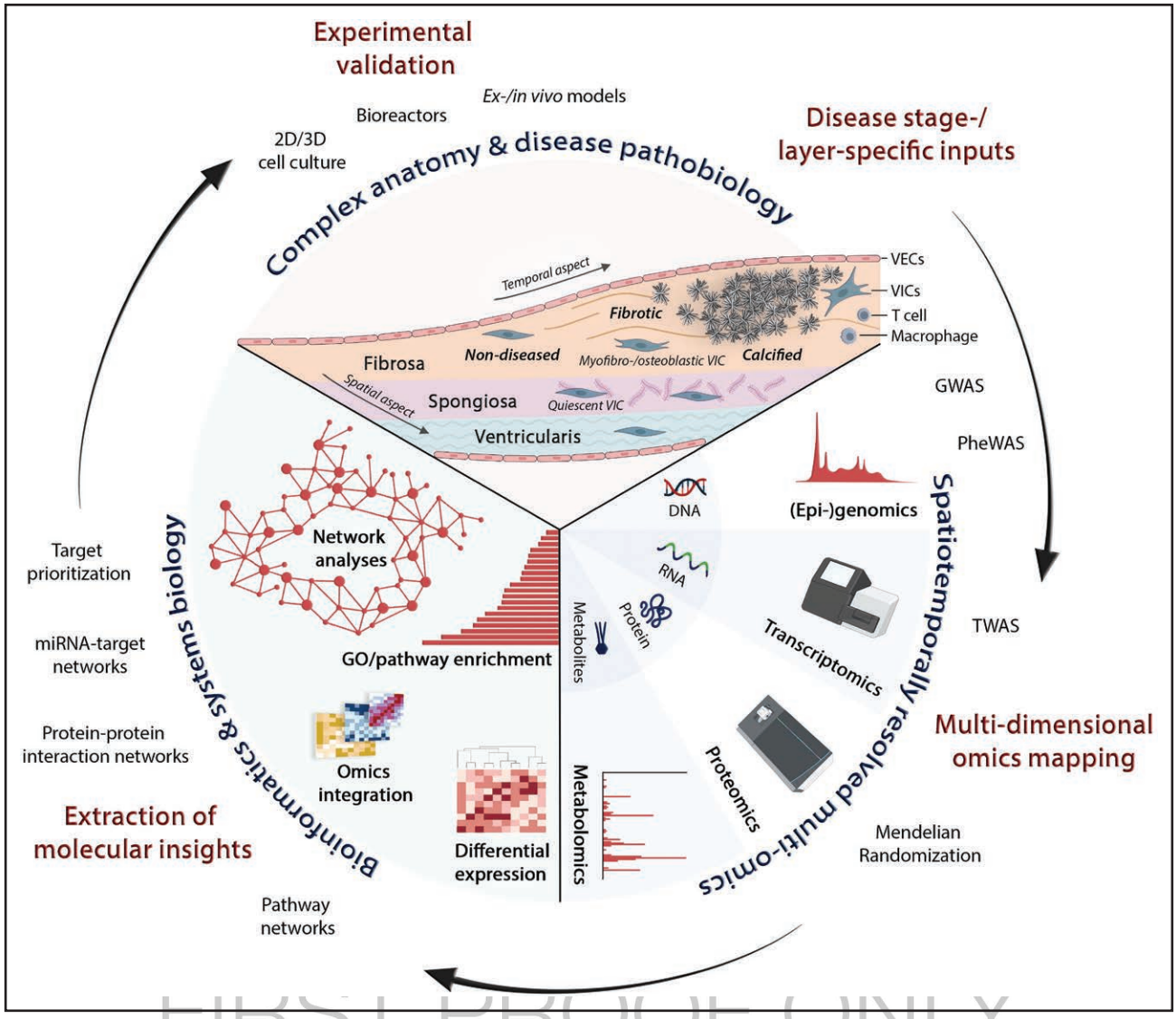

Figure. Multiomics in calcific aortic valve disease (CAVD).
Human CAVD pathogenesis is marked by temporal and spatial heterogeneity of fibrocalcific nodules that impede leaflet opening, impair cardiac function, and accelerate myocardial remodeling. Multiomics approaches enable the holistic assessment of disease-associated DNA, RNA, protein, or metabolite signatures at a bulk or single-cell level. Systems biology and network-based methods enable integration of data from multiple omics layers to identify and prioritize key molecular interactions or disease drivers. Validation of omics-derived insights and targets is an essential step towards clinical translation; 2-dimensional/3-dimensional (2D/3D) cell culture, bioreactors that incorporate physiological biomechanics, and a growing selection of ex-/in vivo models have been successfully employed for this purpose. Created with BioRender.com. GO indicates gene ontology; GWAS, genome-wide association study; TWAS, transcriptome-wide association study; VECs, valvular endothelial cells; and VICs, valvular interstitial cells.

phosphatase-3) was attenuated upon DNA methylation of its intronic enhancer, lysophosphatidic acid–exposure induced a pro-osteogenic response in a largely PLPP3-dependent manner.

Collectively, these findings implicate alterations at genomic levels in valvular calcification across AV morphologies, with certain epigenetic signatures intriguingly interfering with Notch1- and Lp(a)-dependent mechanisms, at least in certain patients. Rapid development, broad availability, and affordability of high-throughput means, including assay for transposase-accessible chromatin using sequencing, bisulfite sequencing, chromatin immunoprecipitation sequencing, and next-generation sequencing open exciting avenues to further our understanding of (epi-)genetic phenomena underpinning CAVD pathobiology.

MAPPING THE VALVULAR TRANSCRIPTOME: INSIGHTS INTO PATHOBIOLOGICAL DRIVERS

Initially informed largely by RNA microarrays, our understanding of the valvular transcriptome (ie, full set of

genome-derived RNAs), builds increasingly on RNA-sequencing (RNA-seq), a high-throughput technology with high sensitivity, specificity, and dynamic range for the quantification of RNA transcripts at both tissue- and single-cell resolution. Pioneering work employed microarrays and normal porcine AV leaflets to assess differences in transcription between the aortic- and ventricular-facing endothelia.[18] These findings revealed 584 genes that were differentially expressed in situ including reduced aortic-sided expression of multiple inhibitors of cardiovascular calcification such as c-type natriuretic peptide, whose receptor was subsequently implicated in murine bicuspid aortic valve formation, leaflet fibrosis, and valvular calcification.[19] The broad availability of bulk RNA-seq has stimulated seminal studies to map the totality of the valvular transcriptome during health and disease, enabling the discovery of novel transcripts while showing superiority in the detection of rare and low-abundance RNAs as compared to microarrays. RNA-seq has been employed to identify gene networks dysregulated by *NOTCH1* haploinsufficiency, a potent risk factor for BAV and CAVD, as noted above,[20] with highest-ranked gene ontology pathways involving endochondral ossification and inflammatory responses.[21] The first spatiotemporally resolved multiomics profiling of CAVD tissues by Schlotter et al[22] revealed disease-stage–specific transcriptional signatures, with *CD74*, *APOE*, and *A2M* ranking among the most abundantly expressed transcripts in calcific as compared to fibrotic and nondiseased portions. RNA-seq has also been used to dissect the impact of sex on myofibroblastic activation of VICs, a potential determinant of sex-specific differences in the fibrocalcific burden of CAVD: network analysis of sexually dimorphic transcriptomes of porcine VICs led to the identification of 2 X-chromosome inactivation escaping genes (ie, *BMX* and *STS*) which fine-tune myofibroblastic activation through a RhoA/ROCK-dependent mechanism.[23] Current (patho-) biological concepts of the noncoding valvular RNAome rely primarily on miRNA-microarray-based work, with more recent reports fueling the growing interest in noncoding RNAs beyond miRNAs.[16] Initial attempts using an miRNA-mRNA-microarray–based approach revealed differential expression of miR-122-5p, miR-625-5p, miR-30e-5p, miR-21-5p and miR-221-3p in diseased leaflets, with ClueGO-assisted analysis identifying 10 pathways, of which focal adhesion, actin cytoskeleton regulation, and ECM receptor interaction showed the strongest association, though a mechanistic link in CAVD-relevant models remains to be established.[24] Microarrays have also been utilized to explore the in vitro effects of the valvular miRNAome, revealing increased miR-486 and decreased miR-204 expression in diseased VICs, with miR-486 antagomir and miR-204 mimic synergistically promoting the myo/osteoblastic transition of human VICs.[25] miRNA-microarray-based work using endothelial-enriched RNA obtained from healthy porcine AV leaflets suggests 7 miRNAs to be lifted in the disease-prone fibrosa, with the manually prioritized miR-214 impinging on profibrotic signaling mediated by TGFβ1.[26]

By harnessing expression quantitative trait loci (ie, genomic loci partly explaining the variation in mRNA expression), transcription-wide association studies can overcome certain limitations inherent to standalone GWAS for the discovery of gene-trait associations. Through the combined analysis of GWAS (1009 cases; 1017 controls) and expression quantitative trait loci data (n=233), a recent transcription-wide association studies corroborated the *PALMD*-CAVD linkage,[11] with risk alleles and CAVD severity associating with reduced valvular *PALMD* mRNA expression,[27] likely perturbing nucleocytoplasmic shuttling through which a CAVD-like phenotype is promoted. Recent advances in sample processing, sequencing technology, and computational analyses have changed the field of transcriptomics, with spatially and temporally resolved approaches representing a much-needed step toward a more holistic understanding of the valvular RNAome. Standardized and fully disclosed workflows, ranging from sample preparation to computational analyses, intertwined with the availability of large-scale biorepositories of human AV tissues will be essential to advance insights into CAVD pathobiology and provide entry points for the development of a much-needed medical therapy.

MASS SPECTROMETRIC APPROACHES TO ASSESS THE VALVULAR PROTEOME AND METABOLOME

Multiple studies have shown a poor correlation between mRNA and protein expression data in several distinct cell or tissue types, emphasizing the importance that post-transcriptional processes play in determining phenotype.[28] This phenomenon persists in the AV and may be particularly exacerbated in this tissue type due to its highly matrix-rich nature.[22] In a seminal work, Schlotter et al[22] quantified abundances of nearly 2000 proteins in 9 human CAVD donors using both global unlabeled and label-based tandem-mass-tagged proteomics on samples divided for layer-specific (fibrosa, spongiosa, ventricularis) and disease stage–specific (nondiseased, fibrotic, calcified) analyses. Along with the generation of a spatiotemporal atlas of CAVD pathogenesis, these efforts identified a novel spongiosa-specific VIC marker (GFAP [glial fibrillary acidic protein]), and the counter-intuitive abundance of VIC myofibrogenesis in the ventricularis, whereas the disease-prone fibrosa was richest in proteoglycans, apolipoproteins, and regulators of bone mineralization, collagen formation, retinoid metabolism, ECM organization, and PI3K-Akt signaling—thus incriminating these processes in early CAVD. As an alternative to difficult microdissection

of spatiotemporal anatomy and disease states, other groups have employed matrix-assisted laser desorption ionization imaging mass spectrometry to deconvolve a spatial proteome from tissue sections. These efforts have identified drivers of osteoblast mineralization (collagen VI, NDRG2) to be particularly enriched at the periphery/leading edge of calcific nodules.[29]

Proteomics has also been applied to cell culture studies, which have shown that plasma-borne drivers of CAVD (eg, Lp(a), apoC-III, etc.) are absent in calcification of side-specific VIC cultures (disease-prone fibrosa vs disease-protected spongiosa VICs), pointing to a need for more complex model systems that mimic non-VIC aspects of the valvular microenvironment.[22] Most recently, global unlabeled proteomics of cellular sub-fractions demonstrated that the long-observed passage-dependent nature of cultured VIC calcification was associated with passage-dependent reductions in TNAP (tissue nonspecific alkaline phosphatase) abundance/activity, highlighting the need for careful standardization of VIC culture conditions during in vitro assessments of VIC pathobiology.[30] The next frontier in proteomics is detection and quantification of covalent post-translational protein modifications including phosphorylation, ubiquitination, and ribosylation which can impact protein function, activity, location, or degradation. Recent initial applications of post-translational protein modification analysis to CAVD revealed that NO modulates S-nitrosylation of the deubiquitinase USP9X and controls anticalcific NOTCH in cultured VICs. Moreover, a matrix-assisted laser desorption ionization imaging mass spectrometry–based inspection of N-glycosylation post-translational protein modifications in pediatric congenital AS identified abundant N-glycosylation in thickened leaflet commissures.[31,32]

Mass spectrometry is also frequently used to assess molecular species most closely associated with phenotype: the metabolome, which is composed of endogenous or exogenous small molecules created via the body's natural metabolism. To date, applications of metabolomics to CAVD remain largely limited to biomarker detection studies that focus primarily on differential responses to valve replacement. Pre-/post-TAVR plasma proteomics in 57 individuals with tricuspid AV–CAVD or BAV-CAVD found BAV-specific reductions in arginine and proline metabolites suggestive of elevated oxidative stress, inflammation, and impaired NO generation. Furthermore, these efforts discovered that upregulated arachidonic acid metabolism was associated with worse recovery in BAVs.[33] Others have queried prognostic biomarkers with predictive utility by studying metabolomic changes pre-to-post-TAVR. Notably, sphingolipid metabolism was quickly and highly elevated after TAVR, while network analyses revealed long-lasting associations between L-arginine/NO/proline metabolism and regression of cardiac hypertrophy; these findings may open exciting avenues for the development of personalized risk scores to assess efficacy of TAVR versus surgical replacement.[34] Recent metabolomic-based efforts have started to move beyond biomarker discovery, and instead, aim to shed light on disease-causing mechanisms and eventual identification of novel drug targets. Untargeted global metabolomics of valve tissues from 96 human donors with mild-to-severe CAVD has identified 72 metabolites and lipids that were altered by disease progression, though none differed between diseased TAVs and BAVs. LysoPA levels were strongly associated with alterations in valve function, and follow-up targeted metabolomics of seven lysophosphatidic acid species in human AV tissue confirmed that lysophosphatidic acid levels correlated with rapid CAVD progression independent of traditional risk factors.[35]

SINGLE-CELL OMICS STRATEGIES AND THE STUDY OF VALVULAR HETEROGENEITY

Bulk omics approaches have limited ability to guide mechanistic insights into spatially or temporally complex diseases such as CAVD. However, over the past years, single-cell RNA-sequencing (scRNA-seq) has revolutionized our ability to delve deeply into these questions and identify low-incidence but high-impact cell populations, examine communication between different cell types, discover novel cell states, and study patterns of cellular differentiation during pathogenesis. Onset of CAVD in adults is hypothesized to be associated with aberrant activation of developmental gene programs, and thus examination of early leaflet remodeling may shed light on disease-driving mechanisms.[36] Drop-seq of pooled cell suspensions derived from enzymatic digestion of murine aortic and mitral valves obtained shortly after birth identified 4 overarching valvular cell types, that is, interstitial, endothelial, immune, and melanocytic cells.[37] Postnatal leaflet maturation was associated most strongly with differentiation of VICs into multiple subtypes specializing in collagen and matrix organization, defense response, and adaptive immunity, while valve macrophages rapidly shifted towards chemokine-expressing phenotypes. Spatial organization was heavily present in endothelial subpopulations, with lymph-like VECs found on the fibrosa and migratory VECs present in areas of leaflet coaptation. Other studies have revealed accumulation of inflammatory VICs and monocyte-derived MHC-II^{hi} macrophages in hyperlipidemic mouse AVs, along with activation of protective PPARγ signaling in VECs.[38]

Two recent studies have begun to characterize the human AV transcriptome at the single-cell level. Although not directly focused on CAVD pathogenesis, the comparative study of distinct cellular compositions of 18 nondiseased human aortic, pulmonary, tricuspid, and mitral valves from end-stage heart failure patients has provided a baseline for valvular heterogeneity and shed light on left/

right-side differences in leaflet composition, mechanobiological regulation of VIC phenotypes, and disease susceptibility of the AV.[39] Xu et al[15] reported the first scRNA-seq study of adult human CAVD, where they sequenced 34 632 cells from 6 donors after mechanical dissociation and enzymatic digestion. Their analyses identified subsets of VICs, VECs, macrophages, and lymphocytes. Onset of CAVD-induced inflammatory cell accumulation and VIC and VEC differentiation associated with endothelial-to-mesenchymal transition towards multiple novel stromal cell subpopulations, although the presence of fibrosis and calcified nodules resulted in a 63% failure rate during single-cell preparation due to contamination of microfluidic-based systems for single-cell library preparations. Indeed, one challenging aspect of this approach is the preparation of single-cell suspensions from solid tissues—tissue debris, lengthy warm enzymatic digestion after surgical removal, and partial or biased isolation of cellular subsets can lead to detection of artifactual gene expression patterns or systemic bias in population proportions.[40] Single nucleus RNA-seq has rapidly gained popularity for its ability to be employed on frozen archival tissue samples while avoiding many of the artifactual drawbacks of scRNA-seq and can be paired with single nucleus assay for transposase-accessible chromatin using sequencing to enable analysis of genome-wide chromatin accessibility in single cells. Broadly speaking, the mouse and human scRNA-seq studies described here identify valvular cell types that can be annotated as VICs, VECs, other stromal cells, and immune cells (generally of leukocytic origins, eg, macrophages, T cells, B cells, etc). Deeper comparisons of specific subpopulations between studies or across species are challenging due to rapid advances in technology and analysis pipelines, but such meta-analyses would be highly beneficial to develop a consensus valve cell atlas or, in the case of mouse vs. human comparisons, for the selection of appropriate in vivo models that best mimic specific aspects of human pathobiology.

Recently, scRNA-seq technologies have evolved beyond atlas-based approaches into a key analytical tool for in vitro drug target discovery. Majumdar et al[31,41] employed scRNA-seq of cultured porcine VICs to reveal that (1) exposure to either a NO donor or induction of S-nitrosylation post-translational modifications suppresses VIC myofibrogenesis, (2) that NO inhibits calcification via regulation of myofibroblast adhesion and matrix remodeling, and (3) that inhibition of the S-nitrosylated deubiquitinase USP9X induces VIC mineralization in vitro. Application of scRNA-seq and machine learning on cultured human VICs has helped to reveal a putative disease-driving $CD44^{high}CD29^{+}CD59^{+}CD73^{+}CD45^{low}$ population.[3] Although single-cell proteomics remains in its infancy, single-cell mass cytometry (cytometry by time of flight) of VICs extracted from calcified and noncalcified areas of human AVs confirmed enrichment of this disease-driving cell population in human CAVD.[3] Omics at the single-cell level is in the midst of a period of rapid advances in technology, capacity, and cost-efficiency. As economies of scale become established, single-cell multiomics represents the forefront of innovation and holds the ultimate promise to revolutionize our understanding of this complex disease.

TOWARDS A HOLISTIC UNDERSTANDING OF CAVD: SYSTEMS BIOLOGY AND MULTIOMICS INTEGRATION

Multiomics integration is the process of combining data from multiple omics types/layers to gain a more complete understanding of biological systems by uncovering complex cross-layer pathways involved in diseased development and identifying key molecular regulators of disease. Network medicine leverages connections between annotated biological factors (eg, physical interactions between proteins, shared pathway constituents, noncoding RNA/target gene interactions, etc) to identify important mechanisms or interactions. Together, multiomics integration and network medicine can provide valuable insights into the complex relationships within biological systems, leading to a better understanding of CAVD initiation, pathogenesis, and treatment.

In an example of the power of open data sharing, Heuschkel et al[42] recently integrated shared differentially enriched molecules from preexisting publicly-available transcriptomic, proteomic, and metabolomic datasets of human CAVD and protein-protein interaction network-based multiomics integration to reveal new evidence of an association between valvular amyloid deposition and calcification. Weighted gene coexpression network analysis generates biological networks based on gene set coexpression, and combinatorial meta-analyses of human tissue microarrays and scRNA-seq positioned macrophage-specific expression of the calcium-handling/osteogenic hub genes *S100A8* and *S100A9* as centrally-important to inflammatory and immune-associated contributions to CAVD.[43]

More recent work involving spatiotemporal transcriptomics and proteomics of human AV with CAVD has utilized betweenness-centrality quantification (shortest paths between network constituents through a node; indicative of bottleneck functionality, high biological relevance, and drug target utility) of protein-protein interaction networks to link the extracellular matrix glycoprotein fibronectin-1 and the protease inhibitor alpha-2-macroglobulin with CAVD progression.[22] In parallel, these efforts employed networks of enriched pathways to combine non-overlapping sets of disease-enriched and leaflet layer–enriched genes and proteins. Care must be taken when working with network-prioritized targets: nodes with high betweenness-centrality (or highly-connected hub nodes) are inherently tied to important cellular functions and are more likely to be dynamic or essential proteins involved in signaling cross-talk.[44,45] As a result, validation experiments to

Table. Exemplary Studies of Multiomics Approaches to CAVD

Study	Samples	Analytical tools	Main findings	Targets
Hadji et al[16]	RNA-seq: 9 CAVD cases and 10 controls Whole-genome DNA methylation profiling: 21 CAVD tissues	*Cuffdiff*, *DESeq*, and *edgeR* for differential expression analysis	DNA hypomethylation-induced lncRNA *H19* upregulation associates with proxies of disease activity; *H19* promotes osteogenic program activation by repressing *NOTCH1* expression	lncRNA *H19* methylation as an epigenetic mechanism driving CAVD pathogenesis
Schlotter et al[22]	RNA-seq and LC-MS/MS: nondiseased, fibrotic, and calcific portions of AV leaflets obtained from AS patients undergoing SAVR (transcriptomics: n=3; proteomics: n=9)	*Qlucore Omics Explorer* and *DESeq2* for differential expression analysis; *ConsensusPathDB* for pathway enrichment analysis and *NetworkX* to calculate network measures (eg, centrality)	First spatiotemporally resolved multiomics study of CAVD; identification of a novel cell-specific cell marker	GFAP as a specific marker of spongiosa-derived VICs; pathways involved in smooth muscle cell activation, inflammation, and calcification signify CAVD progression
Helgadottir et al[11]	Genomics: 2'457 Icelandic AS cases and 349'342 controls with a follow-up study in 4'850 cases and 451'731 controls	*Ensembl* and *Variant Effect Predictor* for annotation; *LD Score regression* for relatedness adjustment; logistic/linear regression for association analysis	Identification of two new variants on chromosomes 1p21 near *PALMD* and 2q22 in *TEX41*; replication of a previously reported AS variant in *LPA*[3]	*LPA*, *PALMD* (intergenic), and *TEX41* as risk loci for CAVD
Thériault et al[12]	GWAS: 1009 AS cases and 1017 ethnically matched controls; 1391 cases and 352'195 controls for replication eQTL: 233 AV tissues obtained from AS patients undergoing SAVR	GWAS: *PLINK* for evaluation of genetic relatedness; additive logistic regression models for association analysis eQTL/TWAS/MR: *PLINK* to estimate effect size; LASSO and elastic net regression to calculate expression weights; AIC-based bidirectional elimination to select SNPs independently associated with *PALMD* expression followed by Wald-based regression for MR	A TWAS linked *PALMD* to CAVD; risk alleles and increasing CAVD severity are both associated with decreased mRNA *PALMD* expression levels in human AV leaflets	*PALMD* as a susceptibility gene for CAVD
Theodoris et al[47]	Targeted RNA-seq: Patient-derived iPSC-derived endothelial cells (*N1*$^{+/-}$ or gene-corrected isogenic cells) Whole-transcriptome RNA-seq: primary human VECs isolated from bicuspid/tricuspid CAVD (n=21) and control tissues (n=5)	KNN or hierarchical clustering algorithms for candidate identification; *Cuffdiff* and *GO-Elite*/*ToppGene* for differential expression and pathway analyses, respectively	A gene network–based screening approach in human iPSCs led to the identification of XCT790, a small molecule with gene network correcting effects; XCT790 attenuated CAVD progression in a mouse model of CAVD	XCT790 (an inverse ERRα agonist) as a possible therapeutic candidate for CAVD
Surendran et al[35]	Nontargeted metabolomics and targeted lipidomics: up to 106 AV leaflets obtained from patients with varying degrees of AS undergoing SAVR	*MetaboAnalyst* for differential expression and pathway analysis; *Cytoscape* to build correlation networks	Untargeted multiomics approach identified 72 metabolites/lipids that are altered across different CAVD stages; levels of lysoPA correlate independently with faster hemodynamic disease progression	AV-derived lysoPA levels as a proxy for hemodynamic CAVD severity
Xu et al[15]	Bulk- and single-cell RNA-seq: a total of 6 AV leaflets of patients undergoing SAVR or aortic dissection repair	*CellRanger*, *STAR* and *Monocle2*	Single-cell RNA-seq identified 14 cell clusters in CAVD vs control tissues (ie, 3 subpopulations of resident VICs, 3 immune-derived cells, 2 types of VECs, and 6 stromal cells found particularly in CAVD leaflets)	Identification of 14 cell subtypes; upregulation of mesenchymal (eg, *COL1A1*, *CNN1*, *COL3A1*) and downregulation of endothelial signature genes (eg, *SELP*, *CD34*, *VWF*) in CAVD tissues implicates EndMT in CAVD pathobiology
Aguado et al[23]	RNA-seq: male and female pig VICs (n=4)	*EdgeR* for differential expression analysis; IPA for network analyses	Female VICs have increased myofibroblastic activation potential relative to males, regulated, at least in part, by X-chromosome inactivating genes such as *BMX* and *STS*	*BMX* and *STS* as sex-specific regulators of myofibroblastic activation

AIC indicates Akaike information criterion; AS, aortic stenosis; AV, aortic valve; BMX, Bmx nonreceptor tyrosine kinase; CAVD, calcific aortic valve disease; EndMT, endothelial-to-mesenchymal transition; eQTL, expression quantitative trait loci; ERRα, estrogen-related receptor α; GFAP, glial fibrillary acidic protein; GO, gene ontology; GWAS, genome-wide association study; lncRNA, long noncoding RNA; IPA, ingenuity pathway analysis; LC-MS, liquid chromatography–mass spectrometry; LD, linkage disequilibrium; lncRNA, long noncoding RNA; LPA, lipoprotein(a); lysoPA, lysophosphatidic acid; MR, Mendelian randomization; PALMD, palmdelphin; RNA-seq, RNA-sequencing; SAVR, surgical aortic valve replacement; SNP, single-nucleotide polymorphism; STS, steroid sulfatase; TEX41, testis expressed 41; TWAS, transcriptome-wide association study; VECs, valvular endothelial cells; and VICs, valvular interstitial cells.

assess specificity and ensure the absence of undesirable off-target side effects are essential. We have also utilized miRNA-mRNA target networks to link AV extracellular vesicle–borne miRs quantified by small RNA-seq to their predicted high-confidence gene targets in recipient cells, and integrated vesicular miRNA-seq and proteomics via

pathways networks to predict the cumulative impact of extracellular vesicle cargoes and prioritize candidate molecules for drug target validation.[46] Others have employed network analyses to screen for compounds with therapeutic potential: targeted RNA-seq coupled with machine learning tactics has been utilized to map the gene network disrupted in human *NOTCH1*-haploinsufficient iPSC-derived endothelial cells and to screen molecules for their network topology-correcting effects.[47] Strikingly, XCT790, an inverse estrogen-related receptor α agonist, showed network-restorative features, sufficient to prevent CAVD initiation and progression in *Notch1/mTR*^{G2} mice. Future efforts to leverage cutting-edge systems biology analyses for target discovery or rational drug design will require careful collaboration between highly interdisciplinary teams with expertise in valvular physiology, molecular biology, systems biology, and clinical medicine (Table).

LOOK TO THE FUTURE OF CAVD RESEARCH AND TARGET DISCOVERY

The dawning era of widespread TAVR in low-risk patients is ironically accompanied by an imminent and severe impact on basic and translational studies of valvular pathobiology, as human tissue samples obtained at surgery become even more scarce. Moreover, access to interventional cardiology or cardiac surgery is extremely limited or completely absent in many areas of the world, and thus the discovery of pharmacotherapeutic approaches to effectively treat CAVD is of utmost importance.[48] Although these precious tissues remain available, multiomics approaches offer an opportunity to comprehensively characterize the molecular fingerprint of human CAVD. It will be incumbent on researchers to embrace open science by making raw data freely available and open-sourcing analytical pipelines to potentiate external validation, data reuse/meta-analysis, and novel repurposing. After target prioritization, rigorous in vitro or in vivo validation studies of omics-derived hits in 2-dimensional/3-dimensional cell culture, bioreactors, or small-animal models of CAVD remain an essential step toward effective and efficient translation to the clinic (Figure).[49] This is followed by high-throughput screening or other rational drug design approaches (eg, virtual screening, ligand- or structure-based design, binding-site identification, etc) to identify drug candidates for preclinical studies of safety and efficacy. Future multiomics studies may shed new light on drivers, predictors, and biomarkers of CAVD progression (particularly important for the design of efficient and economic drug trials) or of successful valve replacement, connections between novel genetic drivers of this polygenic disease and druggable downstream pathobiological mechanisms, or how shared risk factors interact with divergent drivers of atherosclerosis and CAVD. Investigations may also focus on the causes of bioprosthetic valve failure, adult congenital heart valve disease, or even benchmarking novel disease models against human pathobiology. Together, these avenues offer the promise to personalize and revolutionize the diagnosis, care, and treatment of AV calcification while averting an upcoming demographic cliff of disease over the coming decades.

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