Advances in Understanding Cardiovascular Disease Pathogenesis Theme Issue

REVIEWS

Multimodal Analytical Tools to Enhance Mechanistic Understanding of Aortic Valve Calcification

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Calcific aortic valve disease (CAVD) is a rapidly increasing disorder in developed countries. Disease severity ranges from aortic valve sclerosis, marked by leaflet thickening and calcification without major disruption to outflow dynamics, to aortic valve stenosis, marked by leaflet rigidity and flow obstruction. The latter is associated with an increased risk of cardiovascular mortality and morbidity.1 Mortality rates have increased from more than 50,000 cases in 1990 to roughly 125,000 cases in 2019.2 According to a meta-analysis and modeling study,3 the number of elderly patients (age, ≥75 years) with calcific aortic stenosis is expected to double by 2050 in the United States and Europe.4 Although the prevalence of CAVD is an increasing concern, treatment options remain restricted to two techniques for aortic valve replacement (AVR): surgical AVR (SAVR) and transcatheter AVR (TAVR).5–7 However, there is an increasing motivation to treat CAVD independent of such surgical measures, suggestive of pharmacologic treatments.8 For example, statins, via their lipid-lowering mechanisms, have been largely successful at reducing cardiovascular risk, however, to date, have shown no apparent benefit to mitigating aortic stenosis,9 which is indicative of a lack of comprehensive understanding of the underlying mechanisms.10–12 In unison, global transcriptional and protein expression analyses allow for increased understanding of cellular behavior and pathogenic pathways under pathologic stimuli including stress, inflammation, low-density lipoprotein accumulation, increased calcium and phosphate levels, and vascular injury. We also look at how direct investigation of protein signatures paves the way for identification of targetable pathways for pharmacologic intervention. Here, we note that imaging techniques, once a clinical diagnostic tool for late-stage CAVD, have since been refined to address a clinical need to identify microcalcifications using positron emission technology/computerized technology and even detect in vivo cellular events indicative of early stage CAVD and map the expression of identified proteins in animal models. Together, these techniques generate a holistic approach to CAVD investigation, with the potential to identify additional novel regulatory pathways. (Am J Pathol 2023, 184:1–12; https://doi.org/10.1016/j.ajpath.2023.06.017)
disease mechanisms. Strides to elucidate CAVD pathobiology and drivers prompt the implementation of innovative technologies, multi-omics, and network medicine.

Valvular calcification was first recorded in 1697 during an autopsy and, less than a decade later, became clinically associated with symptoms of dyspnea, angina, faintness, and aortic valve petrification. With the advent and implementation of clinical diagnostic tools such as radiography and fluoroscopy, autopsy was no longer the sole method of studying pathologic features of diseased valves. Such techniques now routinely prompt preoperative diagnoses of valvular calcification by visualizing calcified valves using roentgen film and orthodiagrams.

By the mid-20th century, tomographic techniques showed that valvular calcification disturbs valve function by perturbing blood flow. The impact of these radiologic technologies has remained diagnostic rather than etiologic.

Recent basic and clinical research using molecular imaging, multi-omics, and systems biology has provided insight into mechanisms that drive CAVD, resulting in its current classification as a disease driven by inflammatory cell infiltration, extracellular matrix formation, valve thickening, and remodeling, finally leading to aortic valve calcification and stenosis with leaflet rigidity and outflow obstruction (Figure 1). Disease progression leading to aortic stenosis can be broken down into three basic etiologic categories. First, rheumatic heart disease is the leading cause of aortic valve stenosis globally. Generally associated with overcrowding and inadequate access to health care, it is preceded by rheumatic fever and results in commissural fusion, fibrosis, and calcification. Second, congenital abnormal aortic valves (bicuspid and unicusp valves) account for roughly half of all stenotic cases in developed countries. Notably, bicuspid valves develop aortic stenosis 10 to 20 years earlier than tricuspid valves, suggesting a significant role for hemodynamic flow patterns across the valve in pathogenesis. Third, calcification of normal aortic valves is suggested to have origins associated with inflammation, hyperlipidemia, aging, and poor lifestyle. In light of these distinct etiologies, comprehensive characterization of CAVD progression is necessary, thereby providing opportunities to identify therapeutic targets.

This review therefore focuses on technologies at the core of CAVD and drug target research advancement, including transcriptomics, proteomics, and molecular imaging. The authors examine how bulk RNA sequencing (RNA-seq) and single-cell RNA-seq (scRNA-seq) have engendered organismal genomes and transcriptomes, promoting the analysis of CAVD and drug target research advancement. In light of these distinct etiologies, comprehensive characterization of CAVD progression is necessary, thereby providing opportunities to identify therapeutic targets.
of tissue gene expression profiles and cell subpopulations, respectively. They bring into focus how the field also largely is influenced by increasingly accessible proteome profiling techniques. In unison, global transcriptional and protein expression analyses (multi-omics) improve the understanding of cellular and molecular pathway responses to pathologic stimuli including stress, inflammation, low-density lipoprotein accumulation, increased calcium and phosphate levels, and vascular injury. The authors then examine how direct investigation of protein signatures paves the way for the identification of targetable pathways for pharmacologic intervention. Here, they note that imaging techniques, once a clinical diagnostic tool for late-stage CAVD marked by macrocalcifications, has since been refined to identify early stage microcalcifications. Positron emission technology (PET) combined with computerized technology (CT) now can detect \textit{in vivo} cellular events indicative of early stage CAVD\(^2\) and map the expression of identified proteins in animal models.\(^1,6\) Together, these techniques generate a holistic approach to CAVD investigation, with the potential to identify additional novel regulatory pathways.

**Molecular Imaging**

**PET and CT**

Various imaging modalities now are essential to many cardiovascular disease studies. For example, CT scans were among the earliest forms of imaging technology used to analyze CAVD.\(^1\) However, this technology, because of its low spatial sensitivity/resolution, is only capable of identifying late-stage, advanced calcification, not the subclinical microcalcifications that are proposed to precede the more readily detected macrocalcifications.\(^10\) On the other hand, when CT is paired with PET, the combination can detect microcalcifications.\(^14\)

By using \(^{18}\)F-NaF, which binds to hydroxyapatite crystals, CT and PET scans can be used to identify vascular microcalcifications noninvasively \textit{in vivo}, \textit{ex vivo}, and \textit{in vitro}.\(^14,13,37\) In the same light, \(^{18}\)F-fluorodeoxyglucose (\(^{18}\)F-FDG) uptake was examined with PET to assess inflammation.\(^14\) Multiple studies confirmed a relationship between \(^{18}\)F-FDG signal and atherosclerotic plaques, as an early indicator of CAVD, highlighting an overlap between macrophage-positive areas and \(^{18}\)F-FDG-positive areas.\(^33-35\) It has been suggested that \(^{18}\)F-FDG acts as a glucose analog that binds to glucose transport proteins to be taken up by the cell, mimicking the process by which macrophages and other inflammatory cells uptake glucose for cellular processes. The ability of these cells to incorporate higher rates of glucose than neighboring cell types makes \(^{18}\)F-FDG a suitable inflammatory target for PET imaging.\(^34\) Improvements in image quality, acquisition, and quantification make it more likely that clinical studies can be performed at the microcalcification stage.\(^33\)

**Near-Infrared Fluorescence**

A more recent alternative to PET-CT scans is near-infrared fluorescence (NIRF) intravital molecular imaging. Commerically available NIRF dye, IRDye800CW, can be paired with antibodies or nanobodies and injected into a patient to be visualized during, for example, pediatric, oncological, and laparoscopic colorectal surgeries,\(^32,37\) as a real-time guiding agent. Advantages of this procedure include the rapid clearance of the dye, low genotoxicity, high signal-to-background ratio, and deep tissue penetration.\(^38\) In the context of cardiovascular disease—related surgeries, the use of such agents is not yet approved; instead, near-infrared autofluorescence imaging (coupled with optical coherence tomography) has been shown to identify high-risk plaques in patients undergoing catheterization procedures.\(^39\) Nonetheless, a NIRF calcium tracer technique was used to visualize microcalcifications in explanted carotid artery plaques\(^40\); and the imaging modality has been used to characterize plaque morphology in animal models of atherosclerosis.\(^31\)

NIRF imaging also has been used to study disease progression in atherosclerotic plaques, providing insight into aortic valve stenosis.\(^42\) Imaging probes target hydroxyapatite producing an osteogenic NIRF signal, which enables researchers to identify microcalcifications \textit{in vivo}, \textit{ex vivo}, and \textit{in vitro}.\(^15\) Furthermore, intravenous co-injection of two different imaging probes that have excitation in different NIRF spectra allow simultaneous visualization of two different biological processes\(^43\); (eg, calcification and inflammation) (Figure 2).\(^12\)

Colocalization of the signals in atherosclerotic plaques has led to the hypothesis that macrophages themselves undergo the process of calcification through the release of calcifying extracellular vesicles, confirming the notion that calcification is an inflammatory disease.\(^44\) This hypothesis has been supported further through NIRF imaging by using a calcium tracer to identify the calcifying extracellular vesicles that cause the microcalcifications in the macrophages.\(^45\) Results from NIRF imaging were reinforced with gold standard histologic methods.\(^36\) Such corroborative stains often include, but are not limited to, hematoxylin and eosin, von Kossa, Oil Red O, Picrosirius red, and immunohistochemistry, which identify morphologic changes, calcification, lipid deposition, collagen stability, and protein localization, respectively.\(^27-51\) von Kossa substantiates calcification \textit{ex vivo}. When paired with Oil Red O, the two stains validated a relationship between atherosclerosis and calcification in plaques identified with NIRF imaging.\(^17,52\) Immunofluorescence supports NIRF findings on the molecular level. Immunofluorescent staining against both macrophages (eg, anti-CD68 antibody) and calcification (eg, OsteoSense-680 fluorophore) in calcified mouse\(^39\) carotid arteries provided additional evidence to support the hypotheses that calcification is an inflammatory condition.\(^42\) NIRF and immunohistochemical techniques also...
have been applied to mouse valves, confirming the link between inflammation and mineralization in aortic valve leaflets.

Because of the highly specific nature of these probes, NIRF imaging is especially useful when studying inflammation because it is less affected by the autofluorescence of surrounding tissues relative to other imaging modalities. This high specificity enables probes to monitor, over time, enzymes with extremely low abundances. For example, temporal analysis of the low-abundant enzyme, zymosan, showed zymosan-induced inflammation in murine models. This novel application will be useful in understanding the role of low-abundant molecules in CAVD.

NIRF imaging can visualize distinct cellular changes of early aortic valve disease in vivo, validating targets identified by transcriptomics and proteomics (more below). In the aortic valves of hypercholesterolemic apolipoprotein E–deficient mice, macrophage accumulation, an established feature of CAVD, was detected by NIRF imaging. NIRF was also used to detect increased matrix metalloproteinases (MMP-1, -9, and -12) in CAVD, which could be used as a target in stenotic tissue. To confirm their role in CAVD, tracers bound to the activated MMPs were imaged at different stages of the disease. The MMP signal peaked before the valves were fully calcified, suggesting a role of MMPs in early CAVD progression and the potential for MMP imaging to predict CAVD.

Limitations of NIRF imaging include a 24-hour time delay between injection of the agent and detection, suggesting a need for faster-acting agents, and signal attenuation by blood requiring a consensus on correction methods. However, molecular imaging in general is limited because it is not a method for identification of novel disease driver or disease biomarker molecules. Large-scale screening efforts to identify these molecules are undertaken with the assistance of one or more -omics technologies.

Transcriptomics

Transcriptomics is the characterization of multiple gene-expressed RNAs from cells or tissues that relied on microarray or fluorescently labeled nucleic acid hybridization technologies in its infancy. More recently, transcriptomics has transitioned to unbiased RNA-seq methods. Bulk RNA-seq is any sequencing method that provides an average gene expression or abundance profile from a cell population or tissue. scRNA-seq, on the other hand, relies on isolation of individual cells for subsequent gene expression profiling. The latter therefore provides a view of the extent of cellular identity and heterogeneity in a cell population or tissue. Defining the extent of cellular heterogeneity may help identify disease driver cell subpopulations, which, in turn, can be targeted pharmacologically. A comprehensive
review of the various RNA-seq platforms has been reported. The following paragraphs describe the application of this technology to uncover the molecular mechanisms at the root of CAVD.

**Bulk RNA-Seq of Calcified Aortic Valve Leaflets**

Within the past 10 years, a few studies have used RNA-seq to profile the transcriptomes of leaflets derived from patients with calcified tricuspid aortic valve (TAV) and/or bicuspid aortic valve (BAV) disease. BAV is a congenital heart disease that promotes early onset aortic stenosis and calcification in patients. It therefore has been hypothesized that the disease drivers of BAV and TAV are distinct. One study compared calcified TAV and BAV (n = 10 each) leaflets from patients who underwent aortic valve replacement; as controls, normal tricuspid aortic valve was used from patients (n = 10) who underwent orthotopic heart transplantation, but had normal aortic valves. Interestingly, only two genes were expressed differentially when comparing calcified TAV versus calcified BAV: *IGF1* and *RSP02* were increased in calcified BAV. On the other hand, 462 and 329 genes were expressed differentially when calcified BAV and normal TAV, and calcified TAV and different TAV were compared, respectively. An explanation for the limited number of differentially expressed transcripts between the calcified tissues is that they represent the end stage of each disease, which likely have more commonality than in the early stages. This underlying result is not a drawback of RNA-seq itself, rather it is a limitation resulting from the inaccessibility of early stage disease samples. As shown further in the next paragraph, alternative strategies to analyze even late-stage diseased samples have enabled inferences of early stage molecular signatures.

An alternative approach to RNA-seq alone to identify CAVD drivers from late-stage diseased samples is the implementation of genome-wide associations. RNA-seq—dependent expression of quantitative trait loci mapping (n = 233 human aortic valve samples) combined with genome-wide associations, termed transcriptome-wide association study, identified the gene PALMD as a candidate driver of CAVD, particularly when PALMD is underexpressed. This finding inspired a subsequent phenome-wide study (2359 cases and 350,060 controls) of PALMD as a possible therapeutic target, which, in turn, confirmed that aortic valve—specific, genetically deduced expression of PALMD is associated inversely with calcific aortic valve stenosis.

scRNA-seq for the Identification of CAVD Driver Cell Subpopulations

scRNA-seq confronts the limitations of understanding aortic valve heterogeneity, which bulk RNA-seq is unable to accomplish on its own. Resolution at the single-cell level allows for transcriptome analysis of, and distinction between, cell subpopulations. scRNA-seq was introduced in 2009 to determine transcriptomes of single blastomeres and oocytes from mice. As a result of very recent scRNA-seq studies, it now has been established that aortic valve tissue is highly heterogeneous, consisting of distinct extracellular matrix layers with various cell subpopulations.

One such study performed scRNA-seq analysis of aortic valve leaflets from patients with CAVD (n = 4) and patients whose valvular tissues were removed during corrective surgery for aortic dissection (n = 2). The combined patient scRNA-seq data analysis showed 12 candidate cell subpopulations that were ascribed to either valvular stromal, interstitial, or endothelial cells; or monocytes, macrophages, or lymphocytes. Based on differential transcripts, the valvular stromal cells, for example, were classified into five subpopulations, the interstitial cells were classified into three subpopulations, and the endothelial cells were classified into two subpopulations. This study also confirmed that 7 of the 12 cell populations were unique to, or more prevalent in, the CAVD leaflets, and that the monocyte cells were more prevalent in normal leaflets. Subsequent immunofluorescence or immunochemistry was used to localize the transcript products, their proteins, which defined each of the seven CAVD-enriched cell populations in diseased leaflets; for example, the three stromal cell—derived populations that were defined by high expression of either metallothionein-1A (*MT1A*), peptidase inhibitor 3 (*PI3*), or the ribosomal subunit homolog protein (*CMSS1*). Immunohistochemical analysis showed that *MT1A* and *CMSS1* were localized to the spongiosa and fibrosa layers, whereas the PI3 signal was increased in the vicinity of the endothelial layer of CAVD leaflets. Such integrational studies highlight the benefits of this multimodal approach (scRNA-seq to molecular imaging) to generate an understanding of the disease as a system and identify ways to treat it.

A second study used a high-throughput screening—flow cytometry panel strategy to identify a potential subpopulation of progenitor/stem cell—like population of valvular cells that may drive the tissues calcification potential. These candidate disease-driver cells then were isolated from CAVD leaflets using fluorescence-activated cell sorting for subsequent cell culture and scRNA-seq analysis. The isolated disease-driver cells showed an increased potential to calcify in vitro when compared with unsorted valvular interstitial cells. Moreover, scRNA-seq showed that when these cells were cultured in normal versus osteogenic media, each culture condition induced differentiation into multiple yet distinct cell subpopulations. One of the osteogenic clusters contained high levels of a known procalcification enzyme, alkaline phosphatase, and also the newly described amine oxidase-A. Subsequent studies confirmed that loss of amine oxidase-A inhibited the calcification potential in vitro. This scRNA-seq analysis of a cell culture model for valvular calcification being mediated by a progenitor cell...
population provides a means to glean insight into the early mechanism that drives disease progression, even from end-stage diseased tissue.

Proteomics

DNA is transcribed into RNA transcripts, which are translated into proteins.70 Proteins are metabolic and physiological drivers of all bodily functions as well as disease pathogenesis.71 They provide insight into disease mechanisms and can be interrogated on a large scale via proteomic techniques. Proteomic techniques range in complexity and level of data production. The earlier chromatography and blotting techniques are useful for smaller sample sizes but are incapable of depicting protein expression levels. As more advanced technologies such as protein arrays, advanced gel methods, mass spectrometry, and some sequencing methods rose to the forefront, the ability to perform proteomic analysis on more complex samples with a higher degree of sensitivity arose with them.72,73 There are three main technologies that are used to conduct high-throughput proteomics: the aptamer platform,74 the proximity ligation assay,75 and mass spectrometry.73 However, unlike mass spectrometry, which is permissive to unbiased screening of previously uncharacterized proteins and post-translational modifications, the aptamer platform and the proximity ligation assay are detection and quantification panels of characterized proteins. Nonetheless, any of these technologies permit analysis of thousands of proteins simultaneously, increasing the potential for identifying targets for further research and drug therapy tremendously.76

Mass Spectrometry-Based Proteomics of CAVD Tissues

Proteome profiling of CAVD tissues has emerged only within the past 5 to 6 years, enabled by mass spectrometry-based proteomics. The first proteome of human aortic valves was published in 2018.16 Mass spectrometry was used to characterize the differential distribution of proteins across the three layers that make up the human aortic valve—the ventricularis, fibrosa, and spongiosa—to better understand their constituents’ roles in disease pathogenesis. Leaflets obtained from patients who underwent aortic valve replacement surgeries (diseased) or from autopsy (nondiseased) were compared. Their findings identified layer-enriched proteins, including apolipoprotein B, apolipoprotein C-III, and calponin-1, all of which were increased in the diseased compared with nondiseased sample leaflets.16 These and other identified proteins were validated with imaging technologies that, in part, confirmed glial fibrillary acidic protein as a novel marker of the spongiosa layer (Figure 3). This study also noted that in addition to apolipoprotein B, other apolipoproteins, such as ApoA-I and ApoA-II, which are located primarily on high-density lipoprotein (HDL), and ApoC-III, which is located on all classes of lipoproteins (HDL, and very-low-density and low-density lipoproteins), were increased in calcified versus noncalcified samples, leading to the hypothesis that one or more of these apolipoproteins may contribute to the disease pathogenesis.

In a follow-up study, 12 apolipoproteins were investigated in more detail.75 First, the investigators used a targeted proteomics strategy,73 entailing the use of mass spectrometry peptide standards, to precisely quantify and verify the subset of apolipoproteins (8 of 12) that were increased in calcified portions of aortic valve leaflets. ApoC-III was one such apolipoprotein that also was confirmed to co-localize with calcified regions within CAVD leaflets. The potential of Apo-C-III to promote disease progression was shown in vitro, increasing calcification potential, compared with controls, when incubated with human valvular interstitial cells. Subsequent proteomic analysis showed that Apo-C-III induced an enrichment of proteins involved in oxidative stress, inflammation, and removal of reactive oxygen species.76 This study showed the value in conducting disease tissue–sourced proteomics to identify CAVD drivers, but tissue acquisition is dependent on patients reaching stages of morbidity or mortality. Thus, there is an initiative to investigate, at a minimum, the circulatory system for potential prognostic molecules for CAVD development.

Proteomics of Plasma and Urine to Identify CAVD Biomarkers

Urine is a clinically relevant and stable medium for uncovering proteins associated with CAVD. Proteomic analysis of urine from patients with aortic valve stenosis and coronary artery disease identified von Willebrand factor and tissue inhibitor matrix metalloproteinase 1 as signature proteins of diseased samples relative to healthy controls.77 Furthermore, a strong positive correlation was found between von Willebrand factor and HDL-cholesterol and between tissue inhibitor matrix metalloproteinase 1 and HDL-cholesterol in patients with aortic valve stenosis.77 Subsequent confirmation of the strong association between serum HDL and CAVD progression strengthened the proposed relationship between known biomarkers, von Willebrand factor, and tissue inhibitor matrix metalloproteinase 1, and the progression of CAVD.78 This finding not only has implications for therapeutic targeting, but also suggests an influential role of urine for routine screening of early CAVD. As a noninvasive alternative for sample collection, it is particularly appealing for biomarker classification.

When it comes to stable and clinically relevant media on which to perform proteomic analysis, human plasma is the gold standard. Human plasma is a unique compartment for extensive biomarker discovery, drawing from proteins secreted from many organs and tissues throughout the body.79 The high throughput and widespread nature of
plasma proteomics generates efficient yield of hundreds of proteins within thousands of samples: a large-scale performance that rarely is achieved using tissue samples. Applied to cardiovascular disease cohorts, this technology has verified the role of apolipoproteins, among others, in atherosclerosis and other agents of cardiovascular disease. Plasma proteomics also identified novel proteins such as low-density lipoprotein receptor-related protein 2 to be investigated further. With its increasing application among cardiovascular and other diseases, plasma proteomics is the next frontier for extracting information about CAVD biomarkers. Moving forward in this direction, it is important to develop standardized pipelines concerning the design and execution of the study, as well as how the resulting data are processed and analyzed. The Human Plasma Proteome Project is a platform that promotes this sort of systemization, while engendering a space to investigate collective challenges to proteomic studies.

Proteomics at the Single-Cell Level

Proteomics provides valuable insight into the protein profiles of diseased tissue, shedding light on the protein interactions that contribute to CAVD pathophysiology. Integrating proteomic and molecular imaging technologies, as previously mentioned, could serve as an efficient tool to visualize proteome spatial profiles. Until recently, single-cell proteomics or single-molecule proteomics were unventured areas of translational research. Although there have been strides in performing single-cell proteomics, specifically with single-cell proteomics by mass spectrometry, this analysis has been limited by the sensitivity of such high-resolution samples. Even when single-cell proteomic analysis is achieved, drawbacks remain with its low availability and its inability to provide high-throughput results. These limitations, in addition to the importance of understanding expression profiles when investigating CAVD, necessitate transcriptomic techniques. Transcriptional analysis not only resolves concerns surrounding proteomic analysis of high-throughput single-cell resolution, but also provides contextual information behind CAVD expression profiles.

Multi-Omics Applied to CAVD Research

We now understand that transcript levels do not directly translate to protein levels, leaving an imbalance between gene and protein profiles. The poor correlation in the multiple gene-expressed RNA protein relationship can be accounted for by various biological influences including the secondary structure of multiple gene-expressed RNAs, regulatory proteins, regulatory sRNAs, ribosomal availability, and the half-life of individual proteins. In the same study that conducted the first proteome profiling of normal and diseased aortic valve leaflets, it was shown that the accumulation of most apolipoproteins in diseased valves was independent of their transcript levels, supporting the notion that circulating lipoproteins are the likely source of their apolipoproteins.

Integrative approaches also help draw the bridge between transcript or gene levels, and their protein product and localization within the body. Of late, amyloid protein deposition has been implicated in the development of calcification within the aortic valve. Two-dimensional gel electrophoresis has identified serum amyloid P-component up-regulation within calcific aortic valves.
A deeper dive into the role of amyloid protein in CAVD progression was taken with a three-dimensional multi-omics approach that analyzed prior transcriptomics and proteomics studies. This approach found the gene for APP as a connector gene for three input molecules from this transcriptomic and proteomic data, even though the APP gene did not exist within the transcriptomic and proteomic data used to generate this three-dimensional network. Subsequent immunohistochemical staining localized APP exclusively to areas of calcification within extracted aortic valves, illustrating how the integration of techniques allows for a more comprehensive understanding of the interaction between transcripts and protein products within the scope of CAVD.

Frontiers in CAVD Research

Epigenetics

Epigenetics is the study of the factors, independent of mutations, that directly affect a gene’s transcription activity, such as noncoding RNAs, DNA methylation, and heterochromatin components such as histones. The assay for transposase-accessible chromatin (ATAC-seq) is a fast, sensitive technique used to assess genome-wide chromatin accessibility by adding the viral enzyme, transposase, to DNA to tag open chromogen sites that can be mapped to accessible areas of calcification within extracted aortic valves, illustrating how the integration of techniques allows for a more comprehensive understanding of the interaction between transcripts and protein products within the scope of CAVD.

The Potential Impact of TAVR on CAVD Research

TAVR, a catheter-based, less-invasive alternative to SAVR, generates a potential obstacle surrounding sample (explanated valves) accessibility. As the advantages of TAVR are documented, therein lies a decreased dependence on SAVR for AVR procedures, and thus fewer explants for valvular surgeries. A deeper dive into the role of amyloid protein in CAVD progression was taken with a three-dimensional multi-omics approach that analyzed prior transcriptomics and proteomics studies. This approach found the gene for APP as a connector gene for three input molecules from this transcriptomic and proteomic data, even though the APP gene did not exist within the transcriptomic and proteomic data used to generate this three-dimensional network. Subsequent immunohistochemical staining localized APP exclusively to areas of calcification within extracted aortic valves, illustrating how the integration of techniques allows for a more comprehensive understanding of the interaction between transcripts and protein products within the scope of CAVD.

Limitations to Current Animal Models

Apolipoprotein E–deficient mouse models, among other CAVD models in which diet and/or mutations in lipid metabolism (such as low-density lipoprotein receptor and apolipoproteins) induce dyslipidemia, are beneficial because

Figure 4 The multi-omics landscape for calcific aortic valve disease (CAVD). Molecular profiling enabled by the three -omics fields that encompass the central dogma of biology, DNA (epigenetics) to multiple gene—expressed RNA (mRNA) (transcriptomics) to protein (proteomics), and the integration of animal models, imaging technologies, and cell culture models to enable the understanding of CAVD. Molecules of interest, epigenetics, and the further development of animal models represent part of the frontiers for CAVD research. Most studies to date have relied on human aortic valves retrieved from valve-replacement surgeries. ApoC-III, XXX; HDL, high-density lipoprotein; MAOA, XXX; Me, XXX; Mod, XXX; PALMD, XXX; RNA-seq, RNA sequencing; scRNA-seq, single-cell RNA sequencing; vWF, von Willebrand factor.

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they are used commonly to study multiple cardiovascular diseases and thus are readily accessible. Although such disease models may represent certain aspects of the disease, they assume dyslipidemia-dependent etiology that does not exhaustively represent the pathogenesis of any of the three basic etiologic categories of human CAVD outlined above: rheumatic heart disease, congenitally abnormal valvular disease, and atherosclerotic calcification of congenitally normal valves. The effectiveness of statins is the perfect example of the limitations of these mouse models. Statins, which have been proven by randomized trials, the Simvastatin and Ezetimibe in Aortic Stenosis trial and the Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression, only to be effective with dyslipidemia as the disease facilitator, do not act on all pathways of CAVD pathogenesis. When factors such as disease pathogenesis and stage are not controlled, they show no effect on aortic valve stenosis progression. This suggests that CAVD cases of differing etiologic origins likewise have differing underlying mechanisms that require suitable animal models for further investigation. Identifying such cost-effective animal models (Figure 4) will be beneficial for understanding the pathogenesis of atherosclerotic calcification of congenitally normal valves.

Conclusions

Research in the field of valvular calcification has advanced rapidly in recent years. The use of proteomics, transcriptomics, and molecular imaging (Figure 4) has unlocked new avenues of study that enhance the current understanding of CAVD pathobiology and, in conjunction with one another, become even more powerful. Although considerable progress has been made, there still are unanswered questions regarding the disease mechanisms. For example, how can the calcification potential of progenitor-like cells in the aortic valve be mitigated, and is amine oxidase-A a viable clinical target (Figure 4A)? If the documented accumulation of apolipoproteins in the valve (Figure 4) is owing to a life-long deposition of lipoproteins and their constituents within the extracellular matrix, would lipid-lowering interventions be effective?

Multi-omics technologies often are used to discover disease drivers of late-stage CAVD tissue; however, much of the current research fails to investigate the origins of early stage drivers. As a result, therapeutic targeting remains possible only with late stages of the disease, and falls short of thwarting disease progression. To prevent CAVD, it is crucial to continue to examine disease pathophysiology at early stages, which will become increasingly accessible with technological advancement and with suitable animal and cell culture models. Addressing this issue requires coordination between clinical and basic research institutions to ensure the integrity of the discoveries being made via translational research.

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