FROM BIOLOGY TO CLINICAL MANAGEMENT: AN UPDATE ON AORTIC VALVE DISEASE

2nd Edition

EDITED BY: Cécile Oury, Alain Nchimi and Patrizio Lancellotti

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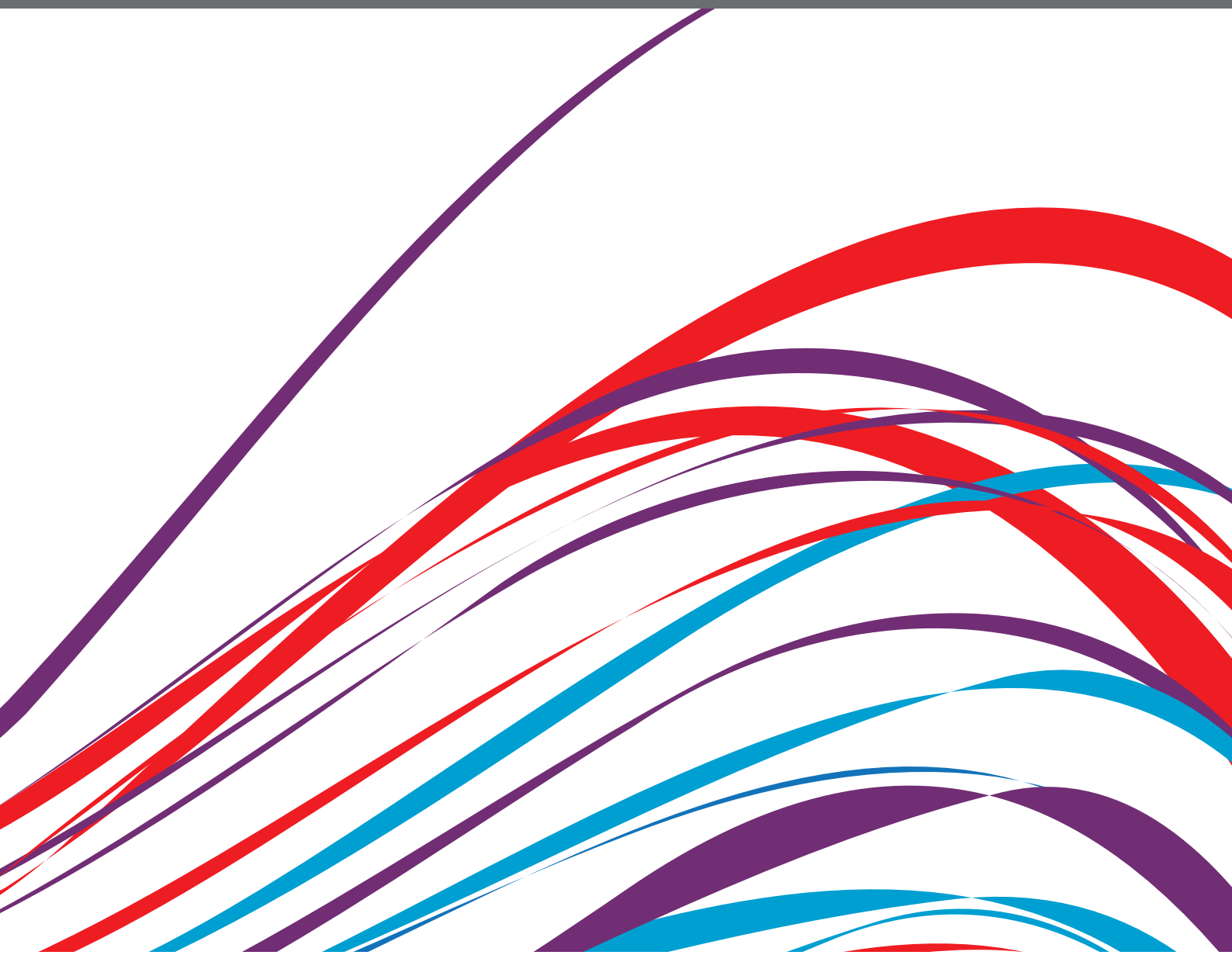

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FROM BIOLOGY TO CLINICAL MANAGEMENT: AN UPDATE ON AORTIC VALVE DISEASE, 2nd Edition

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Calcific aortic valve stenosis is the most frequent valvular heart disease in Western countries, affecting up to 13% of individuals over 75 years. The disease is associated with considerable morbidity and mortality. It is characterized by fibro-calcification of aortic valve cusps and concomitant left ventricular remodelling due to chronic pressure overload, which can evolve into overt heart failure. It progresses very slowly until the onset of symptoms, the indication for aortic valve replacement. Today, about 300,000 aortic valve replacements are performed annually worldwide, either via surgery or transcatheter implantation. This is the only treatment shown to improve survival. There is no pharmacological treatment to prevent or slow disease progression. Major risk factors include older age, congenital anomalies of the aortic valve (bicuspid valve), male gender, hypertension, dyslipidaemia, smoking, and diabetes. However, how these factors contribute to the disease in unclear. Due to the disease itself, patients are at increased risk of both thrombosis and bleeding, which, in addition to advanced age and comorbidities, makes antithrombotic management of these patients difficult. Regarding valve prostheses, the ideal prosthesis either mechanical or biological still does not exist. Clinically available prostheses can lead to major complications, thrombosis or infection, which necessitate reoperation or cause death in 50-60% of patients within 10 years post-implantation. Hence, there are major unmet medical needs in CAVS and more basic and translational research is definitely required. Our Research Topic depicts major challenges and research paths that could be followed to address these major health needs.

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EDITORIAL
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Editorial: From Biology to Clinical Management: An Update on Aortic Valve Disease

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Keywords: aortic valve (AV), aortic valve replacement (AVR), aortic valve calcification, aortic stenosis (AS), TAVI—transcatheter aortic valve implantation

Editorial on the Research Topic

From Biology to Clinical Management: An Update on Aortic Valve Disease

Calcific aortic stenosis (AS) is the most frequent valvular heart disease in Western countries, affecting up to 13% of individuals over 75 years (1, 2). The disease is associated with considerable morbidity and mortality. Major risk factors include older age, congenital anomalies of the aortic valve (bicuspid valve), male gender, hypertension, dyslipidaemia, smoking, and diabetes (3).

The disease is characterized by fibro-calcification of aortic valve cusps and concomitant left ventricular (LV) remodeling due to chronic pressure overload, which can evolve into overt heart failure. AS progresses very slowly until the onset of symptoms (angina, dyspnae, syncope). A large majority of patients remain asymptomatic for a long period, though at increased risk for untoward events (death, heart failure, symptomatic deterioration, LV dysfunction). Development of symptoms is a class I indication for aortic valve replacement (AVR). Today, about 300,000 AVR are performed annually worldwide, either via surgery (SAVR) or transcatheter implantation (TAVI). AVR is indeed the only treatment shown to improve survival. There is no pharmacological treatment to prevent or slow disease progression.

The present research topic provides a comprehensive overview of AS clinical management with a special focus on valve prostheses, imaging and blood biomarkers as well as on recent advances on pathophysiology and valve biology.

Regarding valve prostheses, the ideal prosthesis either mechanical or biological still do not exist. Current prosthesis can cause complications, which necessitate reoperation or lead to death in 50–60% of patients within 10 years post-implantation. In this research topic, Musumeci et al. reviewed the different types of currently available prosthetic aortic valves and their limitations. It appears that thrombosis, infection, bioprosthesis calcification, and degeneration remain major issues, which could be addressed through innovative new generation prostheses.

Rachwan et al. report on a patient who presented with a thrombus on a bicuspid aortic valve in the setting of antiphospholipid syndrome (APLS). APLS is a systemic autoimmune disease defined by thrombotic events in patients persistently positive for antiphospholipid antibodies (aPL). In this case report, 4-months moderate-intensity anticoagulation efficiently eliminated the aortic valve thrombus. However, due to the rarity of this condition, whether conservative anticoagulation or AVR should be recommended remains to be determined. More generally, there is currently no clear recommendation on the choice of antithrombotic regimen for AS patients (4, 5).

Another major challenge in the clinical management of AS is deciding on the correct timing of AVR (6).

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Regarding clinical imaging, echocardiography is central to the diagnosis and risk stratification of patients with aortic stenosis and regurgitation. However, the technique has certain limitations, and aortic valve imaging may benefit from alternative and complimentary multimodality imaging. In the present topic, Nchimi et al. performed a systematic review and meta-analysis in order to evaluate the role of imaging biomarkers in predicting AS progression to clinical symptoms and mortality. Eight studies regrouping 1,639 patients were included in the analysis. This study showed significant associations of computed tomography aortic valve calcification (AVC) and myocardial fibrosis, measured by cardiac magnetic resonance (CMR), with clinical outcomes. The findings on AVC are in line with a recent study showing that sex-specific AVC thresholds accurately identify severe AS and predicts AVR and death (7). Late enhancement gadolinium fibrosis was significantly associated with cardiac mortality, which is in agreement with another recent meta-analysis indicating that LV fibrosis can also have prognostic value after AVR (8). Hence, the prognostic efficacy of these imaging biomarkers for patient management as compared to the current approach that relies mainly on clinical performance need to be tested in large randomized studies.

In addition to clinical imaging, several studies strongly suggest that circulating biomarkers could help for AS patient risk stratification (9). In this research topic, Oury et al. reviewed the role of circulating biomarkers in patients undergoing TAVI. Despite the fact that TAVI offers a marked change in life expectancy and quality of life of high-risk elderly patients, (10) early and late mortality after TAVI still remains relatively high (11–13). Studies indicate that implementing biomarkers of myocardial injury, cardiac mechanical stretch, inflammation, and of hemostasis imbalance in clinical practice might help reducing TAVI-associated complications and mortality. However, the role of these biomarkers has yet to be confirmed in large randomized studies.

Nevertheless, the identification of novel biomarkers will necessitate a better understanding of aortic valve biology and mechanisms of disease. The review by Hulin et al. draws a summary of current knowledge on pathogenic pathways and their potential role as novel therapeutic targets. Heart valve homeostasis is tightly controlled by valve interstitial cells (VICs) embedded in extracellular matrix, valve endothelial cells (VECs) covering the leaflet, and circulating and resident immune cells. AS is now considered as an active multi-step process. Early steps of lesion development would occur through accumulation of lipids and free cholesterol within the fibrosa, followed by infiltration of inflammatory cells, e.g., macrophages and T lymphocytes. VICs then enter an osteogenic program, initiating calcium nodule formation, and valve calcification (2). All these events likely involve mechanical stress and strain, and a major role for valve lining endothelial cells. However, how these complex cellular interplay contributes to AS remains unknown. Furthermore, thorough knowledge of the heterogeneity and function of valve cell subtypes, over the course of the disease, may provide useful informations to develop targeting strategies of diseased cells. In this sense, transcriptional profiling studies during valve development could help to better define valve tissue composition and homeostatic biological pathways. In this research topic, Nordquist et al. compared mRNA expression in postnatal and adult valve tissues. This study nicely unveiled multiple conceivable processes that contribute to postnatal valve maturation and maintenance that may pave the way for elucidating mechanisms underlying valve defects.

Thus, this research topic highlights important unmet medical needs in AS. More basic and translational research is definitely required to clarify disease mechanisms, uncover new multi-biomarker-based diagnostic and prognostic tools, and develop more biocompatible and durable prostheses with the goal of improving patient outcome.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Prosthetic Aortic Valves: Challenges and Solutions

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Aortic Valve Disease (AVD) is the most common Valvular Heart Disease (VHD), affecting millions of people worldwide. Severe AVD is treated in most cases with prosthetic aortic valve replacement, which involves the substitution of the native aortic valve with a prosthetic one. In this review we will discuss the different types of prosthetic aortic valves available for implantation and the challenges faced by patients, medical doctors, researchers and manufacturers, as well as the approaches that are taken to overcome them.

Keywords: aortic valve replacement, mechanical valve, bioprosthesis, percutaneous, surgical, complications

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INTRODUCTION

In Europe alone more than 13 million people (1) are diagnosed with Valvular Heart disease (VHD) each year and 100 million worldwide (2). VHD primarily affects the elderly (>65 years old) in western countries and young people (<30 years old) in developing countries, because of the high incidence of rheumatic heart disease and short life expectancy (3 ESC Guidelines; Supplement).

The deterioration of native heart valves (tricuspid, pulmonary, mitral, aortic) once started is difficult to treat or revert with medications, leaving valve replacement as the only option, whenever valvuloplasty is not possible (4).

Aortic Valve Disease (AVD) is the most common among valvular conditions (44,3% VHD are AVD) (5) and the gold standard treatment was Surgical Aortic Valve Replacement/Implantation (SAVR or SAVI) until the introduction in 2007 of a new revolutionary procedure, Transcatheter Aortic Valve Replacement/Implantation (TAVR or TAVI). TAVI became especially used in inoperable, *i.e.*, high-risk patients, as it is les s invasive than an open-heart surgery (6).

Both SAVI and TAVI are not risk-free, tough, in fact, patients are subjected to life threatening complications associated with the medications given post-implantation and with the deterioration of the implanted valve (7).

In this review we will discuss prosthetic aortic valves, pre and post implantation challenges, and their solutions.

Aortic Valve Disease (AVD)

Aortic Stenosis (AS) accounts for the majority of AVD (almost 50% of all VHD). AS prevalence in Europe is 3–8% among people over 75 years old. If untreated, 90% of patients with severe AS have a life expectancy of less than 10 years, and 50% of the patients will die in the 2–3 years following symptoms onset (3 ESC Guidelines; Supplement).

Calcific Aortic Stenosis (CAS), which is the formation of fibro-calcic nodules on the valve has a prevalence of 0,4% in the general population and 1,7% in the population over 65 years old (8). The pathophysiology of CAS is complex, it involves lipoprotein deposition, inflammation and osteoblast transition of valve interstitial cells (Hulin A et al. in this issue).

Risk factors for AS in the general population are the same as atherosclerotic vascular diseases, *i.e.*, diabetes, hypercholesterolemia, hypertension and tobacco usage (9).

Medication is unable to stop or revert the process of native aortic valve degeneration, with solutions limited to reparation/ reconstruction or, in most cases, replacement.

Aortic Valve Replacement

Worldwide the number of aortic valve replacement in 2003 was 290,000 and by 2050 is predicted to be 850,000 (10).

Prosthetic aortic valves can be of 3 different types: (1) Surgical Mechanical Aortic Valves in different material, including stainless steel, pyrolitic carbon or ceramic, and with different shapes - caged-ball, monoleaflet and bileaflet. They are structurally robust and can theoretically have a long service life (25–30 years). (2) Surgical Biological Aortic Valves are made of biological tissue that can be xenogenic (bovine or porcine) or allogenic (homograft), stented or stentless. Durability is the main problem with these valves, which last between 10–15 years. (3) Transcatheter or Percutaneous Aortic Valves are tissue heart valves and can be of two types: expanded over a balloon or self-expandable. They are inserted percutaneously and are easy to implant, but, like surgical bioprosthesis, they are not long lasting.

The surgical procedure for aortic valve replacement involves an open-heart surgery, the heart is stopped and the patient is attached to a bypass to oxygenate the blood. Since SAVI is quite invasive, it has been slowly replaced by TAVI, which can have 3 different sites of vascular access: transfemoral, subclavian or carotid artery and clinical trials are currently ongoing to evaluate the approach that will give least complications. TAVI is performed in cases where patients are at high risk of death during surgery, due to old age or the presence of additional diseases. Two randomized prospective clinical trials, PARTNER 1 (Placement of AoRtic TraNscatheER) (11) and CoreValve (12) have proven the superiority of TAVI over SAVI in a high-risk cohort of patients. Moreover, in July 2017 - the year of the 15 year anniversary of TAVI (13) - the FDA, based on the favorable conclusions of two trials, the PARTNER 2 (14) and the SURTAVI, has approved the use of TAVI in patients with intermediate risk of a negative outcome during open-heart surgery. But SAVI still remains the reference method, especially in low-risk patients. To be able to extend TAVI to all patients, regardless of surgical risk, more studies, focused on the outcomes of the procedure in the long run, are needed (15).

Both SAVI and TAVI are associated with thrombosis (2), but it is becoming evident that during TAVI there are more periprocedural ischemic and embolic strokes, caused by the dislodgement of debris from the aortic arch, annulus, and native valve (16). To reduce such thromboembolic events, the clinical trial GALILEO (clinicaltrials.gov, NCT02556203) is at the moment recruiting patients to test the hypothesis that, being thrombin a key-player in the pathophysiology of thromboembolic events, patients would benefit from treatment with anticoagulants, like rivaroxaban.

Management of Aortic Valve Replacement

For an aortic valve replacement medical doctors are faced with many decisions, *e.g.*,: define when the aortic valve condition is severe enough to perform the replacement; what kind of intervention - SAVI or TAVI - to perform; and what kind of prosthetic aortic valve to use - mechanical or biological [(17) ESC Guidelines]. This is why it is very important that a multidisciplinary heart team evaluates risks and benefits of all pre and post-procedural decision (18).

Usually, mechanical valves, which are more thrombogenic, but more durable, are implanted in patients younger than 65 years old, which have good hemodynamics, while biological valves are used mainly in the elderly. Although less thrombogenic, tissue valves (surgical or trasncatheter) are prone to structural valve deterioration (SVD), caused mainly by calcification (19) . Nevertheless, more than half of valve replacements are bioprosthetic, especially after the introduction of TAVI in 2007.

Since mechanical valves are thrombogenic, they require long-term vitamin K antagonists (warfarin) and antiplatelet drugs (aspirin) administration. However, such treatments may increase the risk of bleeding. Bioprosthetic, both surgical and transcatheter, valves have better hemodynamic properties compared to mechanical ones, therefore, the antithrombotic treatment is just required for the first months post-surgery (3–6 months) to reduce thromboembolic complications, during the process of prosthesis endothelialization (neointimal coverage of the frame and leaflets) (2).

The choice of the best valve to implant depends mainly on two risk factors: anticoagulation-related bleeding and valve deterioration. Tissue valves are implanted when the risk of bleeding with anticoagulation treatment is high, while mechanical valves are implanted when valve tissue deterioration could be accelerated, *i.e.*, in younger patients.

Antithrombotic management slows down, but does not eliminate the risk of prosthetic valve failure, which depends also on the life-style of the patient as well as on the pre-procedural metabolic profile and inflammatory status, especially for TAVI.

Causes of Failure of Prosthetic Valves

Prosthetic valve dysfunction depends on the valve that has been implanted and on the procedure (SAVI or TAVI). Atherosclerosis risk factors, like diabetes, smoking, hypercholesterolemia, metabolic syndrome may accelerate failure of prosthesis, while dental procedures and other surgeries may increase the risk of valve infection.

Infection

The risk of infection of the prosthetic aortic valve is much higher compared to native valves and can affect all types of prosthesis equally, leading to infective endocarditis. Infections can arise just after surgery (within a week to one month post-surgery), or appear long after surgery (after 6 months). Periprocedural and 30 days infections are more common during SAVI compared to TAVI, although such differences are not statistically significant (7).

Since bacteria colonization of prosthetic valves and, of biomedical devices in general, is difficult to fight, because of biofilm formation (bacteria in the biofilm are more resistant to the usual antibiotics doses), it is important to prevent infections that can arise during dental procedures or other surgeries, with the use of antibiotics [(20) ESC Guidelines].

Thrombosis

Altered local flow due to the presence of prosthetic valve may be a trigger for thrombosis. In fact, high shear stress levels could potentially damage red blood cells (hemolysis) and activate platelets, promoting thrombogenesis.

The disruption of the normal local flow can also be caused by implantation errors *e.g.*, when the transplanted valve does not have the right geometry/model, with a specific annulus size, different for each patient. We talk in this case of a Patient-Prosthesis Mismatch (PPM). To prevent PPM cases, medical doctors should use fluid dynamic computational simulations before valve implantation.

While bioprosthesis are the least thrombogenic, mechanical and transcatheter valves are comparable in terms of thrombogenic potential, due to their similar transvalvular flow gradients (21).

Another important mechanism leading to thrombosis is surface–induced thrombosis, which has been well described in mechanical valves and other medical devices. The contact of prosthetic valves with blood (biomaterial-blood interaction) triggers a thrombogenic process that involves: (1) adhesion of platelets via surface-adsorbed plasma proteins, like lipoproteins, fibrinogen, fibronectin, von Willebrand factor (VWF) or laminin. (2) Activation of the "Contact Activation Coagulation System" via negatively charged surfaces activating Factor XII (FXII). (3) Activation of the "Extrinsic Coagulation System" via adhered microparticles containing Tissue Factor (TF), released by several activated cellular components, like activated leukocytes. (4) Adhesion of leukocytes, in particular neutrophils and neutrophil extracellular traps (NETs), leading to inflammatory reactions, which promote platelet capture and aggregation. (5) Activation of complement via FXII, which further amplifies the coagulation cascade. All these events result in thrombin generation, activation of platelets and formation of platelets-fibrin networks on the prosthetic surface. The fate of such thrombus would be to obstruct blood flow in the place that it was generated or to detach and enter the circulation. To counteract thrombus formation, macrophages can infiltrate the thrombus for the clearance of NETs and provide plasminogen activator, important for fibrinolytic processes (22).

With the more frequent use of transcatheter aortic valves it is becoming important to understand the pathological processes and the triggering mechanisms associated with thrombosis of such valves. In fact, thrombo-embolic events have been reported in TAVI patients especially in the first 3 months post-procedure. One hypothesis is that, since the native valve is not removed, but left in place during TAVI, the leaflets of the stenotic native valve are still rich in TF, which exacerbates platelet activation (2).

Calcification

Calcification occurs more on bioprosthetic valves than on mechanical valves. Bioprosthesis are made of glutaraldehyde-fixed porcine valve cusps or bovine pericardium, composed of devitalized cells valvular interstitial cells (VICs) or fibroblast from porcine or bovine tissues, respectively, embedded in an extracellular matrix of collagen, elastin, and glycosaminoglycans (GAGs). Glutaraldehyde is the primary cause of calcification (23). Although the pathophysiology of valve mineralization is poorly understood, collagen and elastin fibers can serve as nucleation sites for calcium phosphate minerals. Moreover, calcium phosphate minerals have also been observed at the membrane of devitalized VICs (24). The mechanism of formation of calcium deposits in devitalized cells is probably due to calcium influx from the surrounding area of the cells to the inside of cells. The consequence is the formation of hydroxyapatite by reaction of such Ca^{2+} with free phosphate groups derived from membrane's phospholipids.

Procoagulant actors, such as phosphatidylserine-exposing activated platelets and TF-expressing immune cells or microparticles, lipid accumulation and inflammation may also play a role in calcification. However, the relationship between bioprosthesis calcification, lipids, inflammation, and thrombosis has never been established. Whether thrombosis promotes calcification, and/or *vice versa* is unknown.

Outcomes: Challenges and Solutions

Considering all complications of prosthetic aortic valves, there is an urgent need to improve their design, biocompatibility and durability (25).

The development of a prosthetic aortic valve is a very complex matter, achieved with teams of chemists, bioengineers and medical doctors. A prosthetic aortic valve to be clinically safe and durable has to comply to many regulations, pass extensive *in vitro* testing, preclinical studies in animal models (pig or sheep) (**Table 1**) and clinical trials (26).

Biocompatibility and haemocompatibility of the material of the valves is crucial and has to follow the ISO 10993 guidelines. The *in vitro* tests should evaluate the effect of the prolonged contact of the prosthetic valve surface with whole blood at 37°C under shear stress. Lysis of red blood cells can be measured using Lactate Dehydrogenase (LDH) activity, while flow-induced platelet activation can be studied in a perfusion chamber or in a cone and plate device.

An important parameter to determine is the clotting time of plasma that has been in contact with the biomaterial. Such test, if performed using specific inhibitors, allows the discrimination between intrinsic and extrinsic pathways of coagulation, which is important if we want improve prosthetic valve surfaces. Other important tests are cell toxicity as well as immunogenicity of the biomaterial of the valves. The latter evaluated is a measurement of complement (C5a and C3a) activation.

Anti-fouling properties refer to capacity of the material repulse bacteria or other microorganisms. With an anti-fouling biomaterial, microorganisms are not able to adhere and form biofilms of the surface

TABLE 1 | *In vitro* and *in vivo* tests in prosthetic aortic valve development.

Biocompatibility	ISO 10993 tests
Infection	Anti-fouling tests (ISO 14160)
Hemodynamics	Pulse Duplicator (ISO 5840)
Durability	Durability Testers + Shelf life testing (ISO 5840–1 Annex G, H, I, J)
Calcification	*In vivo* animal models 20 weeks (ISO 5840–2)

of the implanted prosthesis. Biomaterials with anti-fouling properties would avoid colonization and accumulation of microorganisms on the surface of the valve (27). Examples of anti-fouling surfaces are poly(ethylene glycol) PEG, oligo(ethylene glycol) or zwitteronic species (28).

Geometry/Design of prosthetic aortic valves is of crucial importance to retain similar hemodynamic properties of native valves. Despite years of studies on the geometrical design of mechanical valves, the super-physiological shear stresses leading to valve deterioration, thrombosis and to a lesser extend calcification, are still detected with this kind of valves. Usually, hemodynamics of prosthetic valves are first tested *in silico*, using numerical simulations, like 2D computational fluid dynamic (CFD) or, more recently, 3D fluid-structure interaction (FSI) simulations. Hydrodynamic performance of a prosthetic valve is then evaluated *in vitro* using a Pulse Duplicator (ISO 5840:2005).

Durability is a critical issue, especially with bioprosthetic valves. An ideal bioprosthetic valve should be like a native valve, extremely durable, going through 40 million cycles a year and 3 billion during a life-time. In native valves durability and strength is given by the flexibility and heterogeneity of the supportive structures (collagen, connective tissue and elastin) and cells (Valvular Endothelial, VECs, and Interstitial cells, VICs). Bioprosthetic valves are far from having similar durability, making this issue an important point to improve for the next generation of bioprosthetic valves. Durability or prolonged accelerated wear testing is mandatory. Prosthesis durability testers can simulate 10 years of valve usage in 6 months.

Sterility is fundamental for implantable medical devices. Sterility is evaluated using the Sterility Assurance Level (SAL), which represents the probability of a single viable microorganism occurring on an item after sterilization. While this probability can be reduced to a very low number, it can never be reduced to zero. Accepted SAL values are 10^{-3} and 10^{-6} for non-implantable device and implantable device respectively. The methods used to sterilize are ethylene oxide, radiation (gamma rays), ozone or addition of antibiotics. It is important to choose the right sterilization method, as it can affect SVD.

CONCLUSIONS AND FUTURE PERSPECTIVE

Although extensive *in vitro* and *in vivo* testing is done prior to releasing a prosthetic valve on the market, prosthetic valve thrombosis, as well as infection and calcification, cannot be avoided.

Several solutions have been proposed to mitigate calcification, like chemical anticalcification agents like deritatives of ammino oleic acid (AOA). Such delipidating agent has been proven effective in removing membrane-bound phospholipids derived from devitalized cells and in reducing calcification (29).

Moreover, alternatives to glutaraldehyde fixation, which is the most used cross-linking agent, have also been proposed (dye-mediated photofication,carbodiimide-based fixation). In fact, glutaraldehyde residues in the bioprosthesis have been implicated in calcification and lack of endothelization (29).

Prevention of prosthesis failure could be achieved with the new generation of smart heart devices, capable of auto-detecting their status or by measuring specific markers in plasma that could predict prosthetic valve failure. For bioprosthesis, for example, several markers have been identified as predictors of SVD: the ratio apolipoprotein B and A-I (apoB/apoA-I) (30); Lipoprotein-associated phospholipase A2 (Lp-PLA) (31); and the ratio of oxidized low-density lipoprotein and high-density lipoprotein (OxLDL/HDL) and proprotein convertase subtilisin/kexin 9 (PCSK9) levels (32).

Lots of hopes lie in Heart Valve Tissue Engineering (HVTE), involving *in vitro* coating of a matrix with appropriate cell types. The matrix can be biodegradable or not and the cell types can be stem or progenitor cells, autologous or allogenic (10). The idea is to develop heart valve substitutes containing living cells able to actively respond and adapt to surrounding mechanical stresses, mimicking more closely the complex functions of native valves (33, 34).

Another alternative is Polymeric Heart Valves (PHV), primarily made of polyurethane (PU-PHV) (35). The geometry of such valves is better controlled (trileaflets) for optimal durability and hemodynamics. Since PU-PHVs are not made of animal tissue, they are safer and less expensive and could be used in TAVI, due to their flexibility. On the other hand, the creation of a flexible polymeric material that can withstand aortic valve flows has proven challenging and resulted in many failures.

To solve geometry issues, like PPM, the latest technologies use stereolithographic 3D printing of models based on X-ray computer tomography (CT) scans of native valves (36). Using this technology it becomes possible to produce a tailor-made prosthetic valve, made of tissue or polymers that would mimic closely the native valve with a minimal impact.

AUTHOR CONTRIBUTIONS

All authors listed have made a direct contribution to the manuscript and have approved it for its publication. LM wrote the manuscript. PL, NJ, AH and AN provided intellectual contributions and edited the manuscript. CO drafted and revised the manuscript.

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frontiers in Cardiovascular Medicine

CASE REPORT
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Complete Resolution of a Large Bicuspid Aortic Valve Thrombus with Anticoagulation in Primary Antiphospholipid Syndrome

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Native aortic valve thrombosis in primary antiphospholipid syndrome (APLS) is a rare entity. We describe a 38-year-old man who presented with neurological symptoms and a cardiac murmur. Transthoracic echocardiography detected a large bicuspid aortic valve thrombus. Laboratory evaluation showed the presence of antiphospholipid antibodies. Anticoagulation was started, and serial echocardiographic studies showed complete resolution of the aortic valve vegetation after 4 months. The patient improved clinically and had no residual symptoms. This report and review of the literature suggests that vegetations in APLS can be treated successfully with conservative treatment, regardless of their size.

Keywords: antiphospholipid syndrome, aortic valve, thrombosis, anticoagulation, echocardiography

INTRODUCTION

Antiphospholipid syndrome (APLS) is a systemic autoimmune disorder characterized by the presence of antiphospholipid antibodies (aPLs) and clinical features, mainly arterial and/or venous thrombosis and/or fetal loss. APLS can be classified as primary in the absence of another autoimmune disease, or as secondary in the presence of an underlying disorder, most commonly systemic lupus erythematosus. aPLs have been found in around 5% of the general population (1); however, only a small proportion will develop APLS. APLS has been estimated to have an incidence of 5 new cases per 100,000 people per year, and a prevalence of 40–50 cases per 100,000 people per year (2). According to the Sydney criteria (3), APLS is diagnosed based on the presence of at least one clinical event (either a vascular thrombosis and/or adverse obstetric event), and the presence of aPL [either anticardiolipin (aCL), lupus anticoagulant (LA), or anti-β2 glycoprotein-1 (anti-β2GP1)] on two or more occasions, with a minimum 12-week interval. Several clinical features associated with APLS have not been included in the Sydney criteria (3). These features include aPL-associated cardiac valve disease (ACVD), nephropathy, livedo reticularis, and thrombocytopenia.

Antiphospholipid syndrome significantly impacts the cardiovascular system. ACVD, presenting with a valvular mass and/or valvular thickening, is often encountered in APLS. Approximately one-third of patients with primary APLS exhibit ACVD (4). The most commonly affected valve is the mitral valve, followed by the aortic valve (5), with regurgitation being the most common functional abnormality (6). These valvular lesions are usually of minor hemodynamic significance, but have been associated with serious thromboembolic events.

There is no general consensus on the definitive treatment of ACVD. Popular regimens used for the treatment of ACVD include the following: warfarin, antiplatelet agents, and low-molecular-weight heparin (LMWH). The efficacy of anticoagulant therapy on valvular masses is controversial. Some believe that ACVD valvular masses are due to inflammation and thus anticoagulation would be ineffective (7), whereas others were successful with anticoagulation in the treatment of these lesions (8). A small minority of APLS patients (4–6%) develop a valve disease that is severe enough to require valvular surgery (9). However, surgical patients had a higher rate of complications, mostly bleeding and thrombosis (10).

Bicuspid aortic valve (BAV) is the most common congenital heart disease, affecting 1–2% of the population with a higher prevalence (2:1) in males (11). Individuals with BAV have a potential risk of complications; the most commonly being aortic stenosis, aortic regurgitation, aortic dissection, and infective endocarditis (IE). Aortic valve thrombosis in the setting of BAV is a rare complication, and only few cases have been reported (12).

To the best of our knowledge, complete resolution of a large bicuspid aortic mass with anticoagulation in the setting of APLS has not been reported in the medical literature. Therefore, this communication explores this rare phenomenon with a review of the literature.

CASE REPORT

A 38-year-old man was referred to us, by his primary care physician, for evaluation of possible aortic valvulopathy. He is known to have dyslipidemia; for which he was not taking any medications. He is a heavy smoker (45 pack-year), drinks alcohol occasionally, and denies drug-use.

Six months before presentation, he started having short episodes (<10 min) of left-arm numbness and weakness with headache and dizziness. He also reported having dyspnea upon exertion. One month prior to his presentation, the patient was hospitalized due to the exacerbation of his clinical symptoms, in addition to a 2-h episode of ataxia and diplopia. Initial workup included computed tomography (CT) and magnetic resonance imaging of the brain, electroencephalogram, and lumbar puncture; all of which were unremarkable. Patient was suspected to have simple partial seizure and atypical migraine, and was discharged on carbamazepine and prophylactic propranolol. Upon follow up with his primary care physician, the patient's symptoms did not improve and a thorough physical examination revealed a cardiac murmur in the aortic region. Based on this new cardiac finding, the patient was referred to us for further evaluation and management.

Upon presentation to our clinic, he was afebrile and hemodynamically stable. Cardiovascular examination revealed a combined systolic–diastolic murmur best heard at the second right intercostal space, suggesting aortic valve disease. The rest of the physical examination was unremarkable.

An electrocardiogram, done at presentation, revealed left ventricular hypertrophy. A transthoracic echocardiogram (TTE) showed an irregular ovoid laminated mass, 3.7 cm × 2.1 cm in size (**Figures 1A,B**). The mass was firmly attached to the aortic valve surface and exhibited no independent motion. Doppler echocardiography revealed Grade II–III aortic regurgitation and a mean gradient of 21 mmHg across the aortic valve. There was also evidence of moderate left ventricular hypertrophy and dilation with a normal ejection fraction (>55%).

Patient was then admitted to the hospital for further workup of his condition. Laboratory studies revealed a normal complete blood count, an erythrocyte sedimentation rate of 64 mm/h, a C-reactive protein of 20 mg/L, and negative blood cultures (three separate sets). Cardiac enzymes were normal and chest radiography showed no significant findings.

Hypercoagulability workup was done. It revealed the presence of aCL (IgG isotype) in serum with a titer of 205 GPL (normal level < 20 GPL) and was positive for LA; anti-β2GP1 was not tested for technical reasons. The levels of protein C, protein S, factor V Leiden, and homocysteine were normal. Serological markers for connective tissue disorders, including antinuclear antibodies, rheumatoid factor, anti-neutrophilic–cytoplasmic antibodies, anti-double-stranded-DNA antibodies, and anti-Smith antibodies were all negative. Serologies for hepatitis B, C, and HIV were negative. Furthermore, CT scans of the chest, abdomen, and pelvis were insignificant.

The diagnosis of non-bacterial thrombotic endocarditis (NBTE) in the setting of primary APLS was suspected. The patient was started on anticoagulation with LMWH and then bridged to oral warfarin; INR 2–3 was maintained. Carbamazepine and propranolol were discontinued. The patient was educated about the importance of smoking cessation and adherence to his statin therapy.

At the 3-month follow-up, only aCL level was repeated and was found to be elevated (170 GPL). Serial TTE controls showed progressive resolution of the mass, with complete regression 4 months after therapy (**Figures 1C,D**). In addition, there was regression in the aortic regurgitation to Grade I, and reduction in the left ventricular hypertrophy and dilation. Interestingly, the resolution of the valvular mass unveiled a BAV that was not previously diagnosed (**Figure 2**).

Furthermore, a cardiac CT was done to rule out APLS-induced coronary artery disease; it showed no abnormalities. The patient was maintained on warfarin, and subsequent follow-ups showed him to be clinically asymptomatic.

DISCUSSION

Cerebral involvement is prominent in primary APLS; with stroke (19.8%) and transient ischemic attack (TIA) (11.1%) being its most common clinical manifestations (13). Hughes et al. (14) reported that in young patients (<45 years), more than 20% of strokes are potentially associated with APLS. Recurrent transient episodes of visual disturbances, numbness, weakness, and dizziness can all be expressions of TIA (15). All these were present in our patient and might explain his symptoms.

Arterial thrombosis involves the brain vasculature in more than 50% of the cases, and it is the main cause of cerebral ischemia in primary APLS (16). However, an association has been reported between ACVD and central nervous system manifestations of

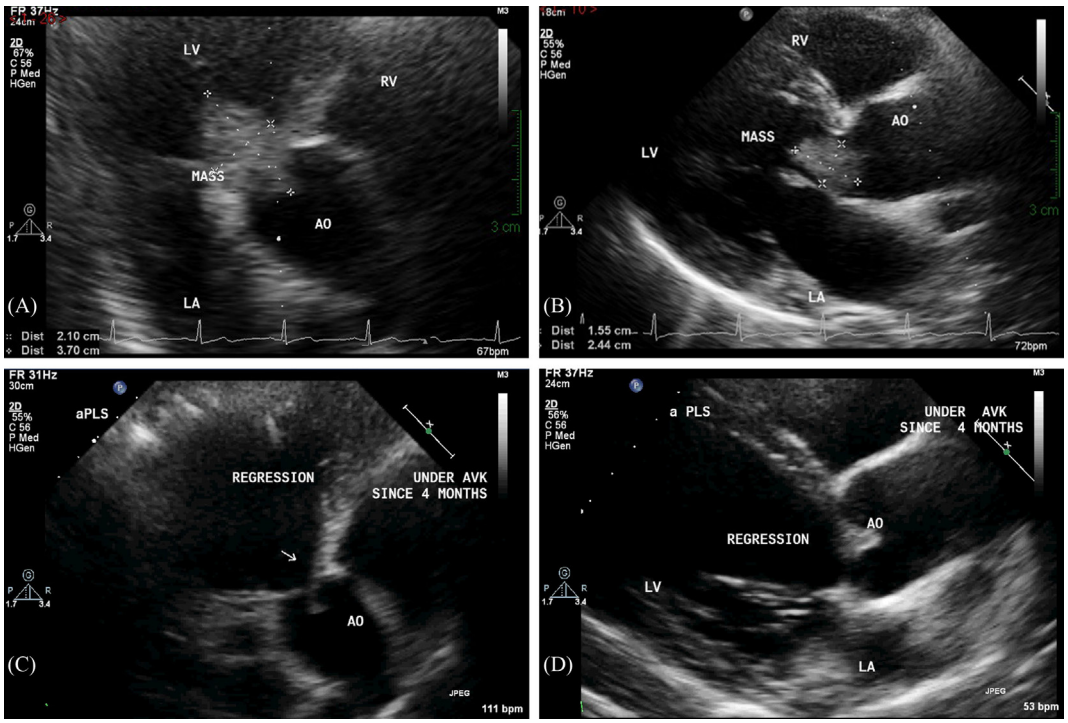

FIGURE 1 | Transthoracic echocardiography performed at presentation reveals a large ovoid laminated mass on the aortic valve, measuring 3.7 cm × 2.1 cm on apical five chamber view **(A)** and 2.4 cm × 1.6 cm on parasternal short-axis view **(B)**. Follow-up transthoracic echocardiography performed after 4 months of anticoagulation shows complete resolution of the valvular mass on apical five-chamber view **(C)** and parasternal short-axis view **(D)**.

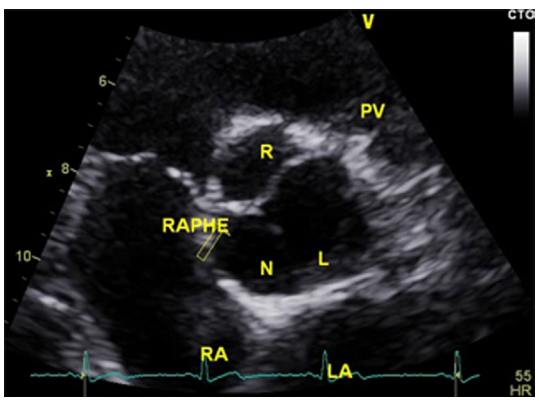

FIGURE 2 | Follow-up transthoracic echocardiography performed 5 months after presentation, showing the presence of previously undiagnosed bicuspid aortic valve.

the syndrome, which suggests that cerebral emboli from non-infectious valvular lesions, often referred to as NBTE, may be a risk factor (17).

Valvular masses have a wide differential diagnosis, which includes NBTE, IE, and cardiac tumor. It is clinically challenging to distinguish between IE and NBTE due to APLS. In fact, both share many clinical features including vascular thromboembolic events, valvular vegetations, and renal and cutaneous involvement. In addition, fever can be present in APLS, and aPL can be frequently found to be temporarily elevated during infections (18). Our patient did not satisfy the modified Duke criteria for IE. Since these criteria are sensitive for disease detection and have a high negative predictive value (19, 20), the diagnosis of IE was rejected.

Aortic valvular masses also raise the suspicion of cardiac tumor, an important differential diagnosis that should not be overlooked. The best way to diagnose a cardiac tumor is by excision and histopathologic examination. However, echocardiography can be used to distinguish between a tumor and a thrombus based on imaging characteristics of the mass. Thrombotic mass is characterized by an irregular or lobulated shape, laminated appearance, microcavitations, and absence of a pedicle (21). In contrast, a cardiac tumor usually appears as a small, mobile, pedunculated or sessile valvular, or endocardial mass (22). In our case, the characteristics of the lesion were typical of that of a thrombus.

The Sydney criteria committee proposes a minimal consensus concerning valvular lesions in APLS but argues against adoption as criteria (3). This consensus defines ACVD as the presence of aPL, in addition to echocardiographic detection of a valvular lesion and/or dysfunction (regurgitation and/or stenosis of mitral and/or aortic valve or any combination of the above) (3). In our patient, such features were present and thus swayed our diagnosis toward ACVD manifesting as NBTE.

Echocardiography is essential in the diagnosis of ACVD. About 30–40% of valvular lesions in the setting of APLS can be

detected by TTE, while 60–80% of lesions can be detected by transesophageal echocardiography (TEE) (23). TTE can be used initially to detect the presence of a cardiac mass. However, if TTE results were non-diagnostic or equivocal, TEE would be a more accurate modality due to its higher sensitivity and specificity (24).

Native aortic valve thrombosis is a rare event. In ACVD and BAV, valvular dysfunction is associated with abnormal blood flow, which can induce endothelial lesion and trigger thrombus formation (25). Furthermore, coagulopathy in APLS can induce aortic valve thrombosis. This is possibly due to particular affinity of aPL to valve endothelium that leads to formation of an immune complex, which can cause an injury to the endothelium (26). Therefore, we cannot be certain about the exact role that each of ACVD and BAV played in the pathogenesis of the thrombotic mass observed in our case.

In terms of treatment, there have been no set guidelines for the definitive treatment of ACVD. Similar to other reports (27–29), our case has shown anticoagulation to be effective in treating valvular vegetation in primary APLS. The optimal intensity of anticoagulation for the prevention of recurrent thrombosis in patients with APLS is uncertain. Two randomized controlled trials found that high-intensity anticoagulation (INR > 3) was not superior to moderate-intensity anticoagulation (INR 2–3) in patient with APLS, and was associated with a higher rate of bleeding complications (30, 31). Therefore, we suggest the use of anticoagulation with an INR target of 2–3 as a standard of treatment. In addition, most specialists recommend lifelong use of anticoagulation due to the high recurrence rate of thrombotic events in APLS (32, 33). Modification of concomitant risk factors for thrombosis, such as hypertension, dyslipidemia, and smoking cessation, must also be addressed.

In conclusion, our report suggests that conservative treatment with anticoagulation along with vigilant observation might be the best therapeutic plan for patients with aortic valvular masses in the setting of APLS. However, these results should be approached with caution as whether conservative management with anticoagulant or aortic valve replacement ought to be recommended remains unresolved due to the rarity of this condition and the lack of trial data.

ETHICS STATEMENT

This case report was exempted from any ethics committee verification due to its retrospective nature. The echocardiographic images and case presentation were approved by the patient to be used for publication.

AUTHOR CONTRIBUTIONS

All authors contributed to the analysis and interpretation of data, wrote the manuscript, approved the final version of the manuscript, and agreed to be accountable for all aspects of the work.

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SYSTEMATIC REVIEW
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Predicting Disease Progression and Mortality in Aortic Stenosis: A Systematic Review of Imaging Biomarkers and Meta-Analysis

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Background: Detecting among patients with aortic stenosis (AS) those who are likely to rapidly progress, yet potentially benefiting from prophylactic aortic valve replacement, is needed for improved patient care. The objective of this study was to evaluate the role of imaging biomarkers in predicting the progression to clinical symptoms and death in patients with AS.

Methods: We searched the Pubmed and the International Clinical Trials Registry Platform databases for studies including patients with AS, and investigating imaging techniques, published in any language until Jan 1, 2018. Eligible sets of data include effect of imaging biomarkers relative to: (1) Overall mortality, (2) Cardiac mortality, and (3) Overall events (Symptom onset and Major Adverse Cardiovascular Events). Meta-analysis was used to examine associations between the imaging biomarkers and outcomes of AS using Random Effect models.

Results: Eight studies and 1,639 patients were included after systematic review. Four studies investigated aortic valve calcification (AVC) whereas the remaining investigated biomarkers provided by cardiac magnetic resonance (CMR). Four articles investigated the presence of midwall fibrosis on late-gadolinium enhancement imaging, three reported its extent (LGE%) and two, the myocardial extracellular volume (ECV). By decreasing strength of association, there were significant associations between cardiac mortality and LGE% [Relative Risk (RR) = 1.05, 95% Confidence Interval (CI) 1.01–1.10]; overall mortality and AVC (RR = 1.19, 95%CI: 1.05–1.36); overall events and ECV (RR = 1.68, 95%CI: 1.17–2.41); cardiac mortality and midwall fibrosis (RR = 2.88, 95%CI: 1.12–7.39).

Conclusion: AVC and myocardial fibrosis imaging biomarkers predict the outcomes in AS, and help understanding AS pathophysiology and setting therapeutic targets.

Keywords: aortic stenosis, meta-analysis, imaging biomarker, myocardial fibrosis, remodeling, calcification

INTRODUCTION

Degenerative aortic stenosis (AS) is one of the most common valvular diseases, affecting up to 6% of subjects over 75 years old in developed countries (1). AS progresses with time in such a way that the only effective treatment is surgical or transcatheter aortic valve replacement (AVR). In the recent years, it has been growingly acknowledged that AS encompasses a wide spectrum of pathways in response to the progressive obstruction of the left ventricular (LV) outflow. These include first adaptive responses such as LV concentric hypertrophy that relieves the wall stress in response to LV overload, then maladaptive responses such as myocardial ischemia and fibrosis that eventually lead to myocardial dysfunction and cardiac output failure (2, 3). The concurrent progresses in computed tomography (CT), cardiac magnetic resonance imaging (CMR) and positron emission tomography (PET) have given rise to imaging biomarkers allowing quantification of the structural remodeling of both the aortic valve and the underlying myocardium (4). On a clinical view, the current indications for AVR are severe AS (peak aortic jet velocity $\geq$4 m/s, mean transvalvular pressure gradient $\geq$40 mm Hg, aortic valve area (AVA) $\leq$1.0 cm2 or $\leq$0.6 cm^2/m^2) causing clinical symptoms, or a decreased LV ejection fraction (<50%) (5, 6). Nevertheless, intervening too late in the disease course (i.e., when adverse remodeling and fibrosis processes have become irreversible) is associated with poor post-operative outcomes (7). Even with severe AS, the symptoms may be difficult to unmask in aged patients, as almost one half report no symptom at the time of diagnosis (8). There is therefore a need to detect from clinical, biological, and imaging tests, patients with AS who are likely to rapidly progress to symptoms, yet potentially benefiting from AVR. Several imaging biomarkers have been or are currently being considered at different levels of evidence to stratify the risk in asymptomatic severe AS. The objective of this study was to determine which imaging biomarkers (derived from CT and CMR) were associated with the prediction of AS progression to clinical symptoms and death.

METHODS

We carried out a systematic review in accordance with the PRISMA guidelines, following a protocol in accordance with the PRISMA-P statement (9). The online free database Medline (via PubMed) was searched for eligible articles. The date of the last search was January 1, 2018. The International Clinical Trials Registry Platform was searched for ongoing studies. The literature search was performed with assistance from an experienced librarian. The search strategy combined four sets of search terms (keywords), in accordance with the "Patient-Intervention-Control-Outcome" methodology. The first set of keywords defined AS (i.e.: aortic valve stenosis…), the second defined imaging techniques (i.e.: Computed Tomography, Electron-Beam Tomography, Magnetic Resonance Imaging, Positron Emission Tomography …), the third defined remodeling processes (i.e.: calcification, hypertrophy, fibrosis, ischemia…), and the fourth defined the clinical outcomes [i.e.: death, mortality, cardiovascular events (decompensation, edema, angina), progression, onset, survival…]. All keyword searches were combined to subject heading searches when appropriate. The full search strategy is provided in the **Supplementary Table 1**. Only original papers, clinical trials and studies, controlled and observational trials, with available full-text in English were included. Studies were eligible if they included only adult patients with AS, and investigated at least one diagnostic imaging technique focusing on the calcific remodeling of the valve, myocardial microvascular obstruction, myocardial fibrosis. Studies that did not relate the imaging results to AS progression or mortality were not included. Reports of pilot studies describing fewer than five patients were excluded.

Data Extraction

Two reviewers with experience in cardiovascular imaging assessed in consensus all titles and abstracts for relevance and eligibility. The full text of potentially relevant articles was retrieved. If full text articles were not available, the corresponding authors were contacted. Reference lists from included articles were searched for other relevant articles. The reviewers extracted and processed the data in standardized extraction forms. Corresponding authors were contacted for additional information if data were unclear or incomplete. Items included the last name of the first author and year of publication, study design, objective, sample size, inclusion, and exclusion criteria, patient characteristics, AS grade, follow-up period, funding source, technical aspects of imaging modalities, methods of measurement, interpretation of imaging results, and quantitative imaging results, measure of effect sizes [as relative risk (RR)].

Risk of Bias Assessment

Study quality and risk of bias was assessed using Cochrane Collaboration's tool for assessing risk of bias (10). Ten specific bias domains were used in the form of answering pre-specified questions about the methods reported by each study in relation to the risk domain, such that the conclusion is either "no" ("–", indicating high risk of bias), "not reported" ("NR", indicating unclear risk of bias), or "yes" ("+", indicating low risk of bias).

Statistical Analysis

Three outcomes of AS were considered for this review: (1) Overall mortality, (2) Cardiac mortality, and (3) Overall events [Symptom onset and Major Adverse Cardiovascular Events (MACE)]. Meta-analyses were done using the *metagen* function from the R package *meta* (11, 12). For each study considered, measures of effect were represented as RR and its corresponding 95% CIs. Conversion of effect sizes were done using the approach of Borenstein et al. (13) where RRs were not reported directly. Biomarkers with more than one effect measures in a study were combined using fixed effect model. The strength of association of each biomarker with the outcomes were quantified by pooling the RRs provided by the original studies using either Fixed or Random effect models and the results are represented as forest plots. Statistical heterogeneity between studies was assessed using the I^2 and Tau^2. To investigate publication bias, funnel plots were produced in addition to the

use of Egger's regression test. In order to rank biomarkers with respect to effects on outcomes, the average RRs and measures of variability were converted to odds ratios (ORs) using the formula at the **Supplementary Information 1**. These ORs were later on transformed to Hedges' g, a common index of effect size, as stated elsewhere (13). With the use of the normal-normal hierarchical model (NNHM), a Bayesian random effect model was implemented using the *bayesmeta* package in R for evaluating the strength of association for each biomarker with the outcome. A normal prior was used for the overall mean whereas a half student-t was used as prior for the measure of heterogeneity among the effect sizes of each biomarker. Posterior predictive *P*-values (PPPV) were computed using 1,000 Monte Carlo sampling. Then, sensitivity analysis for ranking of biomarker effects was done by fitting a consistency random effect network meta-analysis (NMA) assuming a common reference group for each biomarker effect. Hedges' g was computed for each study separately before being combined in the NMA (**Supplementary Figure 1**). The appropriate NMA model was conducted in OpenBugs using 2 chains with different starting values and a burn-in of 50 k after 500 k iterations. Convergence of the model was assessed using history and density plots

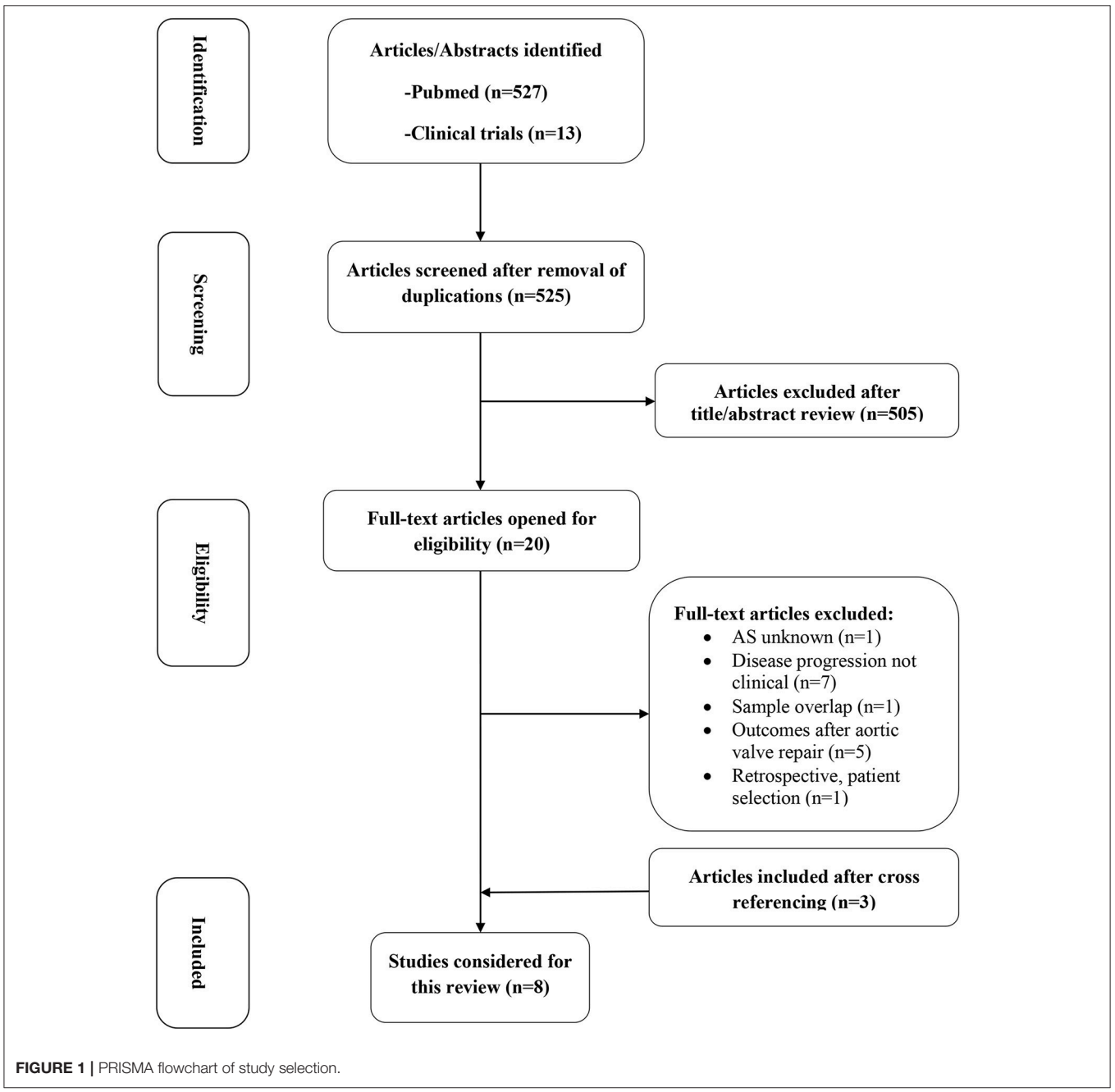

FIGURE 1 | PRISMA flowchart of study selection.

(**Supplementary Figures 3**, **4**). For each Marcov Chain Monte Carlo (MCMC) run, each biomarker is ranked using the absolute value of the Hedges' g. Probabilities of being the first, second and up till the last are estimated and represented on a Cumulative probability plot. These probabilities are used to estimate the surface under the cumulative rank (SUCRA) curve which determined the strength of the biomarker with the outcome. All analyses were done using R studio (R version 3.4.2) and a *p*-value of <5% was considered statistically significant.

RESULTS

The search strategy identified 540 citations (**Figure 1**). After screening of titles and abstracts, twenty articles were selected for full-text review. After full-text review, one study was excluded because it didn't report the patient status regarding AS upon inclusion (14). Seven studies were excluded because they used different endpoints than clinical outcome to investigate imaging results, or mixed (clinical and imaging) data to determine the outcome of AS (15–21). One other study (22) was excluded because it briefly reported the 5-year follow-up of a cohort assessed previously by Dweck et al. (23). Five studies were excluded as they report the outcomes of patients regarding imaging results, after AVR (24–28). One study was excluded because it was retrospective and evaluated only a subset of patients with low-gradient and low-flow (29). Three additional studies were found through cross-referencing (30–32). Subsequently, eight articles were included in this systematic review (**Supplementary Table 2**). All included studies were prospective and their sample sizes ranged from 34 to 794 patients, with a total number of 1,639 patients in this review.

The patient and AS characteristics are summarized in **Table 1**. Four articles investigated aortic valve calcification (AVC) with electron-beam CT (EBCT) (33) and conventional photon multislice CT (MSCT) (30, 31, 34). Four studies investigated LV myocardial fibrosis using late-gadolinium-enhancement (LGE) CMR; all investigating midwall replacement fibrosis (23, 32, 35, 36). Three of these studies investigated replacement fibrosis quantification (LGE%) (32, 35, 36), and two studies evaluated interstitial fibrosis via the extracellular volume (ECV) measurement (35, 36). Lastly, single CMR studies investigated respectively the myocardial perfusion reserve (MPR) as a marker of microvascular dysfunction in AS (36), and native (unenhanced) T1 value as a marker of myocardial fibrosis (32). Included articles reported patient cohorts from Europe, United Kingdom, USA, Canada and South Korea. The overall findings of the risk of bias assessment were: a low risk of selection attrition and outcome reporting bias, and varied risk of detection and commercial bias as the outcome adjudication blinding was nearly systematically unreported, and some investigators related to the industry in four studies (23, 32, 35, 36). All the studies recorded are of moderate-to-low risk of bias with overall quality of 50% or more (**Supplementary Table 3**).

Meta-analyses were restricted to the biomarkers that were reported in at least 2 studies. As listed in **Table 2**, there were variations in study outcomes and studies with several outcomes

TABLE 1 | Patient demographics and characteristics.

References	Study design	Follow-up (years)	N	Age (years)	Male *n* (%)	BAV (%)	AS grade (%)	Peak aortic velocity (m/s)	AVA (cm^2)	Pmax (mmHg)	Pmean (mmHg)
Messika-Zeitoun, et al. (33)	Prospective	2	100	70	58 (58%)	11	Moderate = 71 Severe = 29	2.8	1.8	NR	NR
Feuchtner, et al. (30)	Prospective	1.5	34	70.5	20 (67%)	0	NR	NR	NR	56	36
Dweck, et al. (23)	Prospective	2	143	68	97 (68%)	NR	Moderate = 40 Severe = 60	NR	0.99	70	NR
Utsunomiya, et al. (31)	Prospective	2.4	64	74	28 (44%)	0	Moderate = 55 Severe = 45	3.75	1.14	NR	29
Clavel, et al. (34)	Prospective	3.1	794	73	520 (65%)	NR	Moderate = 57 Severe = 43	3.7	1.10	NR	35
Chin, et al. (35)	Prospective	2.9	203	69	115 (69%)	NR	Mild = 17 Moderate = 22 Severe = 43	3.8	1.0	NR	35
Singh, et al. (36)	Prospective	1	174	66.2	133 (76%)	NR	Moderate = 29 Severe = 71	3.86	0.57	NR	35.4
Lee, et al. (32)	Prospective	2.3	127	68.8	63 (50%)	NR	Moderate = 38 Severe = 62	4.4	0.82	NR	48

N, sample size; AS, aortic stenosis; BAV, bicuspid aortic valve; AVA, aortic valve area; P_{Max}, maximal transvalvular pressure gradient; P_{Mean}, mean transvalvular pressure gradient; NR, not reported; LGE, late-gadolinium enhancement.

TABLE 2 | Associations between imaging biomarkers, effect size (Variability), and outcomes in AS.

References	Imaging method	Biomarker	Outcome	RR	Measure of variability (CI, SE, P)	LnRR	SE(LnRR)
Messika-Zeitoun, et al. (33)	EBCT	AVC	OE	1.06	1.02–1.10	0.06	0.01
			LE	1.11	1.03–1.23		
			ASE	1.05	1.01–1.09		
Feuchtner et al. (30)	MSCT	AVC	MACE	3.18	1.64	1.16	0.49
Dweck, et al. (23)	CMR	Midwall fibrosis	OM	5.35	1.16–24.56	1.79	0.54
			CM	6.68	1.51–29.64		
		LGE %	OM	1.05	1.01–1.09	0.05	0.02
Utsunomiya, et al. (31)	MSCT	AVC	OE	1.09	1.04–1.15	0.09	0.03
Clavel, et al. (34)	MSCT	AVC (severe)	OM	1.75	1.04–2.92	0.73	0.16
		$AVC_{density}$ (severe)	OM	2.44	1.37–4.37		
		AVC	CM	2.14	1.08–4.45		
		$AVC_{density}$	CM	2.28	1.11–4.95		
Chin, et al. (35)	CMR	Midwall fibrosis	OM	8.88		2.18	0.5
		ECV		4.50		1.50	0.5
Singh, et al. (36)	CMR	LGE %	OE	1.06	1.30	0.06	0.26
		ECV		1.43	1.30	0.36	0.22
		Midwall fibrosis		1.16	0.23	0.15	0.23
		MPR		0.62	0.39–0.97		
Lee, et al. (32)	CMR	Midwall fibrosis	OE	1.56	1.05–4.37	0.44	0.36
		LGE %		1.19	1.07–1.90	0.17	0.15
		Native T1		4.45	1.52–12.95	1.49	0.55

AVC, aortic valve calcification; EBCT, electron-beam computed tomography; MSCT, multisclice computed tomography; LGE, late-gadolinium enhancement; ECV, extracellular volume; MACE, major adverse clinical event; OM, overall mortality; OE, overall events; LE, late events; CM, cardiac mortality; ASE, aortic stenosis related-event; CI, confidence interval; RR, relative risk; SE, standard error; Ln, neperian logarithm.

for a biomarker was pooled into one using fixed effect model. The primary outcome was all-cause mortality in three studies (23, 34, 35), and a softer endpoint including cardiac- or AS-related mortality, MACE, AS-related symptoms or AVR in the remaining. Results from the meta-analyses confirmed significant associations between AVC and Overall mortality (RR = 1.19, 95%CI: 1.05–1.36); Midwall Fibrosis and Cardiac mortality (RR = 2.88, 95%CI: 1.12–7.39), LGE percent and Cardiac mortality (RR = 1.05, 95%CI: 1.01–1.10); ECV and Overall events (RR = 1.68, 95%CI: 1.17–2.41) (**Figure 2**). For all the biomarkers, higher values are associated with higher risks of having outcomes of AS, but substantial inconsistency of effect was observed for AVC, and midwall fibrosis with both $\mathbf{I^2}$ >75%. Because of the limited number of studies, assessment of and further correction for bias could not be sufficiently ascertained. Ranking of biomarkers in order of decreasing strength of association with the outcomes resulted in LGE% (PPPV <0.0001) being at the top and midwall fibrosis (PPPV = 0.456) at the bottom (**Table 3**). Similar rankings were observed using the SUCRA values from the NMA (**Supplementary Figure 2**).

DISCUSSION

This systematic review and meta-analysis showed significant associations between imaging biomarkers of aortic valve remodeling and myocardial fibrosis and clinical outcomes in patients with AS. Five years after receiving the diagnosis, approximately two-thirds of conservatively managed patients with asymptomatic AS will develop symptoms, and 75% will have either died or undergone AVR (37). During this time period, it is questionable in how far they would not exhibit raised biomarkers of poor outcome before developing clinical symptoms, altered hemodynamic or performance status. Imaging biomarkers with prognostic value in AS are often correlated with hemodynamic and clinical performance. The aortic valve calcium (AVC) score for instance is recommended in the management of patients with AS, not as a prognostic factor, but to determine the likelihood of severe AS in case of low-gradient, low-flow and preserved LVEF (6), due to its association to AS severity (16, 34). The findings of our analysis advocates for an additional prognostic use this score, as all four articles investigating AVC reported association with mortality; with an overall RR of 1.19, 95%CI: 1.05–1.36) (30, 31, 33, 34). Even though only the largest among these studies (34) introduced $AVC_{density}$ to compensate for differences in aortic annular area, there were little measurement bias, as a highly reproducible and standardized score was systematically reported (16, 17, 38).

Similar risk stratification to AVC is expected from non-invasively assessed LV myocardial fibrosis, a fourfold potential courtesy of CMR using the effects of gadolinium-based contrast agents. These agents strongly decrease the T1 relaxation time of the tissues. As such, they can be used for track-bolus kinetics within the myocardium and assess resting and stress perfusion, thus MPR, which is potentially a marker of microvascular dysfunction (36). Gadolinium-based contrast agents distribute in the plasma and extracellular spaces, which means they do not enter normal cells. Upon equilibrium distribution (i.e.,

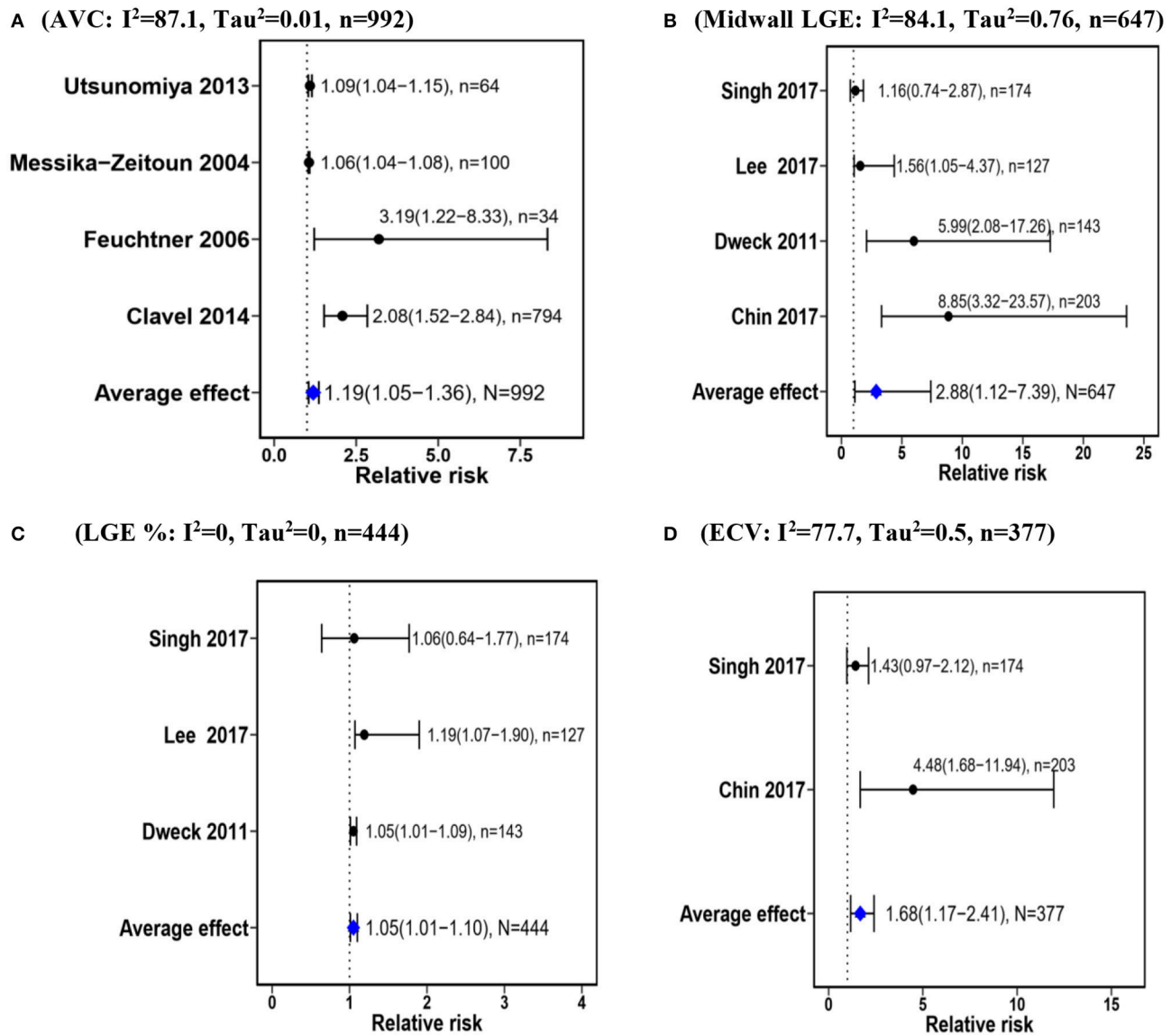

FIGURE 2 | Forest plots showing the average relative risks for the strength of association between imaging biomarkers and outcomes in AS. AVC, aortic valve calcification; LGE, late gadolinium enhancement; ECV, extracellular volume. **(A)** (AVC: $I^2 = 87.1$, $Tau^2 = 0.01$, $n = 992$). **(B)** (Midwall LGE: $I^2 = 84.1$, $Tau^2 = 0.76$, $n = 647$). **(C)** (LGE %: $I^2 = 0$, $Tau^2 = 0$, $n = 444$). **(D)** (ECV: $I^2 = 77.7$, $Tau^2 = 0.5$, $n = 377$).

10–15 min after injection), imaging thus figure the replacement of lost cardiomyocyte by extracellular space expansion (fibrosis), which precludes LV decompensation and arrhythmia (39–41). The presence of midwall fibrosis on T1-weighted imaging is the second biomarker derivable from contrast-enhanced CMR. Recognizing midwall fibrosis on LGE is easy and reproducible, as only requested to differentiate from post-infarct scars that classically involves the subendocardium, and amyloid, which is uncommon. In this meta-analysis, there were contradictory findings regarding the value of midwall fibrosis as a marker of clinical outcome in AS, with overall, a moderate but significant association between midwall fibrosis and cardiac mortality (RR: 2.88; 95%CI: 1.12–7.39). The relative amount of midwall fibrosis similarly accounts for prognostic value, which represents another biomarker provided by LGE. The method of quantification of midwall fibrosis depends on patient- and contrast-specific variables such as enhancement dynamics, CMR equipment and the "density" of fibrosis (42). Various cutoffs to differentiate fibrosis from the surrounding "normal" myocardium were reported across the series analyzed, including Full Width at Mid Height and Standard Deviations from the mean signal intensity histogram. Although conflicting across the series (23, 36), the percent of LGE was overall significantly associated with cardiac mortality in AS (RR:1.05; 95%CI: 1.01–1.09). A step further, assessing interstitial fibrosis necessitates more sophisticated imaging approaches aiming at establishing the T1 relaxation time mapping of the myocardium; the so-called relaxometry. Approaches using unenhanced T1 mapping (32), post-contrast

TABLE 3 | Rank of biomarkers according to posterior predictive *p*-value.

Biomarker	RR	95 % CI	Hedges'g	95% CrI	PPPV	Rank
AVC	1.19	1.05–1.36	0.08	0.01 to 0.13	0.010	2
Midwall fibrosis	2.88	1.12–7.39	0.11	−0.34 to 0.67	0.456	4
LGE%	1.05	1.01–1.10	0.016	0.01 to 0.02	<0.0001	1
ECV	1.68	1.17–2.41	0.15	−0.06 to 0.44	0.134	3

AVC, aortic valve calcification; MF, midwall fibrosis; LGE, late gadolinium enhancement; ECV, extracellular volume; RR, relative risk; CI, confidence interval; CrI, credible interval; PPPV, posterior predictive p-value.

T1 mapping and a mix of both have been investigated and validated against the extent of myocardial fibrosis on histology, each with its own potential advantages and limitations (43). Of these, ECV and derivatives (indexed to the body surface area) were reported in this meta-analysis. ECV represents the volume of distribution of the contrast agent within the myocardium, expressed as the difference of T1 relaxation time changes after contrast administration, corrected for the volume of distribution by using the hematocrit. ECV also showed significant prognostic effect in AS patients in our review (RR:1.68; 95%CI: 1.17–2.41).

Altogether place a special emphasis on the prognostic role of CMR in AS. However, substantial inconsistency of effect was observed for the biomarkers and the cut-offs for patient stratification varied across the cohorts. Valve calcification for instance is an active process independent from the skeletal bone calcification (44), not only associated to local factors like AS severity (15–17) or valve inflammation (45, 46), but also distant influences in relationship with classical risk factors for cardiovascular disease (47). As such, the mechanisms of initiation and progression of this biomarker are neither fully elucidated nor totally predictable. This is epitomized by the fact that females have lower AVC than males even after correction for body surface area, aortic annular area and other risk factors (48), and that severe AS with low AVC is not uncommon (49). Likewise, there is variability on a patient basis regarding both stimuli and responses to myocardial fibrosis. The investigations regarding myocardial fibrosis will need similar levels of standardization as for AVC, and a greater control for the confounders for AS-induced fibrosis or myocardial dysfunction, such as coronary artery disease or myocardial steatosis (50–52). Further studies will be needed to determine the appropriate normal value ranges of above-reported biomarkers and derivatives among subgroups by age, sex, ethnicity, and underlying risk factors and comorbidities. When accounting the current variability of these biomarkers for strength of effect using network meta-analysis, midwall fibrosis, and ECV were the weakest prognostic biomarkers (PPPV 0.456 and 0.134 respectively). This is unsurprising, as midwall fibrosis is too prevalent to make a contributive difference among patient groups, as being present in up to 62% of patients with severe AS (23–25). On the other hand, ECV and its derivatives (including unenhanced T1 values, and partition coefficient) (32, 53, 54) that are potentially reversible and sensitive to earlier adverse remodeling show considerable overlaps between normal and diseased individuals (35).

The association between imaging biomarkers and patient outcome in AS raises the question of a possible paradigm shift in the management of AS. The efficacy of a biomarker-based management as compared to the current approach that relies mainly on clinical performance need to be tested by large randomized studies. Both approaches have nevertheless the potential to be complimentary. Considering this could help refining the risk assessment in severe AS where patients with good symptom/performance status and low level of relevant imaging biomarkers being at low-risk, needing no AVR, whereas those with altered symptom/performance status and high level of the same imaging biomarkers requiring AVR. Consequently, critically evaluating the benefits of AVR in intermediate-risk groups (i.e., patients with either altered symptom/performance status or raised imaging biomarker of poor prognosis) could be a major research issue in the near future.

LIMITATIONS

The aim of this review was to provide an overview of imaging biomarkers that could possibly predict clinical evolution in patients with AS. Our search revealed only a small number of studies, though there are other imaging biomarkers at earlier phases of their development. Some of these newer techniques use radiotracers (46) and others evaluate longitudinal or circumferential myocardial dysfunction (20, 27, 28), or wall stresses flow and deformation pattern changes (55). Our findings link imaging biomarkers with mortality, cardiac mortality or overall events. Nevertheless, AS-related events are often difficult to report and subject to bias. The proportion of patients with severe AS upon enrolment varies from 29 to 71 percent across the series (**Table 2**), indicating some potentially enriched cohorts, though the consecutive enrolment information missed in all but two articles (23, 33). Only one study evaluated potential selection bias via evaluation of the events that occurred >1month after enrolment (33). Most articles did not specify the blinding of the endpoint adjudicator(s). While AVR was often reported in patients who did not experience symptoms, all-cause mortality, cardiac mortality, and symptoms account for other risks than the sole severity of AS. This was underscored in the study of Clavel et al. where the survival after AVR was improved only in patients with high AVC (34).

Lastly, it should be acknowledged that: first, the prognostic value of imaging biomarkers does not necessary outperform clinical test exploring the same pathway when available. Indeed, the only study that evaluated the prognostic value of MPR as a marker of microvascular dysfunction reported a significant prediction for overall mortality (HR: 0.62; 95%CI: 0.39–0.97; $p = 0.035$), which was nevertheless not superior to that of a positive exercise testing (36). The financial burden of risk stratification using imaging biomarkers that uses sophisticated and costly imaging techniques could thus be reduced by developing more cost-efficient clinical or biological biomarkers (19, 56–59). Second, whereas the ideal biomarker for a disease should be sensitive and consistent across age, gender and ethnic groups, the current imaging biomarkers are imperfect by nature, partly due to their specificity to only one of the pathophysiological processes. Indeed, AS is a complex disease process interplaying several pathways, placing emphasis on multi-biomarker prognosis.

In conclusion, AVC and myocardial fibrosis markers are significantly associated with outcomes in AS, and have the added potential to help the understanding of AS pathophysiology and setting therapeutic targets.

AUTHOR CONTRIBUTIONS

AN literature search, article search, data extraction, and manuscript writing. JD statistical analysis, manuscript writing, revision, and summary. LD and LS literature search and manuscript revision. CO and PL study outline, revision, edition, and summary.

SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Can Blood Biomarkers Help Predicting Outcome in Transcatheter Aortic Valve Implantation?

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Transcatheter aortic valve implantation (TAVI) has become the method of choice for patients with severe aortic valve stenosis, who are ineligible or at high risk for surgery. In this high risk patient population, early and late mortality and rehospitalization rates after TAVI are still relatively high. In spite of recent improvements in procedural TAVI, and establishment of risk models for poor outcome, determining individual risk remains challenging. In this context, current data from several small studies strongly suggest that blood biomarkers of myocardial injury, cardiac mechanical stretch, inflammation, and hemostasis imbalance might play an important role by providing informations on patient risk at baseline, and postprocedural progression of patient clinical conditions from days up to years post-TAVI. Although the role of biomarkers for predicting survival post-TAVI remains to be validated in large randomized studies, implementing biomarkers in clinical practice might improve risk stratification, thereby further reducing TAVI-associated morbidity and mortality.

Keywords: TAVI, blood biomarkers, inflammation, myocardial stress, platelet, thrombocytopenia

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INTRODUCTION

Transcatheter aortic valve implantation (TAVI) has changed dramatically the treatment of severe aortic stenosis in inoperable patients or in patients at high risk for surgery. In the high risk population, particularly in the elderly, TAVI can offer a marked change in the life expectancy and quality of life of patients, and even nonagenarian patients can have successful valve replacement with acceptable periprocedural morbidity and mortality rates (1). However, early and late mortality after TAVI still remains relatively high. Results from registries and from the PARTNER trials reported 1 year all-cause mortalities between 22 and 30% (2–4). In order to improve patient evaluation and minimize futility, risk models for poor outcomes post-TAVI have been built and validated, providing Heart teams with important decision-making tools and informations (5–8). Since the prognosis of patients who benefit the most from TAVI is often not only determined by severe symptomatic aortic stenosis (AS), but also by multiple comorbidities, it would still be very useful to have parameters or biomarkers that would help to better predict the risk of major cardiovascular events for these patients.

Here, we present an overview of the role of most studied blood biomarkers for predicting poor outcome post-TAVI (**Figure 1**). Despite recent procedural advances that improved safety and flexibility of TAVI, these studies strongly suggest that biomarkers, in addition to risk scores, might

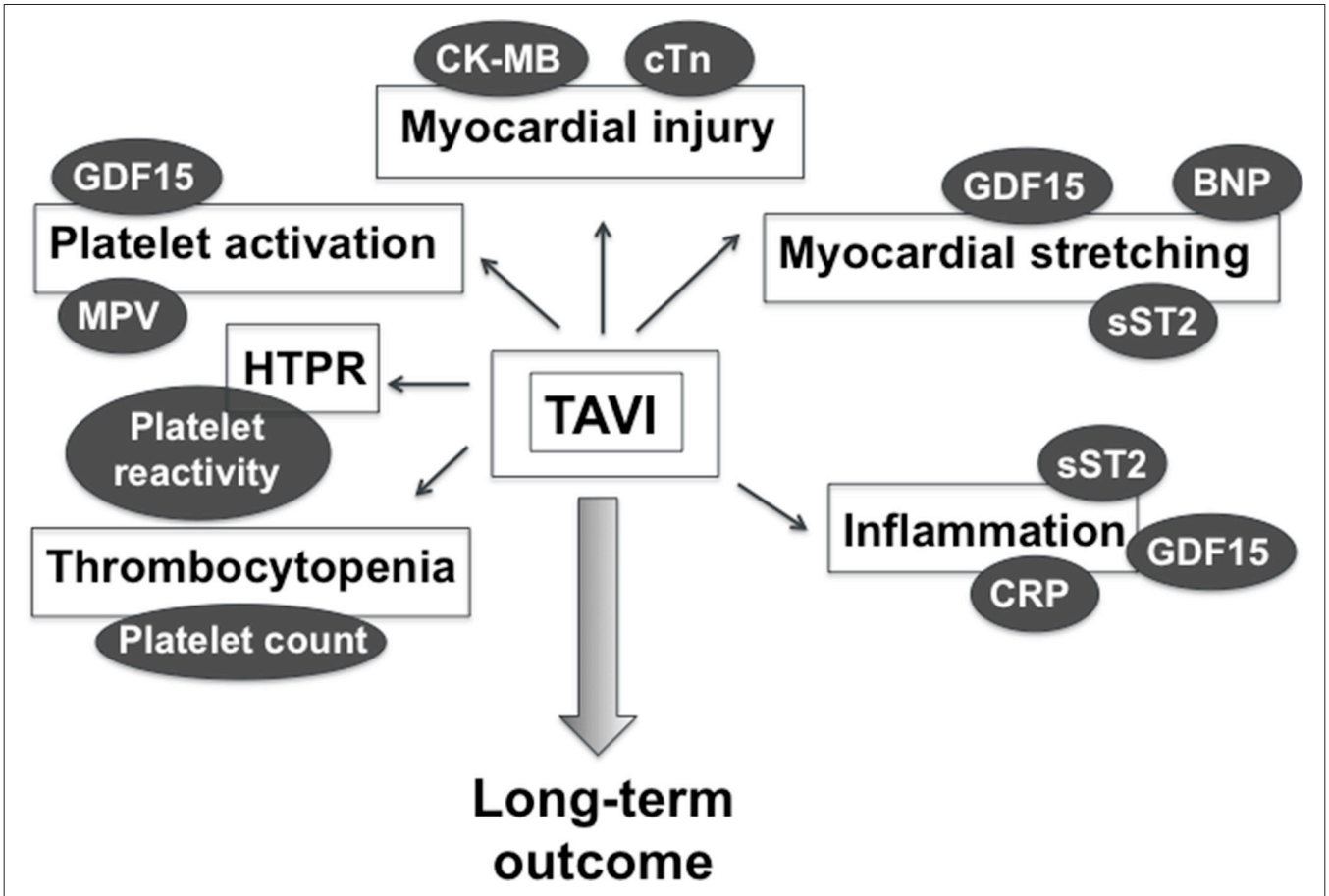

FIGURE 1 | Blood biomarkers of TAVI-related myocardial injury, myocardial stretching, inflammation, and hemostasis imbalance that might provide postprocedural prognostic information. BNP, brain natriuretic peptide; CK-MB, creatinine kinase myocardial band; CRP, C-reactive protein; cTn, cardiac troponin; GDF-15, growth differentiation factor-15; HTPR, high on-treatment platelet reactivity; MPV, mean platelet volume.

help reducing further TAVI-associated morbidity and mortality, in a more personnalized manner.

Markers of Myocardial Injury: Creatine Kinase Myocardial Band, Cardiac Troponin

Periprocedural elevation of cardiac biomarkers of myocardial injury is common in TAVI, with greater values observed following transapical or transaortic approaches compared to transfemoral (TF) approach (9). Higher levels of myocardial injury have been associated with reduced early and midterm survival following uncomplicated TAVI (10–13). Transapical (TA) procedure significantly associates with left ventricular apical fibrosis, contributing to apical wall motion abnormalities, which may, in turn, impair myocardial recovery (14).

TAVI clinical endpoints have been revisited in the current Valve Academic Research Consortium (VARC) –2 document (15), defining specific biomarker cut-off values for clinically significant myocardial infarction post-TAVI. In a large multicenter study of patients undergoing TAVI with different valve types and approaches, myocardial injury, determined by postprocedural rise in levels of creatine kinase myocardial band (CK-MB), was detected in two-third of patients undergoing TAVI, especially through transapical approach (16). Higher peak of CK-MB post-TAVI translated into impaired systolic left ventricle function at 6 to 12 months follow-up, and were associated with greater acute and late mortality (**Table 1**). Regarding cardiac troponin (cTn), correlation with patient outcome is less clear. Two small prospective studies of TF TAVI patients showed that baseline high sensitive TnT (hs-TnT) independently predicted survival in symptomatic high-risk patients with severe AS (18, 22). Post-procedural hs-TnT rose significantly after TF TAVI until day 3, which had prognostic value for 1 year mortality. Determinants of post-procedural hs-TnT were baseline renal function, duration of intraprocedural rapid spacing, as well as pre-TAVI hs-TnT values (18). Despite hemodynamic relief, cTnT levels did not normalize even after months following successful TAVI, suggesting that the prognostic value of cTn for 1 year patient outcome may rely on long-term changes in myocardial texture. A larger study indicated that cTnT elevation above VARC-2 cut-off within 12 h post-procedure was a strong independent predictor of 30 day mortality, and remained significant at 2 years (17). In disagreement with these findings, a more recent study indicated that, in contrast to CK-MB, cTn elevation above normal limit defined by VARC-2 had no impact on late mortality of patients undergoing TF TAVI (23). Notably, VARC-2 cTnI cut-off values

TABLE 1 | Proposed cut-off values of post-procedural biomarkers to predict mortality in TAVI.

Biomarker	Cut-off	Effect	References
CK-MB	>UNL (within 3 days post-TAVI)	↑30 day and late mortality in overall and non-TA TAVI	(11, 16)
	>5 × UNL* (within 3 days post-TAVI)	↑30 day and late mortality in overall and non-TA TAVI	
cTn	>15 × UNL* (within 12 h post-TAVI)	↑30 day and 2 year mortality (overall TAVI)	(17)
	≥166 pg/ml (3 days post-TAVI)	↑1 year mortality (TF)	(18)
BNP	Rise at 30 days post-TAVI	↑1 year mortality in TF TAVI	(19)
	>328 pg/ml (30 days post-TAVI)	↑1 year mortality in TF and transaxillary TAVI	(20)
	≥591 pg/ml (persistent from baseline to discharge)	↑2 year mortality in overall TAVI	(21)

*UNL = upper normal limit based on the 99th percentile values in a healthy population *according to VARC-2*
BNP, brain natriuretic peptide; CK-MB, creatine kinase-myocardial band; cTn, cardiac troponin; TA, transapical; TAVI, transcatheter aortic valve implantation; TF, transfemoral.

failed to distinguish myocardial injury from type 1 myocardial infarction (angiographically high-grade coronary artery stenoses or occlusions) in TF and TA TAVI patients, and therefore could not be used as a marker of periprocedural MI (24). Furthermore, different cut-offs may apply to TA and TF patients. These results should still be confirmed in larger randomized studies.

Markers of Myocardial Stretching: B-Type Natriuretic Peptides

Elevation of circulating B-type natriuretic peptides (BNP) that results from left ventricle myocardial stretching is commonly used in clinics to predict the onset of symptoms and adverse events in patients with severe AS (25–27).

Several studies performed on TAVI patients have assessed the value of preprocedural or serial BNP or of its biologically inactive N-terminal-proBNP (NT-proBNP) as predictors of postprocedural outcome. Initial studies found no association of baseline BNP or NT-proBNP levels and 2 month mortality after TF or TA TAVI (28, 29). A high BNP level in high-risk patients with severe AS was not an independent marker for higher mortality. These two studies showed a transient increase of BNP levels from baseline to discharge, followed by a stepwise decrease until 1 year. The authors related the transient increase in BNP to the transient left ventricle dysfunction with depression of both systolic and diastolic left ventricular (LV) function associated with TAVI (30).

In contrast, a more recent study indicated that a high preprocedural BNP, and a rise in BNP at 30 days independently predicted 1 year outcome post-TF or transaxillary TAVI (20). This result was confirmed in another study from the PARTNER trial (19) showing that an increase of BNP at 30 days was a predictor of 1 year mortality of transfemoral TAVI patients, as was moderate or severe aortic regurgitation over 1 year, and Society of Thoracic Surgeons (STS) score. Therefore, a rise in BNP at 30 days from baseline could provide prognostic information that should prompt careful clinical evaluation of these patients (**Table 1**).

Koskinas et al described an association between a high baseline BNP and a higher risk of all-cause death and cardiovascular death at 2 years, and a more frequent occurrence of VARC-2 clinical endpoints at 1 year (21). In this study, BNP levels increased or remained unchanged from baseline to discharge in 35% of patients, while these levels decreased in 65% of them. A baseline-to-discharge decrease was related to New York Heart Association functional improvement. Patients with persistently high BNP before intervention and at discharge had increased rates of death at 2 years. The same authors compared the prognostic values of BNP and NT-proBNP, revealing superiority of postprocedural NT-proBNP to BNP as a predictor of all-cause mortality at 2 years. Another study analyzed the prognostic value of preprocedural NT-proBNP ratio, defined as the ratio of measured NT-proBNP to maximal normal NT-proBNP values specific for age and gender, on short- and long-term mortality (31). The authors showed that baseline NT-proBNP ratio could predict all-cause mortality at 30 days and 1 year post-TAVI. Finally, in a later study, preinterventional levels of mid-regional (MR), pro-adrenomedullin (MR-proADM), and MR-pro-A-type natriuretic peptide (MR-proANP) and N-terminal pro-natriuretic peptide (NT-proBNP) were associated with 1 year cardiovascular events and all-cause mortality, while no association was found with 30 day outcome (32). Among most recently studied biomarkers, baseline levels of carbohydrate antigen 125 were reported to be superior to NT-proBNP to predict adverse outcome of TAVI (33).

Thus, altogether these studies depict some prognostic value of periprocedural BNP in TAVI that should be validated in larger multicenter studies in order to foster their implementation in current clinical practice.

Markers of Inflammation and Myocardial Stress

GDF-15

A prospective observational study was conducted that compared the prognostic value of risk scores (logistic European System for Cardiac Operative Risk Evaluation [EuroSCORE], EuroSCORE II, Society of Thoracic Surgeons predicted risk of mortality, and German aortic valve score) and circulating biomarkers (high-sensitivity C-reactive protein [hsCRP], growth differentiation factor [GDF]-15, interleukin-6, interleukin-8, and NT-proBNP) to predict all-cause mortality and rehospitalization during the first year after TAVI (34). Strikingly, GDF-15, a cytokine belonging to the family of transforming growth factor-β, appeared to be the best predictor of poor outcome when added to the logistic EuroSCORE and EuroSCORE II.

These results are in agreement with another study in which high preintervention GDF-15 levels were associated with reduced time survival post-TAVI, and were superior to NT-proBNP for patient risk stratification (35). Interestingly, high GDF-15 levels were significantly associated with several variables of poor outcome, such as reduced kidney function, diabetes, STS score, high

creatinine and NT-proBNP levels, and VARC-2 criteria, suggesting that GDF-15 could integrate numerous complicating factors that could contribute to poor TAVI outcome.

Among eight biomarkers measured prior to valve replacement (GDF-15, soluble ST2 [sST2], NT-proBNP, galectin-3 [GAL-3], hs-cTnT, myeloperoxidase, hsCRP, and monocyte chemotactic protein-1 [MCP-1]), Lindman et al identified a combination of elevated levels of GDF-15, sST2 and NT-proBNP as the best predictors of 1 year mortality post-TAVI (36). However, since this study included both TAVI and patients who underwent surgical valve replacement, the utility of these three biomarkers should still be evaluated in specific populations of TAVI patients.

A recent study assessed the association of preprocedural BNP, hs-TnI, CRP, GDF-15, GAL-3, and cystatin-C with LV mycordial recovery with long-term all-cause mortality. Again, GDF-15 was strongly associated with all-cause mortality, as was CRP. GDF-15 improved the risk model when added to the STS score. Though frailty has been associated with worse 1 year outcome post-TAVR, in this study, frailty alone was not superior to GDF-15 and did not significantly improve net reclassification when added to STS score. The authors also found that a lower baseline level of GDF-15 predicted improvement of global longitudinal strain (GLS) at 1 year follow-up, which may partly explain the effect on survival. Notably, GLS at baseline was not as strongly related to outcome as GDF-15 and CRP. GLS at 1 month could, however, predict 1 year mortality. In addition, this study uncovered an intriguing correlation between GDF-15 and left ventricular mass index.

Thus, baseline GDF-15 appears as a promising biomarker that could improve current risk prediction models for patients undergoing TAVI. Furthermore, these findings indicate that inflammation may play a major role in ventricular remodeling and recovery post-TAVI. Performing serial measurements of GDF-15 and CRP would thus be interesting to determine the effect of the TAVI procedure on the progression of the inflammatory process, and its impact on patient outcome.

GDF-15 has been associated with multiple cardiovascular outcomes, possibly due to its pleiotropic effects on inflammation, oxidative stress, endothelial dysfunction, myocardial stress, and aging. Of particular interest, several studies reported an association of GDF-15 with a risk of major bleeding in acute coronary syndrome patients on dual antiplatelet therapy (37, 38). However, no studies have evaluated the possible role of GDF-15 in TAVI-related bleeding events (see below), so far.

Markers of Inflammation and Myocardial Stress

Soluble ST2

sST2 is an interleukin-1 receptor family member that acts as a decoy receptor for interleukin-33, and inhibits cardioprotective IL-33/ST2 signaling (39). Released following hemodynamic stress and cardiomyocyte strains (40), sST2 accurately predicts cardiovascular outcome of patients with acute and chronic heart failure. Consequently, sST2 was introduced in the ACC/AHA guidelines for risk stratification of patients (41). Our team showed an association of sST2 with outcome in aortic stenosis (42).

sST2 levels increase during the 24 h following TAVI, probably related to periprocedular myocardial dysfunction (30). Three studies recently indicated that preprocedural soluble ST2 might have long-term prognostic value after TAVI. The first study showed an association of baseline sST2 with 1 year mortality, with no effect at 1 month (43). sST2 correlated significantly with echocardiographic parameters, CRP, creatinine, and BNP. In a second study, sST2 was independently associated with 1 year mortality after TAVI, as were logistic EuroSCORE, chronic renal failure, and left ventricular ejection fraction (44). However, it was not superior to NT-proBNP or surgical risk scores (STS-PROM) for risk assessment, possibly due to confounding effect of inflammation on sST2 levels. In a third study, sST2 predicted mortality and the occurrence of major cardiovascular events post-TAVI (45). In contrast to the study of Stundl et al, adding sST2 to the STS score improved risk prediction of 2 year mortality.

Again, regarding sST2, future larger studies are awaited to validate these findings.

Markers of Hemostasis Imbalance

In aortic stenosis, high shear stress through aortic valve induces a loss of high molecular weight von Willebrand factor (vWF) multimers (HMWM), platelet activation and release of platelet granule content (46). Increased activation of coagulation with concurrent hypofibrinolysis is also observed (47), all this contributing to the dual clinical picture of AS, characterized by mild bleeding tendency (48), and high thrombotic risk.

Thromboembolic events, primarily stroke, are serious complications of TAVI procedures, occurring in up to 3–5% of patients. In addition, TAVI causes thrombocytopenia in one-third of patients. Importantly, while thrombocytopenia often resolves at discharge, persistent thrombocytopenia accurately predict 1 year mortality post-TAVI (49). Moreover, post-TAVI thrombocytopenia was found to be related to early post-procedural adverse events, including vascular complications, bleeding, and the need for multiple blood transfusions. To prevent TAVI-associated thromboembolic events and thrombocytopenia, a 3- to 6 month dual antiplatelet therapy (DAPT) is currently recommended for all approved balloon expandable and self-expandable transcatheter heart valve prostheses.

To determine which factors may explain the drop in platelet count that occurs after TAVI, Mitrosz et al (50) have prospectivelly analyzed changes in platelet count, along with markers of coagulation activation (F1 +2) and soluble markers of platelet activation (P-selectin, PF4) in a small cohort of severe AS, before TAVI and on the three postoperative days. While platelet reduction shortly after TAVI procedure was mostly influenced by the amount of contrast agent applied during the procedure, levels of PF4 and P-selectin positively correlated with the drop of platelet count, suggesting that thrombocytopenia is secondary to platelet activation. In-hospital major adverse cardiovascular events were observed more frequently in patients with more severe platelet count decrease (51). In another study, levels of thrombin-antithrombin complexes (TAT), plasmin-α_2-antiplasmin complex (PAP), and D-dimers significantly increased after TAVI, and D-dimer as well as PAP remained elevated until day 7, indicative of TAVI-induced increased thrombin formation and fibrinolysis (52).

Post-TAVI thrombocytopenia occurred in one-fifth of patients and was associated with a significantly higher incidence of post-TAVI complications, e.g., acute kidney injury and vascular complications, whereas no impact of activated coagulation on thrombocytopenia was observed.

Thus, altogether these studies indicate that consumption of activated platelets might be the mechanism leading to thrombocytopenia after TAVI. Therefore, periprocedural platelet activation markers may potentially represent predictors of adverse outcome.

Bleeding is a more common complication of TAVI than thromboembolic events, as major and life-threatening bleeding (MLTB) according to VARC-2 can occur in up to 30% of patients (53, 54). Of note, periprocedural bleeding independently predicts all-cause mortality after TAVI (53). High mean platelet volume (MPV) and low platelet distribution width (PDW) were associated with increased risk of any bleeding and MLTB (55). Since larger platelets are more reactive and are believed to increase thromboembolic risk (56–58), this finding may be surprising. However, it is possible that high MPV could be a consequence of patient's health state, making them more prone to bleeding. It has been shown that MPV progressively normalizes during the days following TAVI, in parallel with NT-proBNP and hemodynamic parameters (59), but its relation with patient outcome has not been investigated yet.

Importantly, a decrease of platelet reactivity is probably not the only determinant of bleeding post-TAVI. Acquired von Willebrand disease may also play a role. However, to date, evidence for a link between vWF deficiency and overt bleeding in TAVI is lacking. Indeed, the loss of HMWM does not always associate with bleeding events after valve replacement (48, 60). Though, it has recently been shown that recovery of HMWM levels post-TAVI could be used as a marker of postprocedural paravalvular regurgitation, with a positive effect on 1 year mortality (61).

Finally, a high on-treatment platelet reactivity (HTPR) to clopidogrel, due to impaired response to this antiplatelet medication, appears to be very frequent in TAVI patients (62, 63). Yet, no studies have evaluated the association of HTPR with post-TAVI outcomes. The ARTE randomized clinical trial showed a reduction of death, myocardial infarction, stroke, transient ischemic attack, or MLTB within the 3 months following TAVI with aspirin monotherapy versus DAPT (64). Thus, since there is currently no approved alternative to clopidogrel medication in >75 years TAVI patients (65), larger clinical trials aimed at defining the optimal antithrombotic regimen in these patients are awaited.

Strikingly, a recent study indicated that periprocedural changes in plasma markers of inflammation, interleukin-6 and S100A8/A9, could predict the decline in platelet count in the days following TAVI (66). A drop in platelet count and inhibition of agonist-induced platelet activation occurred in parallel with an increase of the inflammation markers following valve deployment. Thus, the inflammatory process elicited by TAVI may contribute to postprocedural thrombocytopenia. This is in line with a study showing that severe systemic inflammatory response syndrome (SIRS) was related to higher 6 month all-cause mortality after TAVI (67). This concept warrants further investigation.

CONCLUSION

In conclusion, blood biomarkers may enrich current risk scores in the future. BNP is readily available and easy to perform. Large studies will clarify the role of further markers.

AUTHOR CONTRIBUTIONS

CO wrote the manuscript. PL, AN, and JB provided intellectual contributions and edited the manuscript.

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Advances in Pathophysiology of Calcific Aortic Valve Disease Propose Novel Molecular Therapeutic Targets

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Calcific Aortic Valve Disease (CAVD) is the most common heart valve disease and its incidence is expected to rise with aging population. No medical treatment so far has shown slowing progression of CAVD progression. Surgery remains to this day the only way to treat it. Effective drug therapy can only be achieved through a better insight into the pathogenic mechanisms underlying CAVD. The cellular and molecular events leading to leaflets calcification are complex. Upon endothelium cell damage, oxidized LDLs trigger a proinflammatory response disrupting healthy cross-talk between valve endothelial and interstitial cells. Therefore, valve interstitial cells transform into osteoblasts and mineralize the leaflets. Studies have investigated signaling pathways driving and connecting lipid metabolism, inflammation and osteogenesis. This review draws a summary of the recent advances and discusses their exploitation as promising therapeutic targets to treat CAVD and reduce valve replacement.

Keywords: calcific aortic valve disease, calcification, inflammation, oxidative stress, lipids, signal transduction

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INTRODUCTION

Over the course of an average day, aortic valve (AoV) leaflets open and close 100,000 times allowing unidirectionality blood flow from the left ventricle to the systemic circulation. The proper function of AoV is achieved by thin leaflets composed of three distinct layers of extracellular matrix (ECM), rich in fibrillar collagen, glycosaminoglycans (GAGs) and elastin. Calcific Aortic Valve Disease (CAVD) appears first as AoV sclerosis developing into AoV stenosis (1, 2). Macroscopically, leaflets are thickened and progressively calcified resulting into stiff leaflets with restricted movement.

CAVD is one of the most common heart valve disease and its prevalence increases with aging (3). Nowadays, in western countries, 2.8% of the general population aged over 75 years is affected with moderate to severe aortic stenosis (3, 4). With life expectancy increasing, prevalence of heart valve disease is expecting to rise. Nevertheless, due to a lack of drug treatment (5), surgery remains the only way to treat it through surgical valve replacement or transcatheter aortic valve implantation.

The seeking of therapeutic targets relies on mechanistic understanding of CAVD. Due to its association with aging, CAVD used to be considered as a passive disease, but is now established that CAVD is an active cellular-driven regulated process (6). Heart valve homeostasis is tightly controlled by valve interstitial cells (VICs) embedded in ECM, valve endothelial cells (VECs) covering the leaflet, and circulant and resident immune cells. When CAVD develops, lipid deposition, inflammation and angiogenesis occur while VICs are entering an osteogenic program as a response to exposure to risk factors including age, congenital heart defect, male gender, tobacco use, diabetes, hypertension, obesity and dyslipidemia (7–9). As a result, homeostasis is disrupted,

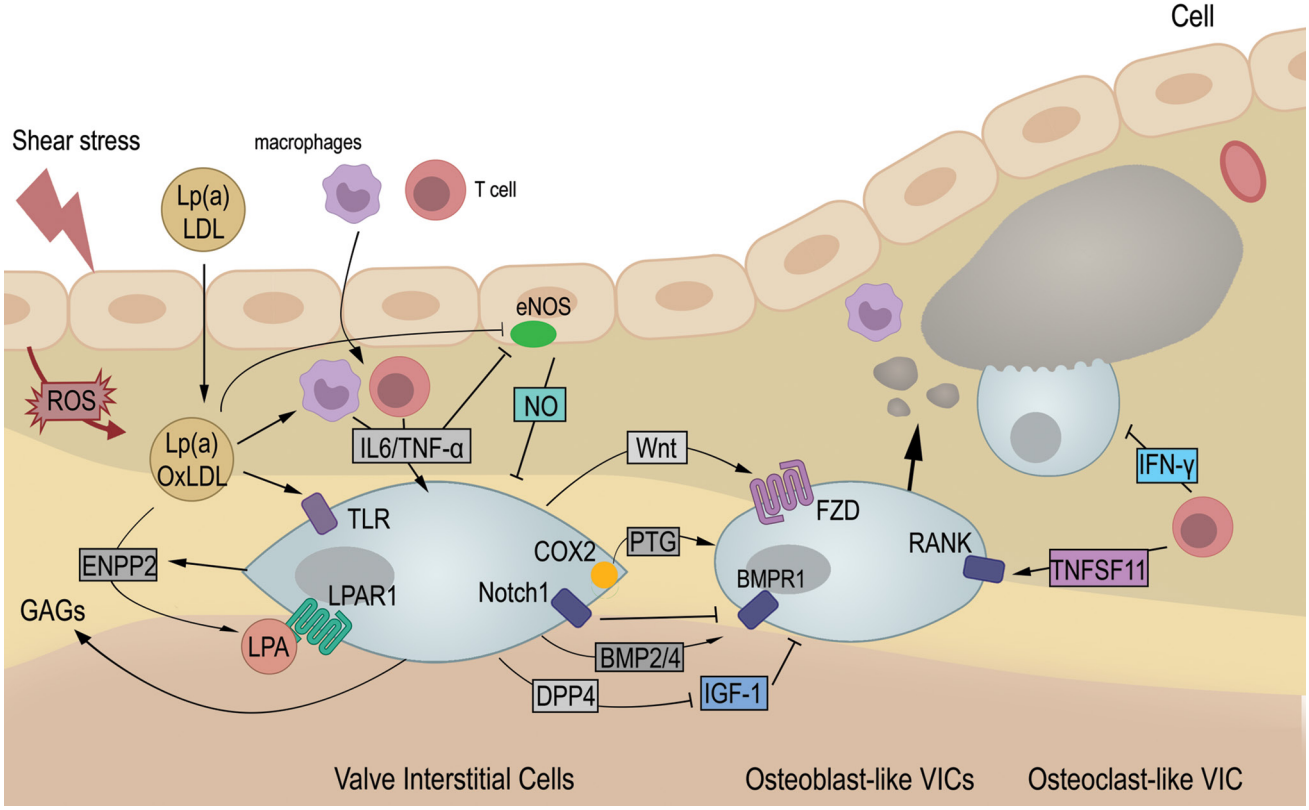

FIGURE 1 | CAVD is a multi-step disease**.** Upon valve endothelium damage, low-density-lipoprotein (LDL) and lipoprotein a [Lp(a)] accumulate. Oxidation of LDL (oxLDL) trigger infiltration of macrophages and T cells that express pro-inflammatory cytokines among which IL-6 and TNF-α. Proinflammatory cytokines impairs protective role of valve endothelial by inhibition of *endothelial nitric oxide synthase* (*eNOS)* and production of nitric oxide (NO). Therefore, oxidative stress (ROS) increases and contributes to enhance oxLDL. Concomitantly, valve interstitial cells (VICs) get activated by cytokines and oxLDL directly, or indirectly through autotaxin (ENPP2) and lysophosphatidyl acid (LPA). Therefore, VICs enter an osteogenic differentiation leading to calcific deposit and nodule formation. Activated VICs secrete glycosaminoglycans (GAGs), favoring further accumulation of oxLDL. Increased cyclooxygenase 2 (COX2) and its product prostaglandin (PTG), Wnt and BMP signaling have been shown to drive osteogenic differentiation while inhibition of IGF-1 signaling by dipeptidyl peptidase-4 (DPP4) contributes as well to pathogenesis. Also, Notch signaling, induced by NO, repress osteogenic differentiation. Finally, T cells favor osteogenesis and osteoclast formation by production of TNFSF11 but secrete Interferon-γ (IFN-γ) which limits calcium resorption. Altogether, aortic valve leaflets gets remodeled and stiffen leading to aortic valve stenosis. ROS: Reactive Oxygen Species. LPAR1: Lysosphosphatidyl Acid Receptor 1. TLR: Toll-like receptor. IL6: interleukin 6. TNF-α: Tumor necrosis factor- α. BMP: Bone Morphogenetic Protein. Morphogen. BMPR1: BMP Receptor 1. Fzd: Frizzled. RANK: Receptor Activator of Nuclear Factor kB. TNFSF11: RANK ligand.

ECM is remodeled, and formation of calcium nodules occurs. Although mechanisms leading to CAVD are still unclear, studies on diseased human aortic valves and animal models of CAVD, reviewed by Sider *et al.* (10), have provided valuable insights into cellular components and signaling pathways involved in the pathogenesis. This review will summarize the current findings with emphasis on valuable therapeutic candidates.

CAVD: Multi-Step Process with Endothelium Damage as Starting Point

Endothelium dysfunction is an early feature of CAVD (11, 12) and likely the result of altered blood shear stress (13). There is indeed a spatial correlation between the calcific lesions, located almost exclusively on the aortic side of AoV leaflet, and the local hemodynamic environment (14–16).The hypothesis of hemodynamic onset is reinforced by the predisposition and accelerated progression of CAVD in patients with bicuspid aortic valve (17) that display different blood flow patterns than observed with tricuspid AoV (18, 19). Endothelium damage favors lipid deposit followed by infiltration of inflammatory cells, two hallmarks of early AoV lesions (20). Therefore, lipids and cytokines will influence neighbored VECs and VICs to promote activation of VICs, ECM remodeling and mineralization of AoV leaflets (**Figure 1**).

Oxidized LDLs Mediate Inflammation and Mineralization

The importance of dyslipidemia in CAVD was confirmed by prevalence of CAVD in familial hypercholesterolemia caused by mutation of *LDL receptor (Ldlr)* and leading to abnormal circulating level of LDL (21–23). Hypercholesterolemia induced in animal models by genetic mutation ($Ldlr^{-/-}$, $ApoE^{-/-}$,

ApoB$^{100/100}$) and/or combined with enriched diet further indicate that increased lipid deposits precede the emergence of inflammatory and calcification processes (21, 24). Due to association between lipid and CAVD, clinical trial using lipid level lowering drug have been carried out, but it has shown negative results in regard with reducing CAVD (5, 25–27). One of the reasons might be that statins are ineffective to reduce Lp(a) level (28, 29) . Lp(a) consists of low density lipoprotein (LDL)-like particle in which apoliprotein(a) is covalently linked to apoliprotein B100 (30). Histopathologic studies demonstrated accumulation of apoliproteins and lipid in early stages of CAVD (31). Genome wide association study further described a SNP in *LPA* gene that was strongly associated with CAVD. Individuals with that SNP had higher Lp(a) plasma level and higher risk of aortic valve stenosis (32–34).

Altogether, Lp(a) appears genuinely to mediate the onset of CAVD. Deciphering the pathogenic mechanisms linking Lp(a) to CAVD has been recently acknowledged as a priority (35). Several studies highlighted a link between lipid metabolism and calcification through oxidation of LDLs. Lp(a) is a carrier of oxidized phospholipids (OxPLs), used by Lp(a)-associated phospholipase A2 (Lp-PLA2), to generate lysophosphatidyl choline (LPC), all highly expressed in human CAVD (36, 37). LPC is then transformed into lysophosphatidyl acid (LPA) by ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), secreted by stimulated VICs (38). LPA is also produced during non- oxidative transformation of LDLs. Therefore, LPA activates VICs through enzymatic LPAR1/RhoA/NF-κb signaling, and mediates mineralization through *BMP2* expression (38, 39). The requirement for RhoA to promote calcific nodule was also illustrated *in vitro* (40). The signaling pathway is confirmed with decreased AoV mineralization when using Ki16425, an inhibitor of LPAR1, in *Ldlr*$^{-/-}$, *ApoB*$^{100/100}$ mice fed with high fat and high sucrose diet (39). It is important to mention that changes in the ECM, with accumulation of glycosaminoglycans, precede and favor oxLDL retention (24, 41, 42).

The findings indicate that lowering Lp(a), OxLDL or targeting LPAR1 are attractive options and might be used to prevent the onset of CAVD. Multiple treatment options are currently suggested to decrease Lp(a). IONIS-APO(a)$_{Rx,}$ and IONIS-APO(a)-L$_{Rx}$, antisense oligonucleotide targeting *Apo(a)* mRNA have been shown to lower Lp(a) level (43). Targeting Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9), a hepatic protease that promotes LDLR destruction, might be a way to decrease LDL and oxidative products. This might be achieved with monoclonal antibodies, Alirocumab and Evolocumab (44), or by using Inclisiran, a small RNAi targeting *PCSK9* (45, 46).

Inflammation Contributes to Calcification

Inflammation occurs after endothelium activation and lipid deposition. Microarray analysis of human CAVD (47) and Rapacz familial hypercholesterolemia swine, an established model of human FH (21) shows upregulation of inflammation-related genes and chemokines. Histological studies present inflammatory cells, composed of macrophages, B and T cells found near osteoblast-like cells and calcified area in human CAVD (20, 48, 49). PET imaging using 18-Flurodexoxyglucose uptake (18F-FDG) to monitor inflammation reports higher 18F-FDG uptake in patients with AoV sclerosis and stenosis and a raise of the activity as the disease gets more severe (50).

Besides activation of endothelial cells (11, 12), OxLDLs trigger proinflammatory cytokines expression and promotes infiltration of immune cells into AoV leaflets (42, 51, 52). In diseased AoV, higher oxLDL content correlates with higher amounts of inflammatory cells (53) . During inflammation, immune cells secrete inflammatory cytokines including IL-2 (54), IL-1β (55), TNF-α (56, 57) , IL6(58) and MMPs (55, 59) than stimulates VICs, ECM remodeling and promote the expression of genes involved in osteogenesis (52). Altogether, data support that CAVD is an inflammatory disease, and inflammation may drive calcification.

Although inflammation precedes ECM remodeling and calcification, inflammation over the course of the disease has not been fully explored yet. Similarly, immune cells display a broad heterogeneity with specific function. Thorough characterization of macrophages, T cells or B cells is now just starting to be done in the context of CAVD. M1 macrophage subset have recently be found to be the predominant macrophage subset in CAVD, promoting osteogenic differentiation of VICs through TNF-α and IL-6 secretion (58, 60). T cells are also reported surrounding calcified area. T cells favor calcification through cytokine TNF-α and TNFSF11 expression (56, 61, 62). Increased T cells in diseased AoV is likely the result of increased circulating CD8 +T cells (63). Activated T cells infiltrate the leaflets and surround calcified area and display high level of inflammatory cytokine IFN-γ (62). Although TNFSF11 promotes osteoclast activity, aberrant IFN-γ level impairs calcium resorption by valve osteoclast. Therefore, calcium accumulates in the leaflets and facilitates nodule formations (62). A similar study indicates that macrophages surrounding calcium deposits in human atherosclerotic are defective and unable to resorb calcification (64). Such role of macrophage in CAVD have not been explored yet. Circulating Tregs are also measured in patients with CAVD and associate with disease progression (65). Although dendritic cells are found abundantly in heart valve and accumulate in AoV stenosis, their contribution to CAVD is still unknown (51, 66).

Deeper understanding of regulation, timing and functional role of immune cells in CAVD will bring valuable information to determine how targeting inflammation might help preventing pathogenesis.

VECs Are Natural Inhibitors of Calcification, Through NO Release, but Activators Through Oxidative Stress

Inflammatory cytokines, TNF-α and IL-6, induce valve endothelial-to-mesenchymal (EMT) transformation through Akt/NF-κb signaling and reduce *endothelial nitric-oxide synthase* (*eNOS*) expression (67). Although some markers of EMT are measured in human calcified aortic valves (67), studies have still to address if EMT contribute to pathogenesis of CAVD.

VECs have the particularity to display side-specific heterogeneity. Endothelium on the aortic side displays an antioxidative and

anti-inflammatory phenotype defined by its RNA expression profile (15). Thus, aortic side of AoV demonstrates protection against repetitive insult in normal AoV. As consequence, VECs are releasing nitric-oxide (NO), a natural inhibitor of pathogenic differentiation of VICs into myofibroblast andosteoblasts (68). Increased NO release has been shown to inhibit calcific nodule formation *in vitro* (69) and *in vivo* with atorvastatin treatment (70). On the opposite, in CAVD, altered mechanical stimulus, oxLDLs or TNF-α impair *eNOS* expression (68, 71, 72). Concomitantly, uncoupling of NO synthase leads to increased production of superoxide and oxidative stress which drives calcification (73). The critical role of endothelium and *eNOS* was further illustrated through modulation of a multifunctional enzyme dipeptidyl peptidase-4 (DPP4) and insulin growth factor-1 (IGF-1). Upon NO depletion, DPP4 increases in human VICs and limits IGF-1 signaling leading to enhanced calcification. Treatment of rabbit and mouse model of CAVD with Sitagliptin, a selective DPP4 inhibitor, was protective against AoV calcification (74). Similarly, the protective role of VECs is illustrated by TGF-β1 expression that translocates Sox9 into VICs nucleus and prevent calcific nodule formation (75, 76). Therefore, enhancing protective role of VECs, during early phase of disease, must be exploited. Notably, increasing NO production with statins or using DPP4 inhibitor ,broadly used as hypoglycemic drugs for treatment of type 2 diabetes mellitus, might mitigate CAVD.

VICs Differentiate Into Osteoblast-Like Cells and Mineralize the Leaflets

Histological studies report the formation of bone nodules in stenotic CAVD resulting from deposition of calcium in the form of hydroxyapatite in the valve leaflet (49). Once heart valve development is complete, VICs become quiescent, but in disease get activated and turn into active phenotype. In response to pathological stimuli, VICs differentiate into osteoblast-like cells with abnormal expression of typical bone genes, including *Runx2, Alkaline Phosphatase (ALP), Osteopontin (SPP1)*, *Osteocalcin* (*BGLAP*) (47) resulting in calcified ECM. Apart from promoting inflammation, OxLDLs and Lp(a) can also directly activate VICs through LPAR1 (38, 39) and TLR activation (52, 77–79), This interaction contributes to trigger GAG accumulation, in a positive feedback loop, and upregulates osteogenic gene expression through *BMP2* and *IL6* expression (38, 42, 80).

Different molecular mechanisms are involved in VICs osteogenic differentiation and shared with bone formation (81, 82). Stimulation of VICs culture with OxLDLs and hypercholesterolemia animal model have been used to investigate signaling pathway underlying osteogenic differentiation. Also studies in *klotho* null mice have been useful to investigate AoV calcification with minimal inflammation (83).*BMP2*, along with osteogenic gene expression, are the usual markers measured to assess VICs osteogenic differentiation. BMP signaling is increased in human CAVD illustrated by increased BMP2, BMP4 ligands and phosphorylation of Smad1/5/8 (82, 84, 85). Downregulation of *Smad6*, an inhibitor of BMP signaling, enhance BMP signaling (84, 86). Inhibition of osteogenic gene expression and calcific nodule formation by targeting Alk3, BMP receptor type-1A, strongly indicate that LDN-193189, a small molecule inhibitor of BMP signaling, should be used to prevent calcification in late stage of CAVD (85).

Mutation in *Notch1* and its association with BAV and AoV calcification highlighted the role of Notch signaling in CAVD (87). Later, studies confirms that Notch signaling represses osteogenic gene expression (88, 89) and is regulated by NO released by endothelial cells (90). Decreased Notch signaling is not just observed in patients with mutated *Notch1* but also in patients with idiopathic CAVD where increased long non-coding RNA *H19,* resulting from hypomethylation, prevents *Notch1* expression (91). The role of prostaglandins has been illustrated in osteogenesis (92, 93), but only recently in CAVD. Prostaglandins are synthesized by COX2, an enzyme highly expressed by VICs in CAVD (94). Pharmacological inhibition of COX2 activity with Celecoxib, a nonsteroidal anti-inflammatory (NSAID) drugs, is sufficient to reduces AoV calcification in *Klotho* null mice (94). Celecoxib is clinically used to treat joint and/or muscle pain(95) but was associated with increased cardiovascular risk (96). Cardiovascular safety of celecoxib is nowadays controversial (97) as recent report indicate that cardiovascular risk associated with moderate doses of celecoxib is not greater than associated with non-selective-NSAID ibuprofen (98). Additional research must evaluate the effectiveness of COX2 inhibitor in human CAVD.

Non-canonical Wnt5b and Wnt11 ligands are found elevated in macrophages of human calcified AoV. Moreover, the ligands stimulate VICs, apoptosis and calcium deposits (99). Abundant expression of Fzd receptors and co-receptors Lrp5/6 also suggest the involvement of canonical Wnt/β-catenin signaling in CAVD (81, 100). *In vitro*, Wnt treatment of VICs inhibit chondrogenic differentiation and promote osteogenic gene expression (101, 102) while Lrp5/6 is required to promote calcification in hypercholesterolemia mouse model (103). In *Axin2* KO mice, increased canonical Wnt/β-catenin signaling promotes ECM remodeling and BMP signaling but fails to calcify AoV (104). The findings illustrate that Wnt signaling is required but might not be sufficient to promote end-stage calcification. These data illustrate the importance to further study the role of Wnt signaling in CAVD as specific inhibitors are being tested (105).

VIC osteogenic differentiation has been one of the most studied process in CAVD due to available cell culture model. However, VIC remains a poorly defined cell type. Heterogeneity of VIC population is underappreciated during heart valve homeostasis and disease. Being able to define which cell type is activated and/or differentiated across disease is a major goal in order to present innovative therapeutic options.

CONCLUSIONS

CAVD is a complex multi-step event that involves numerous biological processes from lipid accumulation, inflammation to osteogenesis. Understanding the underlying molecular and cellular processes is crucial in the establishment of therapeutic targets. Clinical, histological and animal model studies have allowed better characterization of the disease and show the importance of cross-talk between lipids, immune cells, VECs and VICs. As a result, putative molecular targets with available treatments (**Table 1**) emerge for each

TABLE 1 | Putative available therapeutic treatments and molecular targets that might affect the pathophysiology of CAVD. In brackets, species where the drug effect has been reported.

Putative Therapeutic treatments	Molecular Targets	Biological process
IONIS-APO(a)Rx IONIS-APO(a)-LRx	*Apo(a)*	Lp(a) level lowering (human)
Alirocumab Evolocumab Inclisiran	PCSK9	Lipid lowering (human)
Statins	HMG-CoA reductase	Lipid lowering (human) Promotes NO release/ inhibition of calcification (rabbit)
Ki16425	LPAR1	Inhibition of calcification (mouse)
Sitagliptin	DPP4	Inhibition of calcification (mouse)
LDN-193189	BMPR1A	Inhibition of calcification (mouse)
Celecoxib	COX2	Inhibition of calcification (mouse)

stage of CAVD. Giving the multifactorial and complex interplay, timing and combination of therapy should be considered. In the context of appropriate therapeutic timing, accurate biomarkers should be defined. Similarly, thorough knowledge of the heterogeneity and function of valve cell subtype, over the course of the disease, may provide better targeting of the "diseased" cells. Overall, recent advances and future directions bring hope for the development of efficient drug treatment and for the reduction of valve replacement surgeries.

AUTHOR CONTRIBUTIONS

AHu wrote the manuscript. AHe drafted the figure. PL provided intellectual contributions and edited the manuscript. CO drafted and revised the manuscript.

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Postnatal and Adult Aortic Heart Valves Have Distinctive Transcriptional Profiles Associated With Valve Tissue Growth and Maintenance Respectively

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Heart valves are organized connective tissues of high mechanical demand. They open and close over 100,000 times a day to preserve unidirectional blood flow by maintaining structure-function relationships throughout life. In affected individuals, structural failure compromises function and often leads to regurgitant blood flow and progressive heart failure. This is most common in degenerative valve disease due to age-related wear and tear, or congenital malformations. At present, the only effective treatment of valve disease is surgical repair or replacement and this is often impermanent and requires anti-coagulation therapy throughout life. Therefore, there is a critical need to discover new alternatives. A promising therapeutic area is tissue regeneration and in non-valvular tissues this requires a tightly regulated genetic "growth program" involving cell proliferation. To explore this in heart valves, we performed RNA-seq analysis to compare transcriptional profiles of aortic valve tissue isolated from mice during stages of growth (postnatal day (PND) 2) and adult maintenance (4 months). Data analysis reveals distinct mRNA profiles at each time point and pathway ontology identifies associated changes in biological functions. The PND2 aortic valve is characterized by extensive cell proliferation and expression of mRNAs related to the extracellular matrix (ECM). At 4 months, proliferation is not significant and a differential set of ECM-related genes are expressed. Interestingly there is enrichment of the defense response biological process at this later time point. Together, these data highlight the unique transcriptome of the postnatal valve during stages of growth and maturation, as well as biological functions associated with adult homeostatic valves. These studies create a platform for future work exploring the molecular programs altered in the onset of heart valve disease after birth and provide insights for the development of mechanistic-based therapies.

Keywords: aortic valve, RNA-sequencing, mRNA, cell proliferation, extracellular matrix, postnatal, adult

INTRODUCTION

The average heart beats over a billion times during one lifespan to continuously provide blood to every part of the body. Crucial to this task are the four heart valves (aortic, pulmonic, tricuspid and mitral) that function to maintain the unidirectional blood flow. Distinct from the cardiac muscle, the mature valve leaflets are highly organized structures comprised of three layers of extracellular matrix (ECM) components including collagens, proteoglycans, and elastin (1). Formation and maintenance of the valve ECM is mediated by a heterogeneous population of valve interstitial cells (VICs) that are fibroblast-like in phenotype (2). Surrounding the VICs and ECM is a single layer of valve endothelial cells (VECs) that physically protect the valve from external stimuli, and molecularly communicate with underlying VICs to regulate homeostasis of the ECM (3–5). The complex relationship between valve cell populations and the ECM is critical for establishing and maintaining structure-function relationships throughout life. This relationship begins during embryonic development, as mesenchymal precursor cells in the endocardial cushions transition towards an activated VIC phenotype and degrade primitive ECM within the cushions while secreting more diverse ECM components. Elongation and remodeling of the immature valve structures continues for a short time during the postnatal period until around day 10 in the mouse when the ECM components are more defined. Once valve formation is complete, VICs convert to a quiescent phenotype and in the absence of disease, maintain physiological turnover of the ECM to provide efficient function throughout life [reviewed (2, 6)]. While the regulation of valve development is well established, the mechanisms that regulate postnatal valve growth and remodeling, as well as adult homeostasis are poorly understood. Despite constant mechanical demand on the valve leaflets, turnover of valve cell populations in adult valves is relatively low (4). Therefore, it remains unclear how valve cell populations and structure-function relationships are maintained throughout life in healthy individuals, yet dysregulation of these relationships likely underlie the onset and progression of valve dysfunction and disease.

Heart valve disease is a growing public health problem that can affect both adult and pediatric patients. Significant defects during embryonic valve development lead to congenital malformations which compromise the typical structure of the valve, often resulting in reduced ability to function correctly [reviewed (7)]. Distinct from valve disease present at birth, pathology can also be acquired and is most prevalent in the aging population, with up to 13% of people aged over 75 affected by diseases including calcification or myxomatous degeneration (8). Currently, the only effective treatment for valve disease is surgical repair or replacement, resulting in over 90,000 valve replacement surgeries performed in the US each year (9). Surgical treatment comes with many complications including the need for repeat surgeries due to low valve durability and high thrombogenicity, in addition to the large personal and societal economic burdens (10). Therefore, there is a critical need for the development of alternate therapeutics.

A promising therapeutic area is emerging in the field of self-repair and regenerative medicine. Common to both congenital and age-related valve disease is the damage and consequent loss of healthy cell populations alongside the development of pathological cell populations that are therefore unable to preserve valve structure-function relationships (11). The field of cardiac regeneration has recently made significant advances in elucidating the molecular mechanisms of regeneration, and it has been reported that the neonatal myocardium has remarkable regenerative capacity during the first seven days of life (12–14). Furthermore, several pathways have been identified as key players, and the ability to recapitulate these neonatal programs in adults has proven successful in promoting myocardial regeneration after injury and in disease models (15–18).

Neonatal, adult and potential regenerative programs have not been examined in heart valves and therefore the goal of this current study is to initiate this discovery. To do this, we used RNA-seq analysis to explore differential molecular profiles between postnatal and adult valve cell populations. This analysis will help define potential regeneration indicators that in the future might be reintroduced in diseased or aging adult valves to increase their self-repair capacity and improve structure-function relationships. Our study has defined transcriptional differences between postnatal day 2 (PND2) and adult (4 months) aortic valves and identified significant changes in key biological functions related to cell proliferation, ECM, and defense response that may be important for determining the regenerative capacity of the valve to aid in the future development of alternative therapeutics.

MATERIAL AND METHODS

Mice

C57BL/6J mice were fed regular chow mix and housed in a controlled environment with 12 h light/dark cycles at 21°C and 23% humidity and water ad libitum. Animals were euthanized by CO_2 exposure followed by secondary euthanasia by cervical dislocation (adult mice) or decapitation (pups). All animal procedures were approved by The Research Institute at Nationwide Children's Hospital Institutional Animal Care and Use Committee (Protocol # AR13-00054).

Tissue Preparation

Hearts were collected from postnatal day 2 (PND2) and 4 month old *C57BL/6J* mice and fixed in 4% paraformaldehyde/1xPBS overnight at 4°C. For paraffin sections, tissue was embedded in paraffin wax and sectioned at 10 µm. Paraffin was removed in xylene, and tissue sections were re-hydrated through a graded ethanol series and rinsed in 1xPBS as previously described (19). Tissue sections containing aortic valves were then subjected to Movat's Pentachrome staining, EdU staining, or immunohistochemistry/immunofluorescence (described below). For cryo sections, tissue was embedded in OCT and frozen, then sectioned at 7 µm. Prior to staining, tissue was permeabilized using 0.1% Triton-X 100 in 1xPBS and then subjected to immunofluorescence staining.

Immunohistochemistry/ Immunofluorescence

Whole hearts from PND2 and 4 month old *C57BL/6J* mice were collected and prepared according to above methods. Movat's Pentachrome staining was performed on paraffin tissue sections at each time point according to the manufacturer's instructions (Russel Movat, American MasterTech, #KTRMP), then mounted using VectaMount Permanent Mounting Medium (Vector Laboratories, H-5000). For antibody detection, fixed paraffin tissue sections were subjected to antigen retrieval by boiling for 10 min in unmasking solution (Vector Laboratories), and both cryo and paraffin sections were subjected to blocking for 1 h at room temperature (1% BSA, 1% cold water fish skin gelatin, 0.1% Tween-20/PBS) as described (20). Tissue sections were then incubated overnight at 4°C or 1 h at room temperature with primary antibodies against Mmp3 (rabbit, 1:100 paraffin, Abcam ab53015), Nid2 (rabbit, 1:200 cryo, Abcam ab14513), Ptgs2 (Rabbit, 1:100 paraffin, Cell Signaling 12282), and Rarres2 (Mouse, 1:100 paraffin, Santa Cruz sc-373797). For immunofluorescent primary antibody detection of Mmp3, Nid2, and Ptgs2, sections were incubated for 1 h at room temperature with Donkey anti-rabbit or Goat anti-rabbit Alexa-Fluor IgG secondary antibodies (1:500) (LifeTechnologies), then mounted in Vectashield anti-fade medium with DAPI (Vector Laboratories) to detect cell nuclei. For diaminobenzidine (DAB) staining of Rarres2, sections were stained using Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (Abcam, ab64264), counterstained with hematoxylin (Vector Laboratories, H-3404), and mounted using VectaMount Permanent mounting medium (Vector Laboratories, H-5000). Images were visualized using an Olympus BX51 microscope and captured using an Olympus DP71 camera and CellSens software. Image brightness and contrast were edited using Adobe Photoshop CC.

EdU Staining and Quantification

PND2 and 4 month old *C57BL/6J* mice were injected subcutaneously with 10 μg/g body weight EdU (Invitrogen) dissolved in 1xPBS. 24 h later, mice were sacrificed and hearts were collected and prepared according to above methods. Fixed tissue sections were blocked for 1 h at room temperature (1% BSA, 0.1% Cold water fish skin gelatin, 0.1% Tween 20 in PBS with 0.05% NaN_3), followed by use of Click-it EdU Kit (Invitrogen) to detect presence of EdU according to the manufacturer's instructions. Sections were then mounted in Vectashield anti-fade medium with DAPI (Vector Laboratories) to detect cell nuclei. The total number of cell nuclei in one leaflet were counted using ImageJ cell counter. The number of EdU + cells were then counted and calculated as a percentage of total cells. An average of 9 leaflets were counted and averaged for each mouse, with a total $n = 3$. Statistical analysis was performed in GraphPad Prism 7.0a.

Aortic Valve Isolation and RNA-Sequencing

Aortic valves from wild type PND2 and 4 month old *C57BL/6J* mice were isolated with minimal myocardial contamination and immediately flash frozen in liquid nitrogen. Frozen samples were sent to Ocean Ridge Biosciences LLC (Deerfield Beach, FL), where RNA isolation and sequencing was performed as follows. Total RNA was extracted using the TRI Reagent® (Molecular Research Center; Part #: TR118) method, and isolated RNA was quantified using chip-based capillary electrophoresis (Agilent 2100 Bioanalyzer Pico Chip). RNA was digested with RNase free DNase I (Epicentre; Part # D9905K) and purified through minElute columns (Qiagen; Part #: 74204). Final RNA samples were quantified by O.D. measurement and re-quantified using chip-based capillary electrophoresis. Amplified cDNA libraries were prepared from 200 ng on DNA-free total RNA using TruSeq Stranded Total mRNA Library Prep Kit LT (Illumina Inc.; Part #s: RS-122-2101 and RS-122-2102). Chip-based capillary electrophoresis was used to assess quality and size distribution of the libraries. KAPA Library Quantification Kit (Kapa Biosystems, Boston, MA) was used to quantify the libraries. Libraries were pooled at equimolar concentrations and were clustered on an illumina cBot cluster station. Clustering was performed with the HiSeq PE cluster kit v4 and sequenced on an Illumina HiSeq Flow Cell v4 with 50 nt paired-end reads plus dual index reads using the Illumina HiSeq SBS Kit v4. An average of approximately 48.3 million passed-filter 50 nucleotide paired-end reads were obtained per sample (24.1M per direction).

Raw FASTQs were split into files containing 4,000,000 reads and checked for quality using the FASTX-Toolkit. The reads were filtered (removing sequences that did not pass Illumina's quality filter) and trimmed based on the quality results (3 nucleotides at the left end of the R1 reads and 1 nt at the left end of the R2 reads). Sequence alignment was performed using TopHat v2.1.0 to the mm10 genome. BAM files were merged on a per sample. Exon and gene level counting were performed using the easyRNASeq version 2.4.7 package. A binary annotation file, built using the annotation file generation function of EasyRNASeq, was used for this analysis; the Ensembl release 83 GTF file was used as input. Annotation was performed using a Gene Transfer Format (GTF) annotation file for Mus musculus, which was downloaded on February 11, 2016 and contains the current Ensembl Mouse release 83. Filtering of the RPKM values was performed to retain a list of genes with a minimum of approximately 50 mapped reads in 25% or more samples. The threshold of 50 mapped reads is considered the Reliable Quantification Threshold, as the RPKM values for a gene represented by 50 reads should be reproducible in technical replicates. To avoid reporting large fold changes due to random variation of counts from low abundance mRNA, RPKM values equivalent to a count of ≤10 reads per gene were replaced with the average RPKM value equivalent to 10 reads/gene across all the samples in the experiment.

An unpaired two-sample heteroscedastic *t*-test was performed on the $\log_2$ RPKM values to compare the overall effects of age (PND2 or 4 month) on gene expression. Fold changes were also calculated for 4 month / PND2 using the mean of each group being compared. If the mean of both groups considered in the fold change comparison was below RQT, "NA" is reported. All statistical analysis was performed using R version 3.2.2 statistical computing software. A total of 7,496 genes were determined to have a low FDR-value (FDR <0.1) for the unpaired *t*-test. Full dataset is available through NCBI GeoDatasets, accession code GSE108083, "RNA-seq analysis of aortic heart valves in mice".

RNA-Sequencing Data Analysis

A heatmap was generated from 23,303 differentially expressed genes. Log2 transformed RPKM values were utilized and hierarchical clustering analysis was performed with Cluster 3.0 software (21). Genes and samples were clustered using centered correlation as the similarity measure and average linkage as the clustering method. A volcano plot was generated utilizing ggplot2 and is plotted as the -Log10(*p*-value) vs. Log2 Fold Change. The volcano plot highlights the differential gene expression between postnatal day 2 and 4 month aortic valves. A Venn diagram was generated based only on genes with a low *t*-test *p* value ($p < 0.05$), a fold change >2, and RPKM values above the Reliable Quantification Threshold for all biological replicate samples from either group. If at least one of the gene reads from a triplicate set was proven undetectable while all gene reads in the comparative sample set was proven detectable, the gene was considered to be uniquely expressed. If the gene read from both triplicate sample sets had detectable RPKM values about the Detection Threshold, the gene was considered common amongst sample groups. Genes with at least one triplicate below the Detection Threshold in both samples sets are not represented in the Venn diagram.

Functional annotation was performed through the utilization of Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8 (22). 2,082 differentially expressed genes with an FDR <0.05 and fold change >2, were assessed utilizing Gene Ontology (GO) FAT terms, which were employed to filter out broad GO categories based on a measured specificity of each term. Visualization of GO term analysis was performed using the GOPlot R package version 1.0.2 (23). To reduce the redundancy of GO terms, the reduce overlap function was used, with the threshold set to 0.75, which removes GO terms that have a gene overlap greater than or equal to the set threshold. Bubble plots were generated for the reduced GO term list using the GoBubble function, and the top 15 GO terms from biological processes, cellular component, and molecular function were visualized. Each bubble represents a term, where the size of the bubble correlates to the number of genes within the term, and it is plotted as –log (FDR) vs. z-score. The z-score is a crude measurement, predicting if a term will be upregulated or downregulated, and is calculated by taking the number of upregulated genes and subtracting the number of downregulated genes and then dividing this number by the square root of the number of genes in each pathway. The circle plot was generated using the GoCircle function, and highlights the gene expression changes within each of the selected terms. The circle plot highlights the overall gene expression change by showing increased expression in red and decreased expression in blue. The circle plot also highlights the *p*-value of the GO term by the height of the inner rectangle, which is also colored by z-score. A chord plot was generated using the GoChord function, and it represents 59 differentially expressed genes and their correlation to the following associated terms: extracellular matrix, cell proliferation, cell cycle, mitotic cell cycle process, defense response, and regulation of immune system processes. The chord plot also highlights the log fold change of each differentially expressed gene that is shown.

qRT-PCR

RNA was extracted from isolated aortic valves from PND2 and 4 month old *C57BL/6J* mice to validate RNA-seq findings. Briefly, Trizol reagent (Invitrogen) was used to extract RNA according to manufacturer's instructions, and cDNA and PCR reactions were performed as previously described by our lab (24). Primers for genes selected for validation were designed in NCBI Primer-BLAST based on FASTA sequence and shown below:

Gene Name	Forward Primer Sequence (5′ to 3′)	Reverse Primer Sequence (5′ to 3′)
Nid2	AGGAGTGAGCATGTTTCGG	AGGGGTATTGCCAGCTTCAC
Mmp3	TGCATGACAGTGCAAGGGAT	ACACCACACCTGGGCTTATG
Marckls1	CCCGTGAACGGAACAGATGA	CCCACCCTCCTTCCGATTTC
Gsn	GGGACGGCCGGTTACTTAAA	CTTCAGGAATTCGGGGTGCT
Filip1l	AGGCTCCACTGCTGGATTTC	GACTTCTCTGACACGGGACG
Myoc	ACGACACTAAAACGGGGACC	TTCTGGCCTTTGCTGGTAGG
Retnla	GGAACTTCTTGCCAATCCAGC	CAGTGGTCCAGTCAACGAGT
Npdc	GCACTCCCGACACTTTTCTC	GGTACCCACTCCGGGAACT
Sfrp4	CCTGGCAACATACCTGAGCA	AGCATCATCCTTGAACGCCA
Mki67	AGAGCTAACTTGCGCTGACT	ACTCCTTCCAAACAGGCAGG
Nrg1	CCATCTCTCGATGGGCTTCC	ATGCAGAGGCAGAGGCTTAC
Nrep	GCATGATGCCCTTTTTCATCCA	TCCTTAGGCACGGGAAGTCT
Acta2	CCTTCGTGACTACTGCCGAG	GAAGGTAGACAGCGAAGCCA
Dlk1	AGAGTACCCCTCTCCTCACC	CGCCGCTGTTATACTGCAAC
Cfd	TACATGGCTTCCGTGCAAGT	GGGTGAGGCACTACACTCTG

Quantitative real-time PCR using a Step One Plus Real Time PCR system (Applied Biosystems) was used to detect changes in gene expression with Sybr Green reagents. Cycle counts for each target gene were normalized to *β-actin* expression and differences in gene expression were reported as a fold change from the 4 month time point. Statistical analysis was performed in GraphPad Prism 7.0a.

RESULTS

Postnatal Valve Maturation and Adult Maintenance Are Associated with Distinct Transcriptional Profiles

As previously described, the valve structures continue to grow and remodel after birth (1). As shown by Movat's Pentachrome stain, murine aortic valve structures at postnatal day 2 (PND2) are thick and composed of predominantly proteoglycan (blue), with less extensive collagen and elastin (**Figure 1A**). By 4 months of age, the leaflets have elongated and display distinct layers of collagen (fibrosa, yellow), proteoglycan (blue), and elastin (black) (**Figure 1B**).

In order to further define molecular profiles associated with the structural changes in postnatal and 4 month old aortic valves, we performed RNA-sequencing on isolated samples. Overall, RNA expression for samples consistently clustered by time point, as shown several ways including a Pearson's correlation matrix (data not shown), principal component analysis (PCA) (data not shown) and hierarchical heatmap (**Figure 2A**). Of 23,303 detectable genes, 3,659 genes were found to have a *p*-value < 0.05 and a fold change >2, and include 1,858 upregulated and

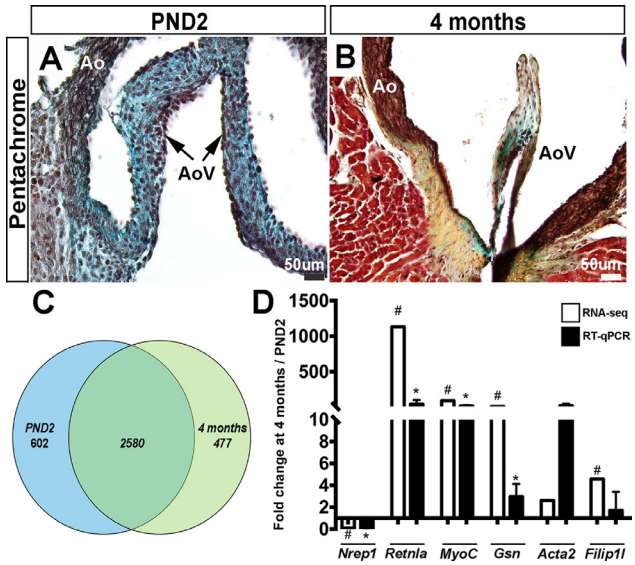


FIGURE 1 | Postnatal and adult aortic vales display differences in ECM composition and gene expression. Movat's Pentachrome staining to show extracellular matrix composition at PND2 **(A)** and 4 months **(B)** (black: elastic fibers and cell nuclei, blue: proteoglycans, yellow: collagen, red: muscle and fibrinoids). **(C)** Venn diagram to show distribution of the detected mRNAs that were uniquely or commonly expressed at PND2 or 4 months. **(D)** RT-qPCR validation (black bars) of RNA-seq findings (white bars) (n = 3, *:$p < 0.05$; two-tailed unpaired t-test, #:FDR <0.05). Ao, aorta; AoV, aortic valve.

TABLE 1 | Top 20 unique genes at PND2 and 4 month time points.

PND2	4 Month
Dlk1	Cfd
Hif3a	2210407C18Rik
Agtr2	Retnla
S100a9	Clec3a
Slc38a5	Adipoq
Col24a1	Ces1d
Bmp7	Thrsp
Vash2	Pck1
Igf2bp3	Mgl2
Stfa1	C7
S100a8	Inmt
Cited1	Cidec
Gm5483	Fmo3
Frem2	Angpt4
Gipr	Hamp
Ube2c	Art1
Dctd	Tmem45b
1110032F04Rik	Olfr224
C1qtnf3	Plin1
Cdkn3	Rpl3l

Genes are listed in order of RPKM with the most highly expressed at the top of the list. Only genes with a low t-test p value (t-test P: Age < 0.05), a Fold Change: 4mo/P2 >2 up/down, and above the Reliable Quantification Threshold in all samples from either group (i.e., the gene RPKM values were >RQT in all three P2 samples or in all three 4mo samples) were retained for further filtering.

1,801 downregulated transcripts. Of these 3,659 differentially expressed genes, 602 were unique to the PND2 time point and include *Dlk1*, *Hif3a, Agtr2* and *S100A9*, while 477 were only expressed at 4 months (*Cfd, Rtn1a, Clec3a, Adipoq, etc.*), leaving 2,580 common to both groups (**Figure 1C**). **Table 1** includes the top 20 mRNAs uniquely expressed at each time point based on RPKM value, which is indicative of mRNA abundance. Additional RT-qPCR analysis of independent cDNA samples validated RNA-seq findings in 10 out of 12 genes (85%) at a significance threshold of $p < 0.05$ (**Figures 1D, 4A and 5A,D** and data not shown).

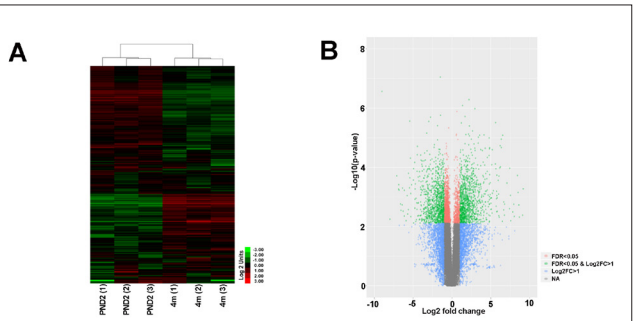
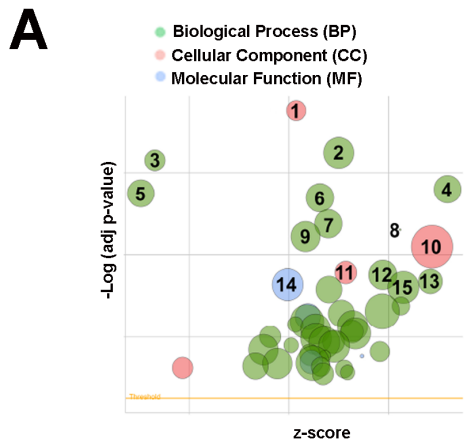


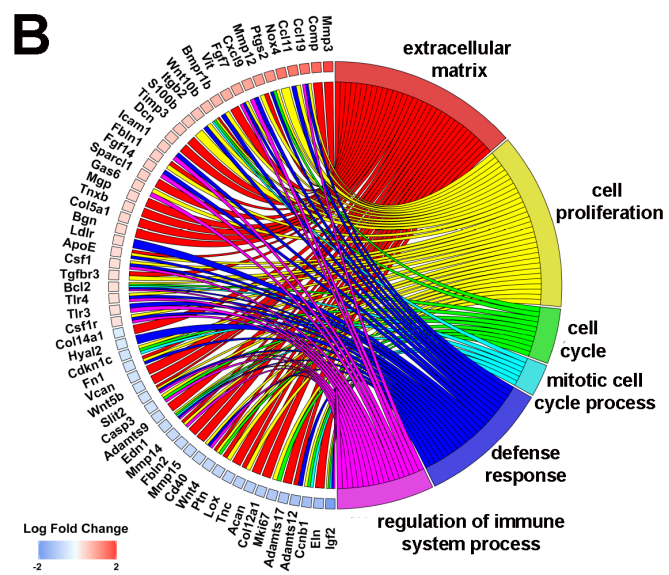
FIGURE 2 | RNA-seq analysis reveals transcriptional differences between PND2 and 4 month old murine aortic valves. **(A)** Hierarchical heatmap cluster shows clustering analysis of biological replicates (n = 3). **(B)** Volcano plot of differentially expressed genes sorted according to fold change and significance (FDR). Green dots represent genes with log2 fold change >1 and FDR <0.05, pink dots represent genes with FDR < 0.05, and blue dots represent genes with log2 fold change >1.

Transcriptional Analysis Identifies Age-Dependent Transcriptional Profiles and Biological Functions

Heatmap hierarchical clustering analysis, where 23,300 differentially expressed genes and samples were clustered using center correlation as the similarity measurement and average linkage as the clustering method, revealed molecular similarities between biological replicates at each time point and distinct differences between PND2 and 4 months (**Figure 2A**). Additional volcano plot analysis graphically displays the differential expression of 23,300 individual transcripts based on significance and fold change (**Figure 2B**). To determine functions associated with differential gene expression changes at each time point, Gene Ontology (GO) pathway analysis was performed. The bubble plot in **Figure 3A** visualizes the biological processes, cellular components, and molecular functions enriched by the differential data set and the table highlights the top 15 GO terms represented. These include biological processes such as cell proliferation, mitotic cell cycle, and defense response, along with cellular components such as extracellular matrix (ECM), indicating that valve maturation involves considerable changes in cell proliferation, ECM composition, and immune system programs. This is further highlighted in **Figure 3B** circle plot displaying genes which are known to be expressed in the heart valves based on previous publications, and their association with



	Category	GO ID	GO Term	Count	FDR	z-score
1	CC	GO:0031012	extracellular matrix	118	1.81E-19	0.368229847
2	BP	GO:0008283	cell proliferation	256	6.40E-17	2.5
3	BP	GO:1903047	mitotic cell cycle process	128	1.83E-16	-6.717514421
4	BP	GO:0006952	defense response	209	1.05E-14	7.954716331
5	BP	GO:0007049	cell cycle	204	1.86E-14	-7.421484445
6	BP	GO:0022610	biological adhesion	219	3.44E-14	1.55419597
7	BP	GO:0051240	positive regulation of multicellular organismal process	215	1.33E-12	1.977783584
8	BP	GO:0048002	antigen processing and presentation of peptide antigen	35	3.11E-12	5.578018081
9	BP	GO:2000026	regulation of multicellular organismal development	241	8.00E-12	0.837403614
10	CC	GO:0044421	extracellular region part	460	3.26E-11	7.180287023
11	CC	GO:0009986	cell surface	142	1.17E-09	2.853216618
12	BP	GO:0048584	positive regulation of response to stimulus	227	1.62E-09	4.712435512
13	BP	GO:0002682	regulation of immune system process	168	4.01E-09	7.097954098
14	MF	GO:0097367	carbohydrate derivative binding	267	6.30E-09	-0.061199006
15	BP	GO:0009605	response to external stimulus	268	9.14E-09	5.741963885



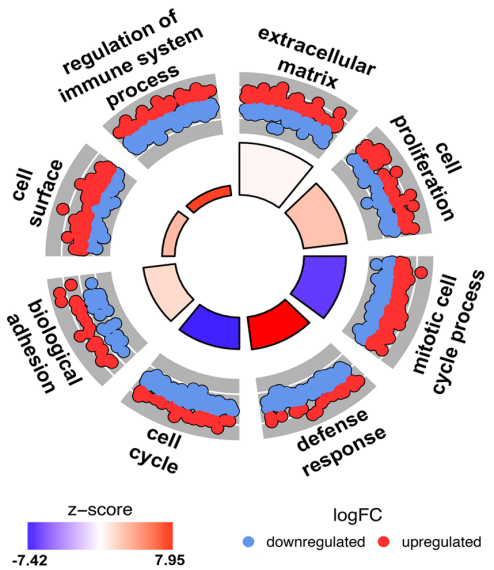


FIGURE 3 | Pathway analysis identifies diverse key biological processes, cellular components and molecular functions between PND2 and 4 month old murine aortic valves. **(A)** Gene ontology (GO) analysis reveals top differentially regulated GO terms, graphically displayed according to significance (*p*-value) and z-score, a measure of overall up or down regulation for the category. Data represented as a bubble plot (left). The size of each circle represents the number of differentially expressed genes in that GO term, while color represents the category (green: biological process pink: cellular component, blue: molecular function). Table (right) lists the top GO terms along with count, FDR, and z-score. **(B)** Chord plot showing 59 differentially expressed genes previously associated with healthy and diseased valves, and their overlap between significant GO terms as determined in **(A)**. Color below each gene corresponds to log fold change, with red representing increased expression at the 4 month time point and blue representing decreased expression at 4 months. **(C)** Circular plot highlighting gene expression differences within each selected GO term, with each red dot depicting a gene upregulated at 4 months in that category, and each blue dot showing a gene downregulated at 4 months. The height of the inner rectangle represents the *p*-value of the GO term and is colored according to z-score, with red being increased at the 4 month time point and blue being decreased at 4 months.

each GO term. More specific trends in these GO terms are shown in **Figure 3C**, with individually upregulated and downregulated genes in each category shown as red and blue dots, respectively. The inner rectangles are sized to positively correlate with the significance of each GO term, and colored to represent the overall direction of change in expression of each individual term. For example, the term "mitotic cell cycle process" has an overall down regulation at 4 months of age, while the "defense response" has an overall upregulation. In contrast, the "extracellular matrix" GO term is overall neither up-, nor downregulated, but the change in many individual transcripts is significant. Together these genomic analyses have defined transcriptional profiles of PND2 and 4 month aortic valve structures, and identified changes in functions associated with these mRNA patterns.

Proliferation Programs Are Downregulated in 4 month Old Aortic Valves

Based on enrichment of cell proliferation-related genes from GO analysis (**Table 2**), we first validated the fold change

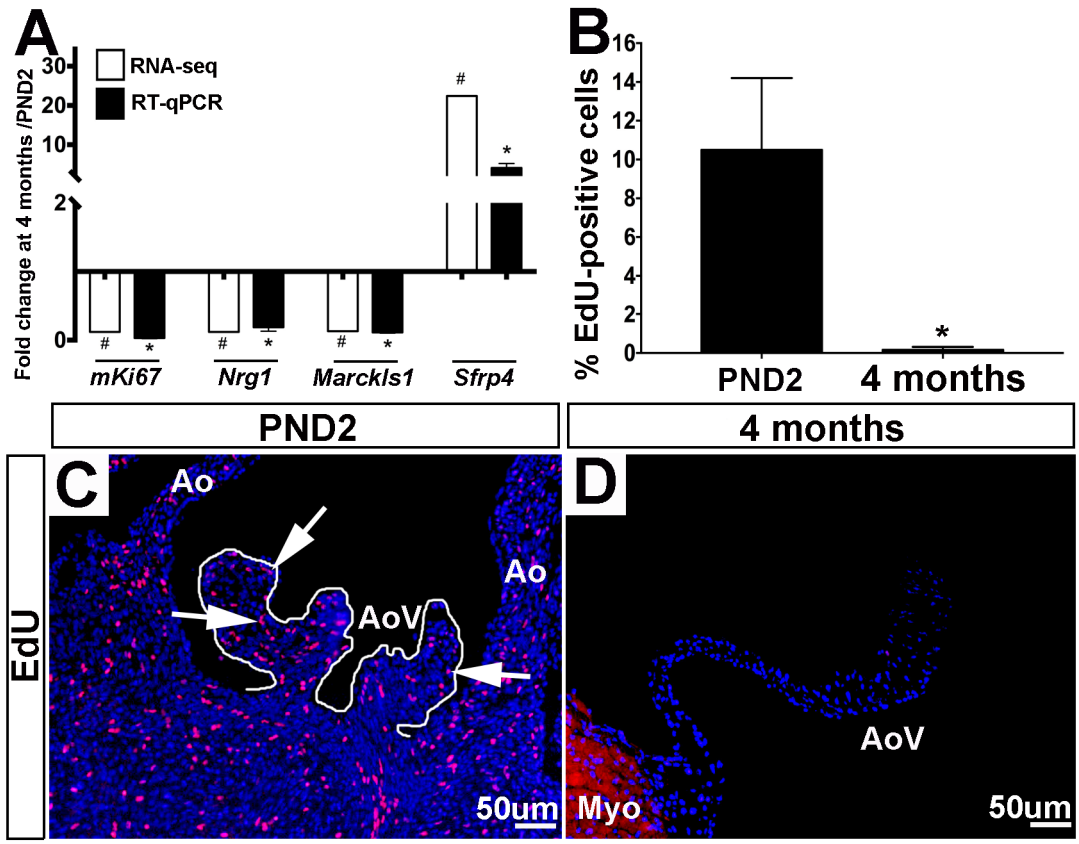


FIGURE 4 | Positive regulators of cell proliferation are enriched at PND2 in murine aortic heart valves. **(A)** RT-qPCR performed on independent samples (black bars) validates the RNA-seq fold change (white bars) trends for proliferation-related genes. Expression is normalized to 1, indicated by the x-axis. (n=3, *:p<0.05; two-tailed unpaired t-test, #:FDR<0.05). **(B)** Quantification of 5-ethynyl-2′-deoxyuridine (EdU) positive cells as a percentage of total cell nuclei (indicated by DAPI, blue) at PND2 and 4 month time points. (n=3, *:p<0.05, two-tailed unpaired t-test). **(C)** Representative images of staining for incorporation of EdU into replicating DNA (white arrows point to EdU-positive cells) reveals high levels of proliferation in PND2 AoV as compared to **(D)** EdU staining in 4 month old aortic valve. Ao, aorta; AoV, aortic valve; Myo, myocardium.

trends observed by RNA-seq using RT-qPCR (**Figure 4A**) on independent biological samples. These validated genes include three positive regulators of proliferation *mKi67, Nrg1* and *Marcksl1*, which all decreased in 4 month old aortic valves, and an anti-proliferative gene, *Sfrp4*, found to have increased expression at this time point (**Figure 4A**) (25–28). To further validate mRNA findings, we utilized 5-ethynyl-2′-deoxyuridine (EdU) to visualize and compare the number of cells actively undergoing mitosis in the aortic valve at PND2 and 4 months of age (**Figure 4B**-D). At the earlier time point, ~10.5% of cells were found to be EdU-positive, while only 0.16% of cells were proliferating at 4 months (**Figure 4B**). These data are consistent with transcriptional changes and previous reports from our lab showing that ~6.2% of VECs (of the total VEC population) and ~3.3% of VICs (of the total VIC population) are proliferative at post natal stages, while in the young adult, proliferation rates of both VECs (~2%) and VICs (~1.1%) were significantly lower (4). Together, these observations suggest that a decline in cell division at 4 months is due to a combination of decreasing expression of postnatal proliferation programs while simultaneously increasing adult programs which inhibit proliferation.

Postnatal and Adult Aortic Valves Have Distinct Extracellular Matrix mRNA Programs

As indicated by the GO term z-score in **Figure 3A**, the overall expression of ECM transcripts does not significantly increase or decrease with age, yet there are considerable differences in the specific ECM-related mRNAs that are expressed between the two time points (**Table 3**). Matrix metalloproteinases, or Mmps, are known to be expressed in both healthy and diseased valves and are associated with physiological and pathological remodeling of the ECM respectively (29–31). Also known as stromelysin-1, Mmp3 targets degradation of proteoglycans, collagens, and

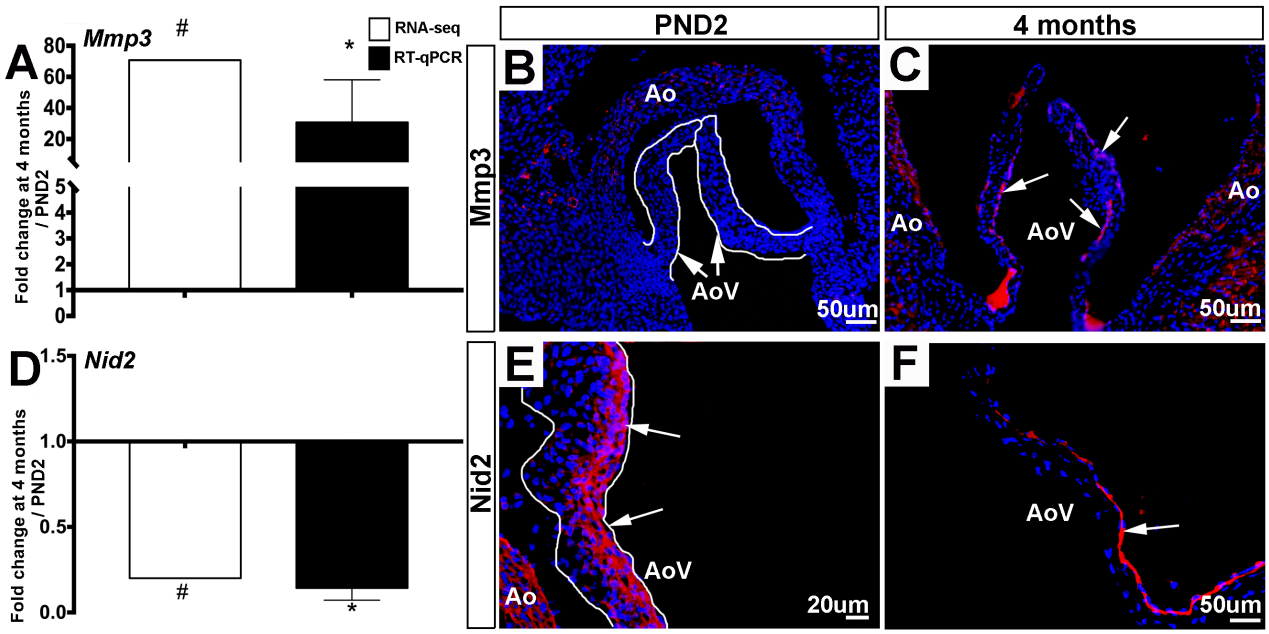


FIGURE 5 | ECM molecular profiles between PND2 and 4 month old murine aortic valves are distinct. **(A)** RT-qPCR performed on independent samples (black bars) validates the trends seen in RNA-seq data (white bars). Expression is normalized to 1, indicated by the x-axis (n=3, *:p<0.05; two-tailed unpaired t-test, #:FDR<0.05). **(B)** Immunofluorescence staining for Mmp3 reveals low expression at PND2 compared to **(C)** 4 months (white arrows indicate Mmp3-positive cells). **(D)** RT-qPCR performed on independent samples (black bars) validates the trends seen in RNA-seq data (white bars) for ECM gene *Nid2*. Expression is normalized to 1, indicated by the x-axis (n=3, *:p<0.05; two-tailed unpaired t-test, #:FDR<0.05). **(E)** Immunofluorescence staining for Nid2 reveals high expression atPND2 (white arrows indicate Nid2-positive cells) compared to **(F)** Nid2 staining at the 4 month timepoint. Ao, aorta; AoV, aortic valve.

elastins (32) and this Mmp family member has been described in cancer (33, 34), but little is known about Mmp3 in mouse valves. In this study, *Mmp3* increased from 1.22 reads per million kilobases (RPKM) at PND2 to 86.14 RPKM at 4 months. This significant increase was confirmed by RT-qPCR (**Figure 5A,B**) and immunofluorescence of aortic valve tissue sections, where Mmp3 is localized primarily to the sub-endothelial region of the leaflet (**Figure 5B**, **Figure S1**). In our previous VEC RNA-seq study, *Mmp3* transcript was not detected in the VEC population at any time point (4), and this is consistent with predominant expression of the protein in VICs; largely those close to the sub-endothelial location at 4 months (arrows, **Figure 5C**, **Figure S1**). In this study we show that the basement membrane ECM protein *Nidogen 2* (*Nid2*) was more highly detected in PND2 samples at 76 RPKM, while only 15 RPKM were detected at 4 months. This expression pattern was confirmed by RT-qPCR (**Figure 5D**) and immunofluorescence data shows broad Nid2 localization in both VECs and VICs at PND2, but more localized within the endothelial cell layer at 4 months (**Figure 5E,F**, **Figure S1**), and this pattern of decreased expression with maturation is consistent at the RNA level in VECs as previously described (4). These data show that PND2 and 4 month old aortic valves have distinct ECM-related transcriptional profiles associated with growth and maintenance respectively.

Gene Ontology Defense Response Markers Are Highly Enriched in 4 month Old Aortic Valves

As shown in **Figure 6A** and **Table 4**, a large majority (77%) of defense response genes are most highly expressed in aortic valve structures at 4 months of age and include *Ccl19, Ptgs2* and *Cxcl9*. The increased expression of *Ptgs2* (also known as *Cox2*) (**Figure 6B,C**) and *Rarres2* (also known as *Tig2*) (**Figure 6D,E**) are confirmed here by immunofluorescence. Ptgs2 is an enzyme involved in the synthesis of prostaglandins which are known to mediate pain and inflammation responses (35), and has been described in the valve as a pro-osteogenic marker (36). Consistent with this previous valve study, we observed expression towards the endothelium at 4 months of age (**Figure 6C**, **Figure S1**), and this is consistent with RNA expression in the VEC population (4). Like Ptgs2, Rarres2 is also known to regulate inflammation and has been linked with hypertension (37), a known risk factor of aortic valve stenosis (38). By immunofluorescence, Rarres2 is widely expressed throughout the valve leaflet including VECs and VICs at 4 months of age, which is a significant increase compared to PND2 (4). Overall, our RNA-seq data shows that expression of defense response-related genes increases with age in the murine aortic valve.

TABLE 2 | Genes included in the "Cell Proliferation" GO term.

Downregulated at 4 months	Upregulated at 4 months
AGTR2	ADIPOQ
H19	HSPA1A
BMP7	CD74
VASH2	PLA2G2D
IGF2	RBP4
IGF2BP1	H2-AA
FAM83D	H2-AB1
CTHRC1	VSIG4
CRH	CCL19
CDK1	CRLF1
NRK	CCL11
BEX1	NOX4
BUB1	SFRP4
IL31RA	CD209A
MELK	PTGFR
SCUBE2	BCL6
CCNB1	ITGAX
FIGNL1	KCNA1
AURKB	LGI4
UHRF1	ATF3
AGER	PTGS2
CDCA7L	AR
SHCBP1	ESR1
WISP1	CFB
NRG1	CEBPA
MKI67	APOD
HELLS	MMP12
ASPM	IL2RA
MCM10	RUNX3
IQGAP3	NR4A1
KIF20B	HSF4
MARCKSL1	CD274
BIRC5	ALDH3A1
RACGAP1	IFIT3
ROR2	FGF7
CHEK1	IL7R
TNC	CLEC11A
CDH3	WFDC1
SFRP2	AGAP2
E2F8	FLT3L
E2F7	FOLR2
SOX4	LIMS2
CDC20	GAPT
CENPF	SERPINF1
PTN	NR1D1
WNT4	FGF16
IRF6	BMPR1B
F2RL1	SERPINE2
FOXM1	IGHD
HMGA2	F3
MYCN	WNT10B
CD40	TRPV2
CDC6	CD28
MMP14	MLXIPL
TACC3	SIX1
EDN1	CPEB1
HMGB2	CYR61
SMARCA1	PTGIR
LAMC2	ECM1
GPC3	THPO
PROX1	CX3CL1
VASH1	ITGB2
CD276	ABCB1A
RNASEH2B	THRB
NASP	H2-M3
BLM	TOB2
ORC1	CHD5
NKX2-5	MUSTN1
CDK2	SAMD9L
TFRC	FTH1
LOXL2	PODN
CXADR	PTPRC
CASP3	FCGR2B
SLIT2	S100B
CAV3	FOSL2
DBN1	ATF5
EFNB2	COL18A1
FN1	BTG2
CDH5	PTPRU
SMYD2	COL4A3
TNFRSF13C	IL15
CDKN1C	NOV
DOT1L	FBLN1
RPS6KA2	JUN
SNAI2	PTAFR
MEG3	ADRB2
RIAN	SPN
RRM1	HAVCR2
HAS2	IL33
GM13275	RORA
DDR1	LRP1
ATPIF1	NTN1
GATA6	ITGAM
ERBB2	LEFTY1
MCM7	PID1
DISC1	GAS6
SCARB1	CST3
CTPS	EGR3
PTPRK	NAMPT
PTPRF	PDGFD
EDNRA	CD37
CD24A	CD9
SLC25A5	SLC11A1
TEK	CNN1
TRIM35	MVP
CNOT6	ANGPT1
KDM5B	H2-T23
PICALM	DOCK8
	TSPO
	CD86

TABLE 2 | Continued

Downregulated at 4 months	Upregulated at 4 months
	NACC2
	SPHK2
	HCLS1
	IRS2
	ZFP36
	CDKN1A
	TRF
	SLFN1
	IGFBP4
	RHBDD1
	DPT
	TNS2
	DOCK2
	NCF1
	PIK3CB
	APOE
	PAWR
	CD46
	CSF1
	IRF1
	CNTFR
	TACC2
	TGFBR3
	NUPR1
	BCL2
	VIPR2
	PTTG1
	TLR4
	RUNX2
	HYAL1
	IGFBP5
	CSF1R
	NCOA3
	PAK1
	PDE5A
	BRIP1
	CORO1A
	IFITM3

Genes are sorted according to fold change with the highest fold change at the top of the list.

DISCUSSION

This current study explores transcriptional differences in PND2 and 4 month old murine aortic valve expression profiles with the goal of identifying genetic programs representative of valve growth and valve maintenance, respectively. Long term, this may be important for the development of alternative therapies; specifically, those exploring the growth or regenerative capacity of adult diseased valves. Our results indicate that at PND2, dynamic leaflet growth is associated with a unique transcriptional profile compared to homeostatic adult valves at 4 months. Of the 23,303 detectable genes in our RNA-seq data, the number of differentially expressed transcripts found to be up and down regulated at each time point were approximately equal, suggesting that gene transcription patterns were not overtly altered,

TABLE 3 | Genes included in the "Extracellular Matrix" GO term.

Downregulated at 4 months	Upregulated at 4 months
S100A9	MYOC
COL24A1	MMP3
BMP7	GLDN
2010005H15RIK	CHAD
CTHRC1	MMP10
FREM2	COMP
COL26A1	ENTPD2
ELN	LAMC3
COL9A1	CILP2
FREM1	SOD3
ADAMTS12	FBLN7
FRAS1	NPNT
MFAP2	PRELP
ADAMTS17	MMP12
WISP1	COL4A6
HMCN1	VIT
LAMA1	CCBE1
COL12A1	CPXM2
ACAN	OPTC
TNC	SERPINF1
FREM3	SERPINE2
LAMB1	F3
LOX	CRISPLD2
EMILIN3	WNT10B
TFPI2	EPYC
NID2	AEBP1
PTN	CYR61
WNT4	ECM1
MMP15	ECM2
FBLN2	PODN
MMP14	SMOC2
TPSB2	COL5A3
PXDN	TIMP3
LAMC2	SMOC1
GPC3	COL18A1
ADAMTS9	DCN
GPC2	HIST1H4C
NID1	COL4A3
LOXL2	NOV
ANGPTL4	FBLN1
SLIT2	SPARCL1
WNT5B	SPN
VCAN	NTN1
CMA1	RARRES2
FN1	CST3
COL5A1	LGALS3BP
COL5A2	IGFBP7
LAMA3	VWA1
P3H1	MGP
TUBB5	TNXB
AGRN	COL15A1
SFPQ	BGN
ITGA6	THSD4
LAMC1	HIST1H4A
SLC25A5	TRF

Continued

TABLE 3 | Continued

Downregulated at 4 months	Upregulated at 4 months
CD93	LAMB2
COL4A1	DPT
	LMCD1
	APOE
	ADAMTSL5
	TGFBR3

Genes are sorted according to fold change with the highest fold change at the top of the list.

but rather transitioned from a postnatal to adult expression profile. Such profiles are related to enriched biological functions including cell proliferation at PND2 and defense response at 4 months of age. Interestingly, ECM components were enriched at both time points, but the associated mRNA profiles were unique. Together these analyses contribute to the current knowledge and further advance our understanding of the molecular signatures and biological functions characteristic of the whole aortic valve structure at PND2 and 4 months. provide critical information related to genetic programs in the growing and homeostatic heart valve.

PND2 murine aortic valves are defined by a specific transcriptional profile including the unique expression of 602 genes (**Figure 1C**, **Table 1**) that were not detected at 4 months of age. In addition, 1,801 transcripts were upregulated at PND2, while 1,858 were decreased compared to the adult homeostatic valve. According to Gene Ontology analysis, many of the transcripts enriched at PND2 suggest an overall upregulation of active cell proliferation by a specific set of genes. These include increased expression of those associated with active cell division (*Ki67, Aurkb*), pro-proliferation markers (*Bub1, Cdk1, Foxm1, Nrg1*) (27, 39, 40) and decreased expression of proliferation inhibitors (*Nox4, Sfrp*) (41–44). This specific expression profile of cell proliferation markers supports EdU observations and represents active elongation of the immature valve structure. It will become important to understand how this molecular signature of cell proliferation is downregulated after maturation is complete and explore the potential of re-introducing key regulators to stimulate cell division and replenish dysfunctional cell populations in the adult following injury or disease.

In addition to high levels of cell proliferation, the PND2 valve is characterized by a specific ECM mRNA profile (**Table 3**) which likely corresponds to the mechanical demands during the postnatal period. When comparing PND2 to 4 months, RNA-seq analysis reveals significant enrichment of highly expressed fibrillar collagens including *Col24a1*, *Col9a1*, and *Col5a1*, indicating the need for additional stability as the growing postnatal valve adapts to hemodynamic changes in response to closing of the foramen ovale (45, 46). RNA-seq analysis also uncovered higher PND2 levels of ECM proteins such as *Frem1*/*Frem2* and *Nidogen2* (*Nid2*), which have been shown to stabilize basement membranes underlying endothelial cells and may provide further structural integrity to the developing valve (47, 48). Besides identifying the enrichment of differentially expressed fibrillary collagens and specific proteoglycans, RNA-seq analysis shows that the PND2 valve also expresses a distinct profile of ECM enzymes, such as *Mmp15* and *Adamts17*,

TABLE 4 | Genes included in the "Defense Response" GO term.

Downregulated at 4 months	Upregulated at 4 months
AGTR2	CFD
S100A9	ADIPOQ
CITED1	HAMP
IGF2	HP
COLEC10	CD74
NGP	CCL8
C1QTNF3	H2-AA
CRH	H2-EB1
S100A8	H2-AB1
IL31RA	CLEC10A
ADAMTS12	GM2564
CHAF1B	CCL19
AGER	CCL11
PBK	C4B
ULBP1	C4A
RNASEL	PTGFR
RAET1E	BCL6
ELF3	ITGAX
BRINP1	PTGS2
RAET1D	ESR1
F2RL1	CFB
CD40	APOD
LCK	IL2RA
MASP1	H2-Q7
TPSB2	FFAR4
MDK	CXCL9
EDN1	ITIH4
HMGB2	SLAMF7
SLFN9	C1S1
CPA3	IFIT3
CD276	C1S2
TSPAN6	GBP2
TYRO3	FGF7
CASP6	C1RA
CCR1	C1RB
SLIT2	WFDC1
FN1	OAS2
GM13275	GPR17
GGT5	PELI3
HYAL2	MYLK
EDNRA	CFH
CD24A	ISG20
FANCA	H2-K1
TRIM35	SERPINF1
SUSD4	NR1D1
CD93	CLEC7A
SLC35B3	BMPR1B
	C1RL
	F3
	LYZ1
	TGTP1
	SLAMF8
	TGTP2
	ITK
	HRH1

Continued

TABLE 4 | Continued

Downregulated at 4 months	Upregulated at 4 months
	ALOX5
	CD28
	NR1H3
	PSTPIP1
	IRAK3
	LYZ2
	H2-D1
	TAP2
	APOBEC1
	GNG7
	PTGIR
	ECM1
	CX3CL1
	ITGB2
	CD14
	CCRL2
	HIST2H3C2
	GBP6
	GBP10
	H2-M3
	C3
	MILL2
	SERPING1
	CYBB
	PTPRC
	FCGR2B
	RAB7B
	HIST1H2BE
	TRIM30A
	NFKBIZ
	ICAM1
	SLFN8
	IFIH1
	HIST1H2BJ
	FAS
	IL15
	NOV
	PTAFR
	MAP1A
	CASP1
	MYO1F
	ADRB2
	FGF14
	HIST1H2BC
	SPN
	HAVCR2
	IL33
	B2M
	COLEC12
	RORA
	ITGAM
	TAP1
	RARRES2
	CST3
	ZC3H12A

Continued

TABLE 4 | Continued

Downregulated at 4 months	Upregulated at 4 months
	IRGM2
	NAIP5
	HIST1H2BL
	IGTP
	PNMA1
	HIST1H2BK
	CD37
	TLR8
	SLC11A1
	MGLL
	IL17RE
	HIST1H2BN
	CADM1
	H2-T23
	HERC6
	CD86
	SETD6
	ZFP36
	THEMIS2
	LDLR
	HFE
	C1QA
	IGFBP4
	LMCD1
	ALOX5AP
	NCF1
	GBP8
	APOE
	TNFRSF25
	SERPINB9
	CD46
	CSF1
	IRF1
	NUPR1
	BCL2
	STAR
	DRD1
	PIK3AP1
	IRF8
	TLR4
	TLR3
	BIRC3
	HYAL1
	CSF1R
	C1QB
	UNC93B1
	TRIM21
	PDE5A
	PTGIS
	CORO1A
	AOAH
	IFITM3

Genes are sorted according to fold change with the highest fold change at the top of the list.

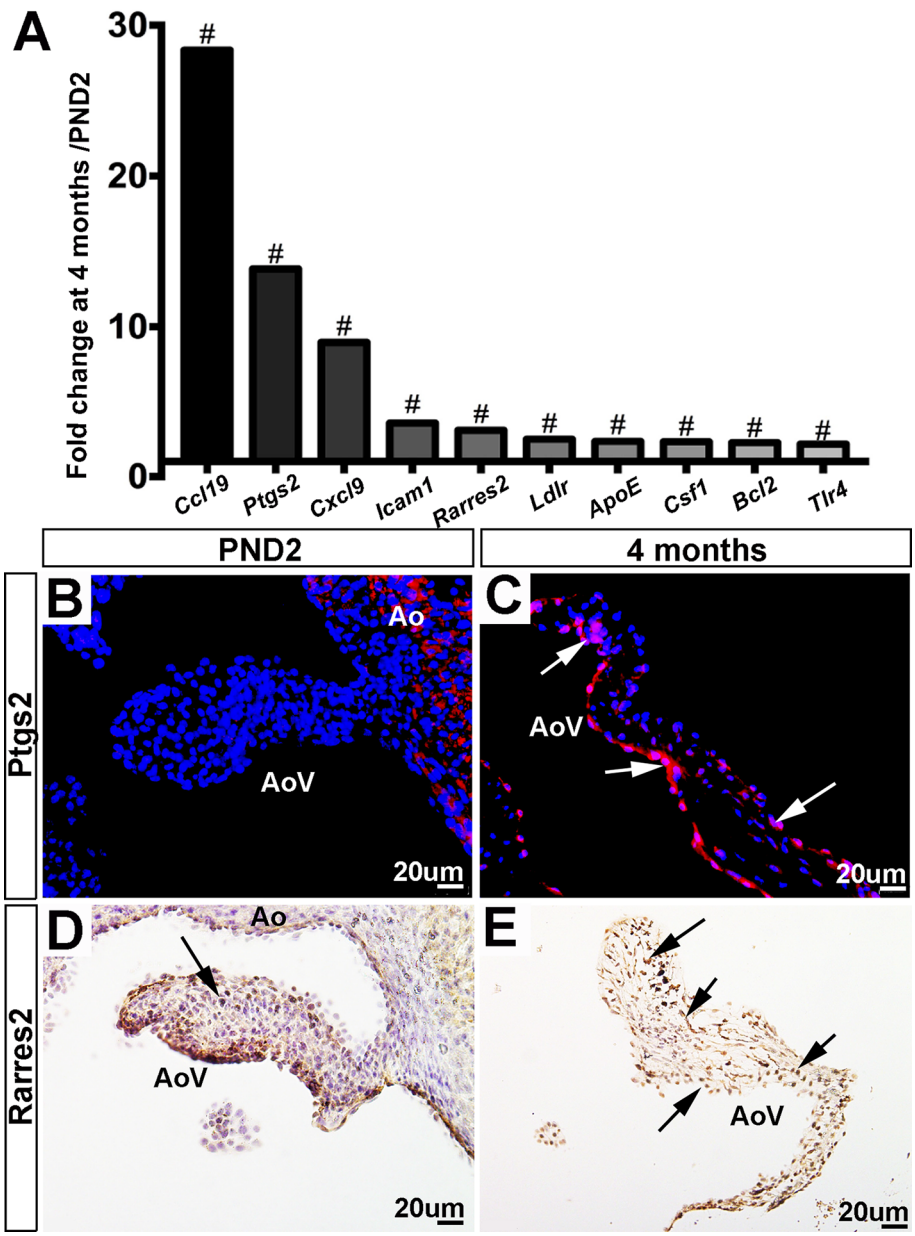


FIGURE 6 | Expression of defense-related genes increases with valve maturation. **(A)** RNA-sequencing fold changes of defense-related genes. Expression is normalized to 1, indicated by the x-axis (#:FDR < 0.05). **(B)** Immunofluorescence staining for Ptgs2 validates RNA-seq trends, showing low expression at PND2 compared to **(C)** Ptgs2 staining at the 4 month time point (white arrows indicate Ptgs2-positive cells). **(D)** Immunofluorescence staining for Rarres2 validates RNA-seq trends, showing low expression at PND2 compared to **(E)** Rarres2 staining at the 4 month time point. Ao, aorta; AoV, aortic valve.

indicating the need for physiological remodeling during growth and maturation

The 4 month adult valve is physically and molecularly distinct from the postnatal valve, with elongated leaflets containing distinct layers of collagen, proteoglycan, and elastin (**Figure 1B**). At this time point, 477 transcripts were found to be uniquely expressed, with the most abundant unique mRNAs including *Cfd*, *Retnla*, and *Clec3a*. In contrast to the PND2 aortic valve, the adult valve displays significantly decreased levels of cell proliferation, likely due to increased expression of proliferation inhibitors and lack of enrichment of positive regulators. At 4 months, the valve ECM is diverse compared to PND2 (**Table 3**) and likely reflects differences in biomechanical demand in response to the adult circulatory system (49, 50). Similar to the PND2 valve, collagens and proteoglycans are predominant. However, the most highly differentially expressed collagens are those associated with basement membranes, including *Col4a6* and *Col18a1*, which act as a cell scaffold to maintain current cell populations and cell integrity as opposed to providing support for high cell turnover. In addition, the proteoglycan profile is moved towards enrichment of decorin (*DCN*) and biglycan (*BGN*) consistent with previous studies in aging pigs (51). Furthermore, the contribution of ECM remodeling enzymes is shifted to *Mmp3* and its

inhibitor *Timp3* at 4 months, possibly indicating a differential need of the ECM to sustain homeostasis. Previous studies have suggested correlations between VIC phenotype and ECM composition (52, 53) and therefore we anticipate that our findings at 4 months are related to the quiescent VICs, while the diversity at PND2 is dictated by proliferative and active VICs.

One of the most prominent differences in expression profiles between the PND2 and 4 month aortic valve is the considerable upregulation of defense-related transcripts at the older time point, indicating increased immune system activation with valve maturation (**Figure 6**, **Table 4**). Previous studies from other groups have shown that the appearance of immune markers such as *Ptgs2* and *Rarres2* precedes the onset of disease both in the heart valve and other cardiovascular systems (36, 37). In addition, there is increasing evidence to suggest that inflammation in the valve is an initial homeostatic repair mechanism activated in response to minor valve injuries sustained throughout life, but that this repair mechanism may become pathogenic if overactive or long-lasting [reviewed (54)]. Our study suggests that some level of activation of the immune system in the valve is present at 4 months of age under homeostatic conditions, however, it is not clear whether these defense markers are an early indication of valve degeneration, or a root cause of disease themselves. Further investigation into target genes such as *Cfd* and *Adipoq* will give insight into the possible role of defense response genes in valve disease therapeutics.

Our data shows that postnatal heart valves contain highly proliferative VICs producing a distinctive set of postnatal ECM proteins, while adult VICs are mainly quiescent and are associated with a very different ECM composition as well as increased defense response markers. A limitation of our study is that RNA-seq analysis was performed on whole valve tissue and therefore RNA profiles cannot be distinguished between VEC and VIC populations, however IHC studies shown here, in combination with a previous study from our group (4) support enrichment towards one cell type. Nonetheless, we have unveiled multiple conceivable processes that contribute to postnatal valve maturation and maintainence that pave the way for elucidiating mechanisms underlying valve defects present at birth and those acquired later in life. Furthermore, the basic priciples of cell proliferation and ECM remodeling may also be applied to valvulogenesis and congenital valve malformation. From here further research is needed to determine how findings in this study can be used to develop alternative therapeutic strategies to promote self-repair, replenishment and regeneration of valve cell populations in pathogenesis.

ETHICS STATEMENT

All animal procedures were approved by The Research Institute at Nationwide Children's Hospital Institutional Animal Care and Use Committee (Protocol # AR13-00054).

AUTHOR CONTRIBUTIONS

The experimental data was collected by EN, and RNA-seq performed and analyzed by CN. Additional analysis of RNA-seq data was undertaken by SL. EN and JL generated the manuscript with input from SL. The entire study was overseen by JL.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcvm.2018.00030/full#supplementary-material

FIGURE S1 | Cell localization of Mmp3, Nid2 and Ptgs2 in the aortic valve at 4 months of age. Immunohistochemistry to show Mmp3 **(A)**, Nid2 **(B)** and Ptgs2 **(C)** expression (red) relative to CD31 (endothelial cells, green) in 4 month old aortic valve leaflets. Arrows indicate protein expression in VICs, arrowheads denote staining in CD31-positive VECs.

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