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THE PRESENT AND FUTURE

STATE-OF-THE-ART REVIEW

Calcification in Aortic Stenosis

The Skeleton Key

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ABSTRACT

Aortic stenosis is a common, potentially fatal condition that is set to become an increasing public health burden. Once symptoms develop, there is an inexorable deterioration with a poor prognosis. Despite this, there are no medical therapies capable of modifying disease progression, and the only available treatment is aortic valve replacement, to which not all patients are suited. Conventional teaching suggests that aortic stenosis is a degenerative condition whereby "wear and tear" leads to calcium deposition within the valve. Although mechanical stress and injury are important factors, it is becoming increasingly appreciated that aortic stenosis is instead governed by a highly complex, regulated pathological process with similarities to skeletal bone formation. This review discusses the pathophysiology of aortic stenosis with an emphasis on the emerging importance of calcification, how this can be visualized and monitored using noninvasive imaging, and how our improved knowledge may ultimately translate into novel disease-modifying treatments.
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Aortic stenosis is the most common form of valve disease in the Western world and is set to become an ever-increasing public health burden (1,2). Despite this, there are no medical therapies to halt or delay disease progression, and the only available treatment is aortic valve replacement or implantation, to which not all patients are suited. There is, therefore, a major unmet clinical need to identify pharmacological treatments capable of modifying this disease process.

Aortic stenosis was long considered to be a degenerative condition whereby "wear and tear" resulted in progressive calcium formation within the valve. Although mechanical stress and injury remain central to its pathophysiology, emerging evidence has indicated that aortic stenosis develops as part of a highly complex and tightly regulated series of processes, each of which may be amenable to medical intervention (3). In particular, aortic stenosis can be divided into 2 distinct phases: an early *initiation phase* dominated by valvular lipid deposition, injury, and inflammation, with many similarities to atherosclerosis, and a later *propagation phase* where pro-calcific and pro-osteogenic factors take over and ultimately drive disease progression (**Figure 1**) (4). This review discusses the pathophysiology of aortic stenosis, with an emphasis on the emerging importance of calcification, how this can be imaged with modern noninvasive techniques, and how our improved knowledge might ultimately lead to the development of novel therapies.

PATHOLOGY OF AORTIC STENOSIS

INFLAMMATION, LIPIDS, AND THE INITIATION PHASE OF AORTIC STENOSIS. Under normal circumstances, the aortic valve is composed of 3 leaflets, each of which is a thin (<1 mm), smooth, flexible, and

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ABBREVIATIONS AND ACRONYMS

BMP = bone morphogenetic protein

CT = computed tomography

FDG = fluorodeoxyglucose

LDL = low-density lipoprotein

OPG = osteoprotegerin

PET = positron emission tomography

RANK = receptor activator of nuclear factor kappa B

RANKL = receptor activator of nuclear kappa B ligand

TGF = transforming growth factor

VIC = valvular interstitial cell

mobile structure (3). In aortic stenosis, these leaflets become thickened, fibrosed, and calcified, resulting in reduced leaflet mobility and progressive valvular obstruction.

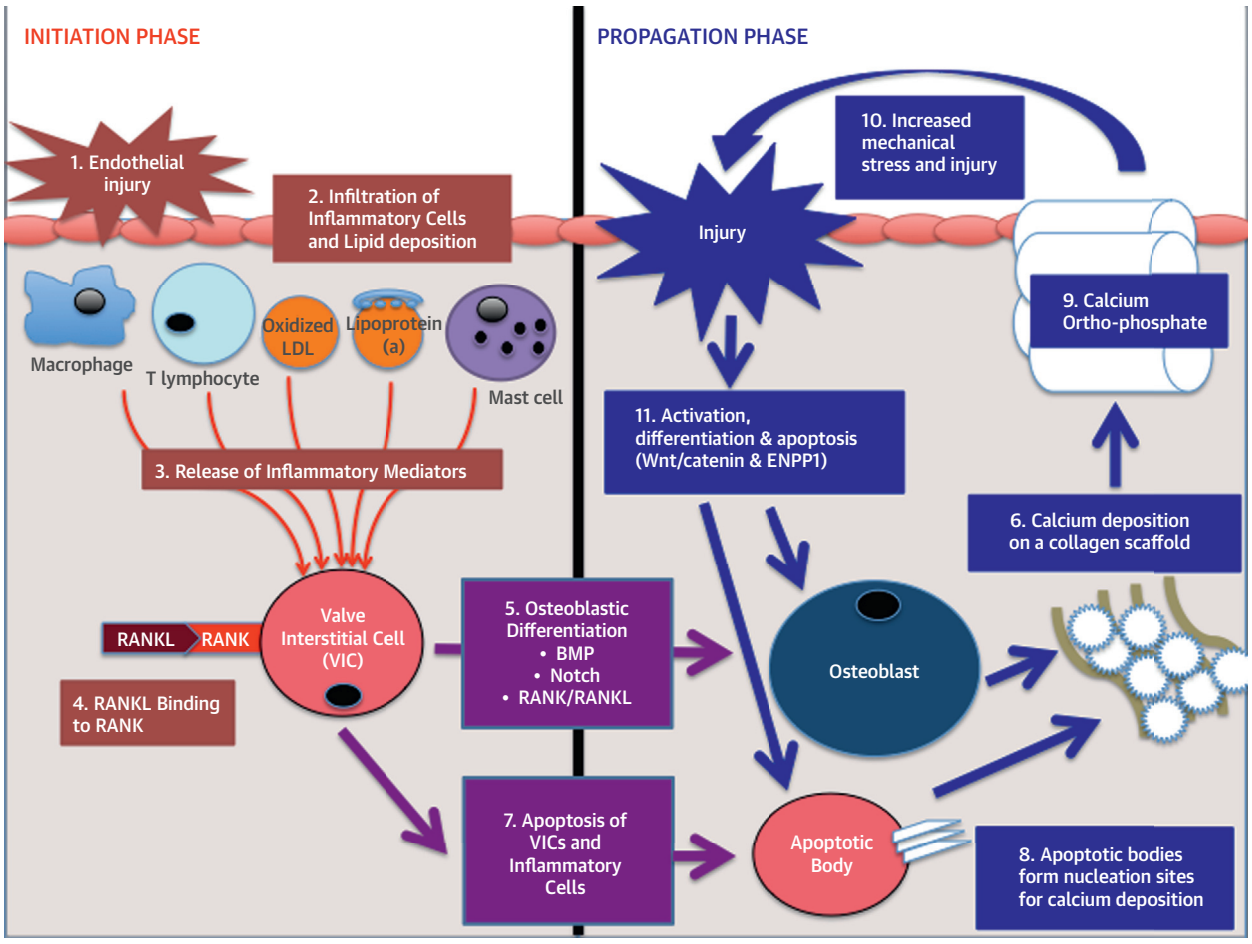
The early stages of aortic stenosis are in many ways similar to atherosclerosis. Indeed, the 2 conditions share many common risk factors, with large longitudinal studies consistently demonstrating that the *incidence* of aortic stenosis is linked to factors such as smoking, age, and hypertension (5–7). As in atherosclerosis, endothelial damage due to increased mechanical stress and reduced shear stress is believed to be the initiating injury, perhaps best illustrated by bicuspid valve disease. The characteristic 2-leaflet structure of these valves results in less efficient dissipation of mechanical stress and accelerated endothelial damage, so that patients almost universally develop aortic stenosis and display more rapid disease progression (8).

Following endothelial damage, the same lipids implicated in atherosclerosis infiltrate the valve, in particular, lipoprotein(a) and oxidized low-density lipoprotein (LDL) cholesterol. Consequently, observational studies have identified cholesterol and its related lipoproteins as independent risk factors for the development of aortic stenosis (5–7,9). Indeed, a strong genome-wide association was recently established between a single-nucleotide polymorphism in the locus of lipoprotein(a) and the incidence of aortic valve calcification (10). Progressive endothelial injury and lipid oxidization then establishes an inflammatory response within the valve that is characterized predominantly by infiltration of macrophages, but also involves T lymphocytes and mast cells (11). At this early stage, regions of stippled microcalcification that colocalize with sites of lipid deposition are observed (11). The formation of these microcalcifications may be mediated by cell death and the release of apoptotic bodies in these areas. Such apoptotic bodies are similar to the matrix vesicles found in bone, which contain the prerequisite components for calcium crystal deposition (including calcium and inorganic phosphate ions) and facilitate the formation of needle-like crystals of hydroxyapatite (4,12). In bone, as these hydroxyapatite crystals expand, they pierce the outer membrane of the vesicle and become exposed to the extracellular environment, thereby forming nucleation sites for further calcium deposition. It is probable that similar processes also occur within the valve (13). Furthermore, hydroxyapatite deposition evokes further proinflammatory responses from macrophages, creating a positive feedback loop of calcification and inflammation in the early stages of disease (14). It seems likely that these mechanisms underlie early calcium formation in aortic stenosis and its association with lipid and inflammation.

The apparent link between lipid, inflammation, and calcification in these early stages and the pathological similarities with atherosclerosis led to the hypothesis that statins might be beneficial in patients with aortic stenosis. This was supported by encouraging nonrandomized human data (15) and studies in hypercholesterolemic animal models demonstrating that lipid deposition and oxidative stress precede the conversion of valvular interstitial cells to those with an osteoblastic phenotype, and that this process is inhibited by atorvastatin (16,17). However, when statins were formally tested in 3 independent randomized controlled trials of patients with aortic stenosis, each demonstrated a failure of this therapy to halt or retard aortic stenosis progression, despite reducing the serum LDL cholesterol concentrations by more than one-half (18–20). This has led investigators to re-examine the pathophysiology underlying aortic stenosis and to the realization that although inflammation and lipid deposition may be important in establishing the disease (the initiation phase), the later stages are instead characterized by an apparently self-perpetuating cycle of calcium formation and valvular injury (the propagation phase) (4). Indeed, once this propagation phase has become established, disease progression is dictated neither by inflammation nor by lipid deposition, but rather by the relentless accumulation of calcium in the valve leaflets. This may explain the failure of statins to modify disease progression in aortic stenosis, which commonly presents beyond the initiation phase. Moreover, there is some data that statins may even be procalcific in the vasculature (21,22).

CALCIFICATION AND THE PROPAGATION PHASE. Skeletal bone formation is characterized by the initial deposition of collagen matrix, which provides a scaffold upon which progressive calcification can develop. With time, this calcium acquires a more ordered crystalline structure until the characteristic features of lamellar bone are finally observed. Similar structural processes are believed to occur in the aortic valve, with many of the same cell mediators and proteins implicated (23). Indeed, in aortic stenosis, collagen is deposited in anticipation of the procalcific processes that subsequently dominate. This fibrotic process within the valve may be mediated, in part, by reduced nitric oxide expression following endothelial injury (24); however, the renin-angiotensin system (RAS) is also believed to play a central

FIGURE 1 The Pathophysiology of Aortic Stenosis



Initiation phase: endothelial injury **(1)** facilitates the infiltration of oxidized lipids and inflammatory cells **(2)** into the valve and the release of proinflammatory mediators **(3)**. These trigger the very early stages of valve calcification. The propagation phase: these proinflammatory processes subsequently induce VICs to undergo osteogenic differentiation **(5)** via several different mechanisms, including the binding of RANKL to RANK **(4)**. Differentiated cells within the aortic valve first lay down a collagen matrix and other bone-related proteins causing valvular thickening and stiffening before producing calcium **(6)**. Additionally, apoptotic remnants of some VICs and inflammatory cells **(7)** create a nidus for apoptosis-mediated calcification **(8)**. Calcification of the valve **(9)** induces compliance mismatch, resulting in increased mechanical stress and injury **(10)**. This results in further calcification via osteogenic differentiation and apoptosis **(11)**. Hence, a self-perpetuating cycle of calcification, valve injury, apoptosis, and osteogenic activation is established that drives the propagation phase of the disease. BMP = bone morphogenetic protein; ENPP1 = ectonucleotide pyrophosphate 1; LDL = low-density lipoprotein; RANK = receptor activator of nuclear kappa B; RANKL = receptor activator of nuclear kappa B ligand; RAS = renin-angiotensin system; VIC = valvular interstitial cell.

role. Angiotensin-converting enzyme (ACE) is up-regulated in calcific aortic valve disease and is likely to be delivered to the valve by LDL, its natural vehicle (25). Here it facilitates the conversion of angiotensin I to II, which mediates profibrotic effects via the angiotensin II type 1 (AT_1) receptor. Although angiotensin II is also able to mediate antifibrotic and anti-inflammatory effects via angiotensin II type 2 (AT_2) receptors, differential expression of these receptors in favor of AT_1 has been demonstrated in calcified aortic valves, so that a profibrotic profile dominates. Likewise, although angiotensin-converting enzyme type 2 (ACE-2) exerts antifibrotic and anti-inflammatory influences via the Ang1-7/Mas pathway, this pathway is down-regulated in calcified aortic stenosis, with reduced expression of both ACE-2 and Mas receptors in calcified valves compared with control subjects (26). Increased RAS expression is, therefore, implicated in the development of fibrosis within the valve. On a systemic level, RAS is implicated in the development of hypertension, which often accompanies aortic stenosis and

may accelerate its progression given the increased mechanical stress it imposes upon the valve (27).

Beyond this initial fibrosis, valvular calcification in aortic stenosis ultimately dominates and appears dependent upon the presence of osteoblast-like cells that develop an osteogenic phenotype. In support of this hypothesis, gene-profiling studies have demonstrated increased valvular expression of several osteoblast-specific proteins, including the Cbfa1/Runx2 transcription factor, essential for osteoblastic differentiation and regulation of osteoblast function (28,29). A number of other extracellular matrix proteins closely associated with osteoblast function and more commonly associated with skeletal bone formation are also up-regulated in calcific aortic valves. These include osteopontin and bone sialoprotein, which are facilitators of the attachment of osteoblasts to the bone matrix, and demonstrate up to a 7-fold elevation in gene expression at sites of developing calcification (30,31). Importantly, valvular ossification also appears to be dependent upon angiogenesis, supporting the hypothesis that this is an active, highly regulated, pathological process (23).

The source of osteoblast-like cells within the aortic valve remains controversial. In vitro, multiple cell types present in the vasculature are capable of undergoing differentiation into those with an osteoblast-like phenotype. The most likely candidate appears to be the myofibroblast, a highly plastic cell that is also commonly referred to as the valve interstitial cell (VIC) (32). The differentiation of this cell into an osteoblast phenotype is not fully characterized, but appears to be a central step in the development of aortic stenosis and is regulated by a rapidly growing list of molecules and complex pathways. In vivo molecular imaging has demonstrated that in the early stages of aortic stenosis, this differentiation appears coordinated by macrophages (33,34) via the action of proinflammatory cytokines (interleukin [IL]-1β, IL-6, IL-8, tumor necrosis factor [TNF]-α, insulin-like growth factor-1, and transforming growth factor [TGF]-β) (4,35,36). However, in the later stages, this differentiation again appears to be dominated by calcific pathways, including the Notch, Wnt/β-catenin, and receptor activator of nuclear factor kappa B (RANK)/receptor activator of nuclear factor kappa B ligand (RANKL)/osteoprotegerin (OPG) pathways, which we discuss here.

Notch belongs to a family of cell surface receptors (Notch 1 to 4) that are highly expressed in the aortic valve, playing an important role in its morphological development (37). Individuals with loss-of-function mutations in Notch-1 have higher rates of cardiovascular calcification and aortic stenosis. In 2 unrelated families with a high incidence of congenital aortic valve disease, genome-wide linkage analysis identified loss-of-function Notch-1 mutations as the cause (37). In particular, Notch-1 appears to be important in establishing osteogenic cells in the valve via the action of bone morphogenetic protein (BMP)-2 (38). BMP-2 is a potent osteogenic differentiation factor and part of a family of multifunctional cytokines belonging to the TGF-β superfamily. Expression of BMP-2 is increased in calcified atherosclerotic lesions and aortic valves (29,39), and it appears to have a central role in the differentiation of plastic cell populations toward an osteogenic phenotype. Indeed, exposure of normal human VICs to BMP-2 induces osteoblastic features in these cells (40,41). In addition, binding of Wnt to LDL receptor-related protein 5 receptors may activate the canonical Wnt/β-catenin pathway that is also implicated in osteogenic cell differentiation (42). Similarly, TGF-β1 is able to induce nuclear translocation of β-catenin and increased Wnt signaling, stimulating the osteogenic differentiation of mesenchymal progenitor cells (43). The latter process can increase in response to mechanical stress and may therefore explain, in part, the self-perpetuating and exponential increase in calcification activity observed once osteogenic differentiation has occurred and the propagation phase is established (**Figure 1**) (43,44).

Systemic regulators that govern calcification activity, both in the bone and in the vasculature, tightly control calcium homeostasis; consequently, there is an inverse correlation between bone mineral density and vascular calcification. Osteoporosis is associated with age-independent increases in vascular calcification and even cardiovascular mortality (45). A prospective study of 25,639 men and women demonstrated an inverse correlation between bone mineral density and incident aortic stenosis in older women (46). Moreover, other disorders of bone turnover, including chronic kidney disease and Paget's disease, also manifest changes in the vasculature (47–50). This dichotomy has been termed the "calcification paradox" and is likely to be explained by common pathological pathways having reciprocal effects on the bone and vasculature simultaneously.

A potential mechanism for this association lies in the activity of the RANK/RANKL/OPG pathway (**Figure 2A**). In bone, RANKL (a member of the TNF cytokine family) binds to RANK (a transmembrane protein expressed on marrow stromal cells and pre-osteoclasts), acting as a potent inducer of osteoclast

differentiation and activity. This drives demineralization of bone, but is policed by osteoprotegerin (OPG), a soluble decoy receptor, which binds RANKL and prevents it from activating RANK (**Figure 2**). In contrast, RANKL appears to have the opposite effect on cells in the vasculature, inducing an osteoblastic phenotype in human VIC cells that results in increased matrix calcification, the formation of calcific nodules, and increased expression of alkaline phosphatase and osteocalcin (**Figure 2A**) (51). RANKL also promotes the osteogenic properties of vascular smooth muscle cells, once again via the up-regulation of BMP-2. As a consequence, whilst OPG-deficient mice develop osteoporosis, they simultaneously accelerate vascular calcification in association with increased expression of RANKL in both regions (52). A potential explanation for the differential effects of RANK/RANKL/OPG in these 2 tissues is that in bone there is an abundance of pre-osteoclasts that favors the pro-osteoclastic properties of RANKL (36). In contrast, this pool is absent in the vasculature so that RANKL's pro-osteoblastic effects on myofibroblast and smooth muscle cells predominate.

Imbalances in RANKL/OPG signaling have been demonstrated in calcific aortic valves. In human valve tissue taken from patients with aortic stenosis, immunochemistry revealed less OPG-positive cells in areas of focal calcification, whereas western blotting demonstrated that OPG was not expressed at relevant levels in aortic stenosis, but was detectable in control subjects. The converse is true of RANKL, with increased levels observed in stenotic aortic valves (51). In combination, these data support the hypothesis that the RANK/RANKL/OPG axis is implicated in the development of aortic valve calcification and provide 1 explanation for the link between aortic valve calcification and bone mineral density. Other investigators have suggested that the differential effects of oxidized LDL might also be of importance, with this molecule appearing to promote calcification and the osteoblastic differentiation of vascular cells in vitro, whilst inhibiting these processes in a bone-derived pre-osteoblast cell line (4,53,54).

Fetuin-A is a circulating protein that can exist in isolation or as a complex with matrix γ-carboxyglutamic acid protein (MGP). Both are powerful guardians against ectopic calcification and simultaneously inhibit many of the procalcific processes discussed earlier (55). MGP needs to be both carboxylated and phosphorylated to be activated, a process dependent on vitamin K. There is speculation that use of the vitamin K antagonists, such as coumarins, may be associated with increased vascular calcification (56). The actions of fetuin-A and MGP include inhibition of BMP2 and TGF-β, reduction of apoptosis-mediated calcification, and direct prevention of calcification by binding to calcium crystals. Reduced circulating levels of Fetuin-A and MGP are thought to explain the vascular calcification seen with end-stage renal failure. Moreover, plasma fetuin-A concentrations are decreased in aortic stenosis and inversely associated with the rate of disease progression (57,58). Interestingly, this association was seen only in older patients (>70 years of age) (59). Conversely, increased plasma dephosphorylated (inactive) MGP was a strong independent predictor of faster stenosis progression, but only in younger patients (≤57 years of age) (60).

WHY DOES CALCIUM BEGET CALCIUM? Once calcification is established in the valve, it would appear to initiate further calcium formation. This self-perpetuating cycle of calcification and valve injury appears to be the central driver of *disease progression* and the propagation phase of aortic stenosis (**Figure 1**).

The mechanism for this may, in part, relate to the compliance mismatch caused by calcific deposits in the leaflets that results in increased mechanical stress, injury-induced activation of the Wnt/β-catenin pathway, and further osteoblast differentiation. However, it may also be explained by the actions of membrane-bound ectonucleotidases. These are produced by VICs and regulate the extracellular production of inorganic phosphate (a promoter of calcification) and inorganic pyrophosphate, an inhibitor of pyrophosphate. Ectonucleotide pyrophosphatase 1 (ENPP1) is highly up-regulated in calcific aortic valve disease, with a polymorphism associated with increased transcripts of ENPP1 identified in stenotic valves (61). Hydrolysis of extracellular adenosine triphosphate (ATP) by ENPP1 produces a net increase in inorganic phosphate, thus favoring calcification and promoting the production of further ENPP1 in a positive feedback loop (61). Moreover, because ATP acts as a cell survival signal for VICs via the $P2Y_2$ receptor, its depletion also triggers apoptosis of these cells, providing a further key stimulus to calcification (61). Finally, loss of $P2Y_2$ signaling increases the secretion of IL-6, a cytokine that promotes further osteogenic differentiation of VICs via the actions of BMP (62). Thus, via these multiple mechanisms, the ectonucleotidase pathway appears to have a central role in amplifying procalcific processes within the valve during the propagation phase of aortic stenosis.

Given that the pathophysiology and the progression of aortic stenosis are dominated by calcification,

FIGURE 2 Mechanisms Underlying the Relationship Between Bone Mineral Density and Valvular Calcification and Potential Mechanisms of Action for Denosumab and Bisphosphonates

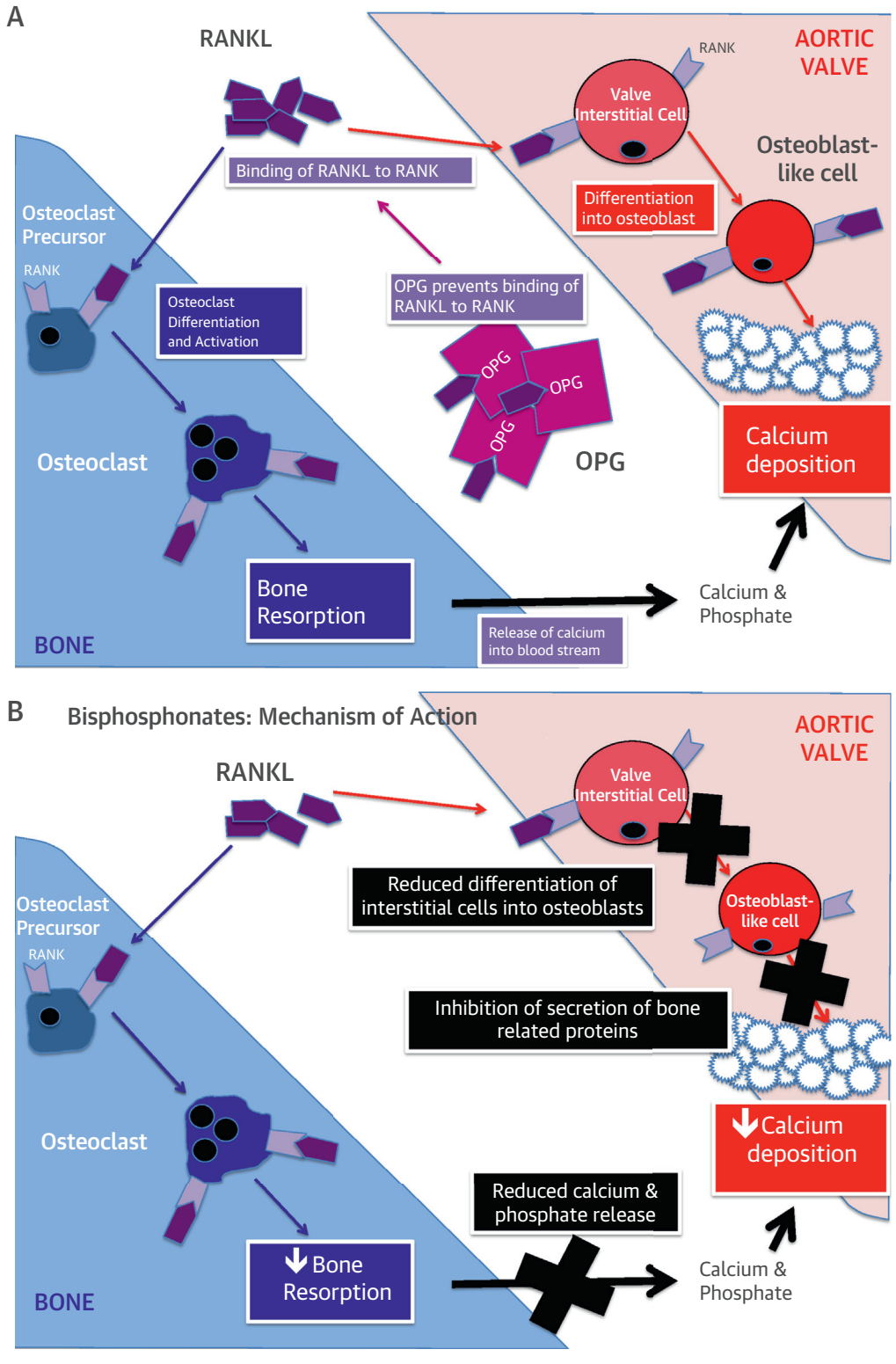


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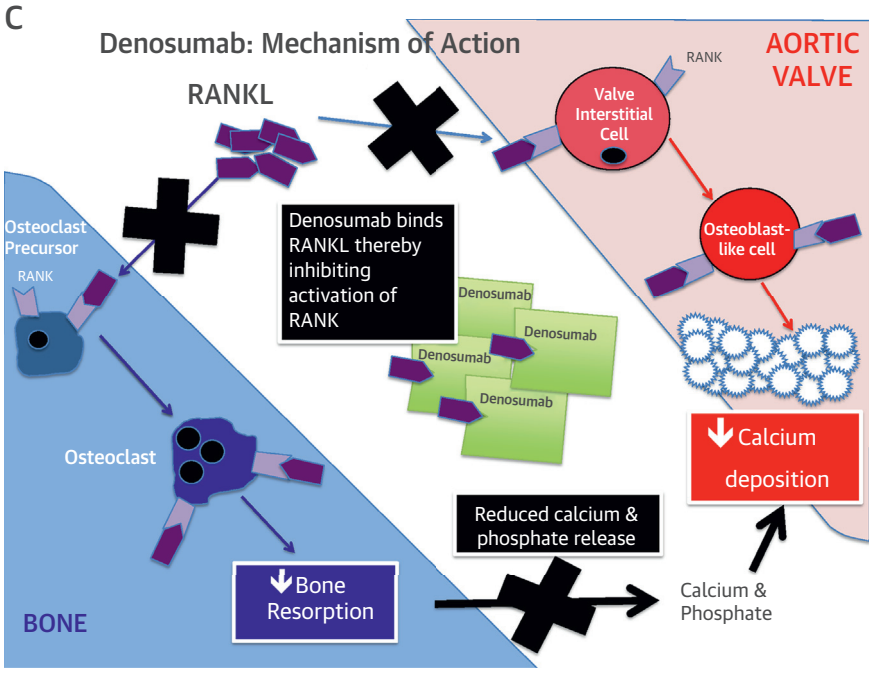
FIGURE 2 Continued



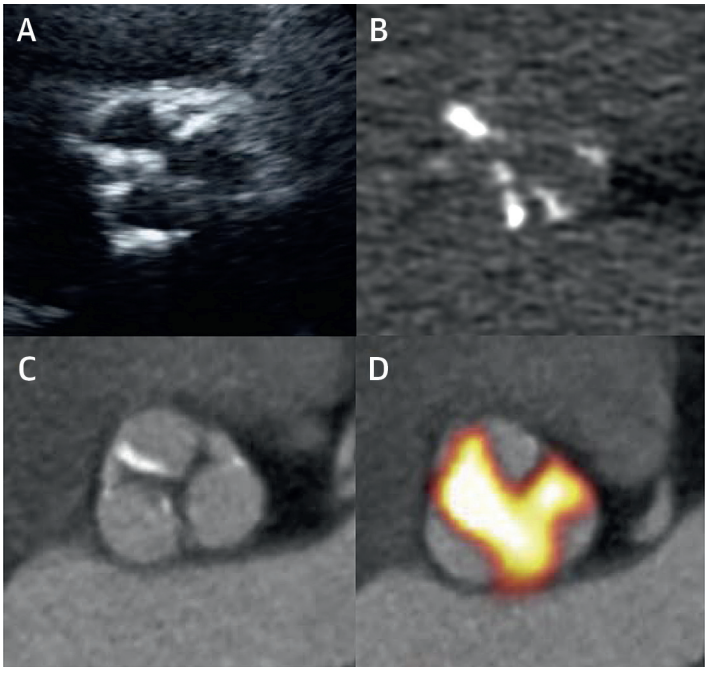
(A) Schematic representation of the differential effects of the RANK/RANKL/OPG axis upon skeletal bone and the calcifying aortic valve. Aortic valve: binding of RANKL to RANK on valvular interstitial cells induces osteogenic differentiation and calcium deposition. Bone: binding of RANKL to RANK on osteoclast precursors induces osteoclastic maturation and differentiation, resulting in bone resorption and increased availability of calcium and phosphate. Osteoprotegerin binds to RANKL, preventing it from binding to RANK, thus reducing both bone resorption and calcium deposition within the valve. **(B)** Proposed mechanism of action of bisphosphonates in the treatment of aortic stenosis. Bisphosphonates reduce the production of inflammatory cytokines, thereby reducing osteogenic differentiation of VICs. They also inhibit the production of matrix metalloproteinases and regulate extracellular mineralization within the vasculature. Simultaneously, the prevention of bone resorption by bisphosphonates also reduces the availability of procalcific substances. In combination, this is likely to result in reduced calcium deposition in the valve, along with reduced bone resorption. **(C)** Proposed mechanism of action of denosumab in the treatment of aortic stenosis. Binding of denosumab to RANKL prevents its binding to RANK, which inhibits the osteogenic differentiation of valvular interstitial cells. It simultaneously prevents osteoclast differentiation in the bone, and inhibits bone resorption and availability of procalcific substances. In combination, this is likely to result in reduced calcium deposition in the valve and reduced bone resorption. OPG = osteoprotegerin; other abbreviations as in Figure 1.

we will next discuss how this process can be imaged to better understand the pathophysiology of aortic stenosis, to predict disease progression and clinical outcomes, and to help develop novel treatments strategies for this common and potentially fatal condition.

CLINICAL IMAGING OF AORTIC VALVE CALCIFICATION

The burden and activity of aortic valve calcification can be measured using noninvasive imaging. In particular, echocardiography, computed tomography (CT), and positron emission tomography (PET) can all be used to provide progressively more detailed assessments of the calcific processes occurring within the valve (Figure 3). These techniques have not only informed our understanding as to the importance of calcification in aortic stenosis, but have also aided our ability to assess disease severity and to predict progression and adverse cardiovascular outcomes. The latter is of particular importance. Aortic stenosis progression frequently does not occur in a linear or predictable manner, making estimation as to when valve replacement will be required challenging. Annual or biannual clinical review is generally required, with serial echocardiography performed to track progressive valve narrowing. The development of a noninvasive method capable of predicting the future natural history of aortic stenosis and the likely timing of valve surgery would represent a major advance and help streamline patient care. Given

FIGURE 3 Different Methods for Imaging Calcification in a Single Subject With Aortic Stenosis



(A) 2-dimensional echocardiography. **(B)** CT calcium scoring. **(C)** CT angiography. **(D)** 18F-fluoride PET-CT. CT = computed tomography; PET = positron emission tomography.

the central role that mineralization plays in disease progression, it is, perhaps, not surprising that assessments of aortic valve calcification have, to date, provided the best prediction.

ECHOCARDIOGRAPHY

Echocardiography is a cheap, safe, and widely used method of assessing aortic stenosis severity in the clinical setting. International guidelines recommend grading aortic stenosis severity using the following hemodynamic echocardiographic assessments: the peak velocity, the mean gradient, and the aortic valve area (63). However, echocardiography can also be used to categorize valves according to their degree of valvular calcification into those with no, mild, moderate, and severe calcification. Indeed, in a series of 128 patients with severe, asymptomatic aortic stenosis, this semiquantitative assessment provided powerful prognostic information, acting as a strong independent predictor of death or aortic valve replacement that outperformed the more conventional hemodynamic measures (64). Although this observation has been confirmed in another study of 141 asymptomatic patients (65), the clinical utility of this approach has been limited by disappointing interobserver agreement in grading the calcification (66,67).

CT CALCIUM SCORING

CT provides a much more detailed, reproducible, and accurate assessment of the calcific burden in the aortic valve than echocardiography (66). Using the same protocols used for coronary calcium scoring, electrocardiography-gated noncontrast CT can provide information with respect to the density, volume, and mass of macroscopic calcium deposits within the valve (66). However, as in the coronary arteries, the aortic valve calcium burden is generally described using Agatston units (AU), which take both the radiodensity and volume of calcium into account. In a series of explanted aortic valves, scores of 500, 1,100, and 2,000 AU approximated to 300, 1,100, and 1,200 mg of aortic valve calcium, respectively (66).

Early studies demonstrated that CT calcium scoring of the aortic valve could be used as an alternative marker of stenosis severity, demonstrating a good relationship with hemodynamic echocardiographic assessments (66,68,69). However, until recently, we lacked appropriate thresholds that might differentiate patients with and without severe aortic stenosis, thereby limiting its utility (**Table 1**) (70). These thresholds are now available as a consequence of a landmark series of papers published by Clavel et al. (71–73). Across 3 sites in Europe and North America, they performed both echocardiography and CT calcium scoring in 646 patients with moderate or severe aortic stenosis and good left ventricular function. In those subjects whose severity of stenosis was not in doubt on echocardiography (n = 460), the authors examined the optimal CT calcium score for differentiating moderate from severe aortic stenosis. Interestingly, female subjects required less calcium to develop severe hemodynamic stenosis than male subjects (even after correcting for body surface area and the left ventricular outflow tract area calculated by echocardiography), so that the optimal thresholds were found to be 1,275 AU in women and 2,065 AU in men. These thresholds then appeared to be of use in adjudicating the severity of the stenosis when echocardiographic markers were discordant. More importantly, the authors went on to demonstrate that, in a population of 794 patients, these thresholds predicted all-cause mortality independent of all other markers of an adverse prognosis (73). Standard hemodynamic parameters on echocardiography were included in this analysis, suggesting that CT can provide additional, complementary information to

TABLE 1 Studies Attempting to Define Computed Tomography Calcium Scoring Thresholds for the Diagnosis of Aortic Stenosis

First Author (Ref. #)	Year	n	Method and Criteria for Defining Severe Aortic Stenosis	Calcium Score Threshold (AU)	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
Cowell et al. (107)	2003	157	Echocardiography aortic valve velocity >4 m/s	>3,700	100	50	39	100
Messika-Zeitoun et al. (66)	2004	100	Echocardiography aortic valve area <1 cm^2	>500	100	69	57	100
Koos et al. (74)	2004	72	Cardiac catheter aortic valve area <1 cm^2	>563	85	92	95	77
Clavel et al. (72)	2013	460	Echocardiography aortic valve area index ≤0.6 cm^2/m^2, mean gradient ≥40 mm Hg	>1,274 (women) >2,065 (men)	86 89	89 80	93 88	79 82
Cueff et al. (108)	2011	179	Echocardiography aortic valve area <1 cm^2	>1,651	82	80	70	88
Cueff et al. (108)	2011	20	Echocardiography low flow/low gradient severe AS Aortic valve area <1 cm *and* ejection fraction ≤40% *and* mean pressure gradient ≤40 mm Hg^2	>1,651	95	89	97	80

Values are % unless otherwise indicated.
AS = aortic stenosis; AU = Agatston units.

that obtained during routine clinical care, as had previously been hinted at by earlier studies (**Table 2**) (66,73-75).

An expanding body of published data has also demonstrated the ability of CT calcium scoring to predict disease progression in aortic stenosis. Initial studies indicated that the aortic valve CT calcium score progresses fastest in patients with the highest baseline calcium burden (76). We have recently confirmed this predictive ability in a large prospective study of patients with the full range of calcific aortic valve disease. The fastest rates of progression were again observed in subjects with the most advanced disease. Indeed, a good correlation was observed between the baseline calcium score and disease progression at 1 year ($r = 0.58$; 95% confidence interval [CI]: 0.15 to 0.82; $p = 0.01$) (77), which strengthened further after 2 years of follow-up ($r = 0.90$; 95% CI: 0.84 to 0.93; $p < 0.001$) (67). Moderate associations between the baseline CT calcium score and echocardiographic measures of disease progression were also observed (e.g., change in mean gradient; $r = 0.40$; 95% CI: 0.21 to 0.56; $p < 0.001$) (67), and very similar observations were recently reported in a different patient population (**Figure 4**) (78).

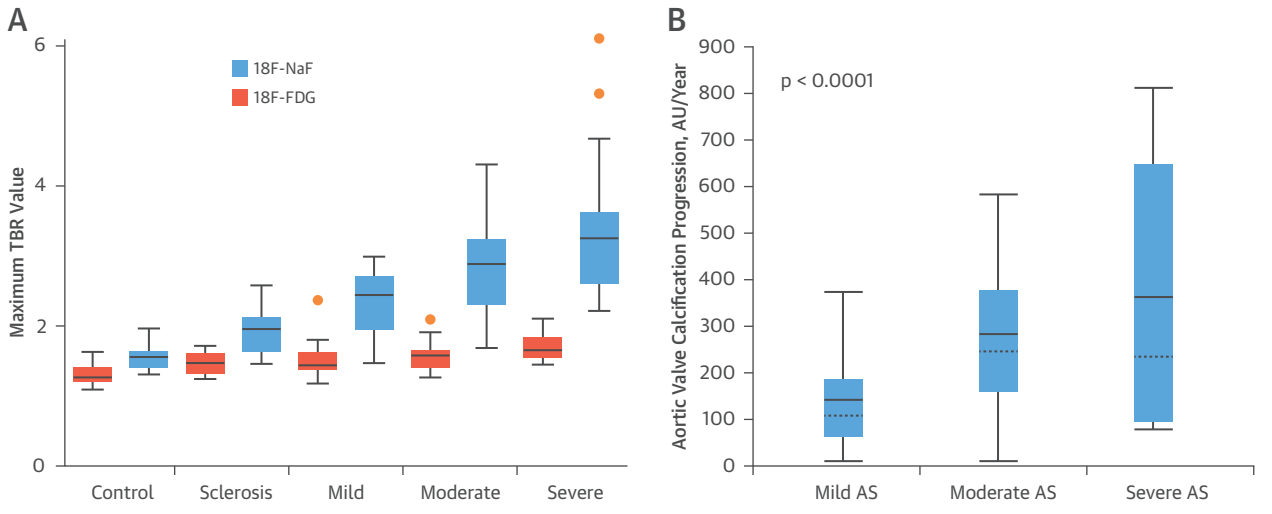
In summary, CT calcium would, therefore, appear to be a useful alternative method for grading disease severity in aortic stenosis, offering powerful

TABLE 2 Studies Using Aortic Valve Computed Tomography Calcium Scoring to Predict Outcomes

First Author (Ref. #)	Year	n	Duration of Follow-Up	Outcomes	Key Findings
Messika-Zeitoun et al. (66)	2004	100	2.0 ± 2.3 yrs	Event-free survival Survival without dyspnea, angina, syncope, heart failure, or need for surgery	AVC independently predicted event-free survival, with an adjusted relative risk of 1.06 (95% CI: 1.02–1.10) per 100-AU increment ($p < 0.001$). 5-year event free survival rate was 90 ± 4% for those with AVC <500 AU vs. 29 ± 14% for those with AVC ≥500 AU ($p < 0.0001$).
Feuchtner et al. (73)	2006	34	18–24 months	Major adverse clinical event Symptoms due to hemodynamic progression Cardiac death	AVC strongest predictor of a major adverse clinical event ($p < 0.001$) among all parameters assessed (1,928 ± 789 AU vs. 5,111 ± 2,409 AU).
Utsunomiya et al. (109)	2013	64	29 months	Cardiac events Cardiac death, AVR, nonfatal MI, and heart failure requiring urgent hospitalization	AVC predictor of cardiac events (HR: 1.09; 95% CI: 1.04–1.15) per 100-AU increment AVCS ≥723 (the median value) had significantly worse outcomes than those with AVCS <723 ($p < 0.0001$)
Clavel et al. (75)	2014	794	3.1 ± 2.6 yrs	Mortality	Severe AVC (defined as ≥1,274 AU in women and ≥2,065 AU in men) was an independent predictor of overall mortality (HR: 1.71; 95% CI: 1.12 to 2.62; $p = 0.01$)

AU = Agatston units; AVC = aortic valve calcium; AVCS = aortic valve calcium score; AVR = aortic valve replacement; CI = confidence interval; HR = hazard ratio; MI = myocardial infarction.

FIGURE 4 Relationship Among Baseline Disease Severity, Disease Activity, and Disease Progression



(A) Studies using PET have demonstrated that calcification activity in the valve (as measured using ^{18}F-fluoride) steadily increases with disease severity. As a consequence, activity is highest in those with the most advanced disease, and a good correlation exists between ^{18}F-fluoride activity and the baseline CT calcium score (79). **(B)** Subsequently, this increased calcification activity appears to translate to more rapid disease progression (as measured by both echocardiography and CT calcium scoring) in patients with the most advanced forms of aortic stenosis (78). Reproduced with permission from Nguyen V, et al. (78). AS = aortic stenosis; AU = Agatston units; CT = computed tomography.

prediction of both disease progression and adverse clinical events. It is potentially complementary to standard echocardiographic assessments and may have some advantages, most notably that it is not dependent on cardiac loading conditions, geometric assumptions, or on the presence of other cardiovascular conditions, such as mitral regurgitation and hypertension. Further work is now required to validate the proposed thresholds in other patient populations and to explain the observed sex differences.

POSITRON EMISSION TOMOGRAPHY

PET is a noninvasive imaging technique that allows the activity of specific biological processes to be measured in vivo within specific structures, including the aortic valve. In principle, any disease process can be evaluated dependent on the availability of a suitable tracer. To date, studies in aortic stenosis have largely investigated tracers targeted to inflammation (^{18}F-fluorodeoxyglucose [FDG]) and calcification (^{18}F-fluoride), aiming to establish the relative contributions of these processes to disease development and progression (79–81).

INFLAMMATION. The PET radiotracer ^{18}F- FDG is a glucose analog taken up by metabolically active cells. Because it is unable to proceed through the glycolytic pathway, it accumulates within these cells without further metabolism. Because vascular macrophages have higher metabolic requirements than the surrounding tissue, ^{18}F-FDG has emerged as a useful tool for the identification of vascular inflammation. Uptake in regions of carotid atheroma correlates well with macrophage density (mean percent staining of CD68-positive cells, $r = 0.85$; $p < 0.0001$) (82,83) and is modifiable with statin therapy (84).

To determine the contribution of inflammation to the pathogenesis of calcific aortic stenosis, we performed PET imaging of the aortic valve using ^{18}F-FDG in a prospective cohort of 121 patients with the full spectrum of calcific aortic valve disease (including 20 patients with aortic sclerosis and 20 control subjects) (79). ^{18}F-FDG activity was increased in patients with aortic stenosis compared with control subjects (1.58 ± 0.21 vs. 1.30 ± 0.13; $p < 0.001$), and this correlated with disease severity (79). However, unlike previous work on carotid atheroma, the ^{18}F-FDG signal did not correlate with macrophage (CD68) staining, raising the possibility that ^{18}F-FDG may not be acting as a marker of inflammation in the calcifying aortic valve, but instead might reflect glucose utilization by other metabolically-active cells, such as myofibroblasts or differentiated osteogenic cells (77).

CALCIFICATION. ^{18}F-fluoride has been used safely as a bone tracer for more than 40 years, exchanging with hydroxyl groups in hydroxyapatite to form fluoroapatite. Similar hydroxyl bonds are also present in the different forms of calcium in the vasculature (including hydroxyapatite and amorphous calcium) so that ^{18}F-fluoride binding acts as a marker of vascular calcification. In particular, the binding of ^{18}F-fluoride to calcium appears to be critically dependent upon the surface area of calcium orthophosphate available for incorporation. ^{18}F-fluoride, therefore, preferentially binds regions of newly developing microcalcification (beyond the resolution of CT), which have a nanocrystalline structure and very high surface area, rather than to large, established, macroscopic deposits, where much of the calcium is internalized and, therefore, not available for binding (85). On this basis, increased ^{18}F-fluoride uptake is observed in regions of actively developing calcification, demonstrating a close association with alkaline phosphatase staining ($r = 0.65$; $p = 0.04$) on excised aortic valve tissue removed at the time of surgery (77).

When the same cohort of 121 patients was imaged with ^{18}F-fluoride, the observed PET signal in the aortic valve was stronger and more clearly demarcated than was seen with ^{18}F-FDG (**Figure 3**). Moreover, the spatial distribution of the ^{18}F-fluoride signal was often discrete from the macroscopic calcium deposits identified by CT, indicating that ^{18}F-fluoride uptake provides distinct, but complementary information to CT alone. Uptake was increased in patients with aortic stenosis compared with healthy control subjects (2.87 ± 0.82 vs. 1.55 ± 0.17; $p < 0.001$) and correlated with disease severity ($r = 0.73$; $p < 0.001$) (79). Indeed, the highest calcification activity, as measured using this tracer, was observed in patients with the most advanced disease (**Figure 4A**). Again, this supports the hypothesis that calcification begets calcification activity in aortic stenosis and would explain the rapid rates of disease progression in those at the severe end of the spectrum.

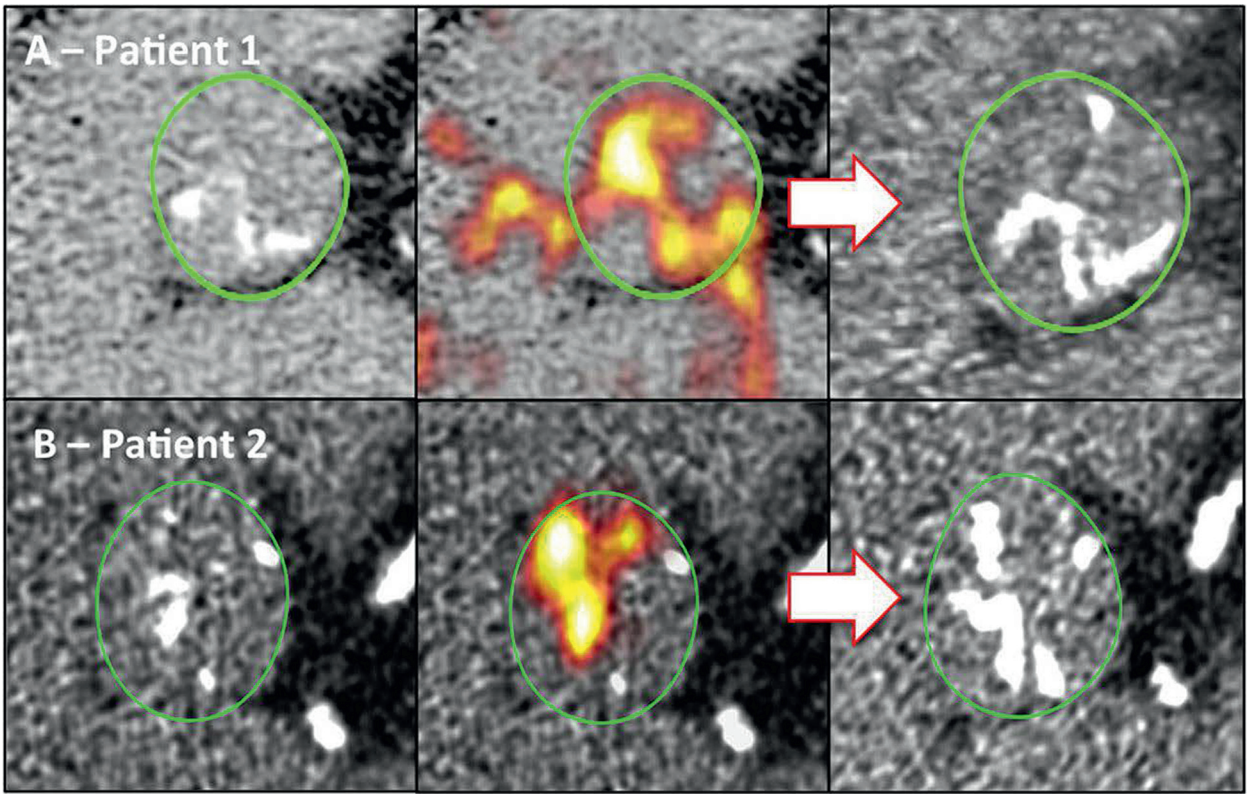
When patients were recalled for repeat CT calcium scoring of the valve at 1 and 2 years, new calcium could be observed in the areas of increased ^{18}F-fluoride activity seen on the baseline scan (**Figure 5**). As a consequence, a close correlation was observed between the baseline valvular ^{18}F-fluoride uptake and the progression of the aortic valve CT calcium score ($r = 0.80$; 95% CI: 0.69 to 0.87; $p < 0.001$), with PET appearing to offer some additional predictive information over and above the baseline calcium score. Moreover, this translated into an ability to predict valve hemodynamic progression, with moderate correlations also observed between ^{18}F-fluoride activity and the mean ($r = 0.32$; 95% CI: 0.13 to 0.50; $p = 0.001$) and peak ($r = 0.32$; 95% CI: 0.12 to 0.49; $p = 0.002$) aortic valve gradients (67). Finally, after a median of 1,526 days of follow-up, ^{18}F-fluoride emerged as a prognostic marker serving as an independent predictor of the combined endpoint of aortic valve replacement and cardiovascular mortality (hazard ratio: 1.55; 95% CI: 1.33 to 1.81, after adjusting for age and sex; $p < 0.001$).

In summary, these data highlight the potential application of ^{18}F-fluoride as an immediate, noninvasive measure of disease activity in aortic stenosis with the ability to predict its natural history. The instantaneous readout of disease activity holds particular promise in assessing the early efficacy of novel therapeutic agents, in which treatment effects are likely to be discernible over a much shorter time period than could be resolved using clinical endpoints, echocardiography, or CT.

Should ^{18}F-fluoride PET be used as a clinical tool? Although PET performed well in predicting the natural history of aortic stenosis, the far simpler technique of CT calcium scoring appeared to provide almost equivalent prediction of disease progression. Moreover, in agreement with the work by Clavel et al. (72,73), CT again provided incremental prediction of clinical outcomes to even echocardiographic assessments of hemodynamic severity. Whilst supporting a greater role for CT, this would argue against the use of PET in the routine clinical arena. It also raises the question as to why an anatomic measure of calcium burden can provide such effective prediction of future disease progression? We believe that this reflects the close association between calcification activity in the valve (as assessed by ^{18}F-fluoride) and the baseline calcium score ($r = 0.80$; $p < 0.001$), and provides further evidence for the model of calcium begetting further calcium formation in the propagation phase of the disease (**Figure 1**). Regardless of the mechanism, the close link between calcium burden and calcification activity in the valve ensures that even the simplest methods of aortic valve calcium burden provide a surrogate of disease activity and effective prediction of disease progression.

The imaging techniques described previously allow us to image calcification in the valve in progressive detail. They have helped to confirm the important role that calcification plays in driving aortic stenosis and have allowed us to both characterize the severity of disease and to better predict disease progression. We anticipate that CT calcium scoring will assume a greater clinical role, whereas PET will prove a powerful research tool, in particular as an endpoint

FIGURE 5 Change in Aortic Valve CT Calcium Score and ^{18}F-Sodium Fluoride PET Activity After 1 Year



Baseline CT calcium scores **(left)** for patients 1 and 2 **(top and bottom)**. Fused coaxial ^{18}F-fluoride PET-CT scans **(middle)** show fluoride uptake in **red** and **yellow**. The 1-year follow-up **(right)** suggests that the baseline PET signal predicts the spatial distribution of subsequent macrocalcification (77). Abbreviations as in **Figure 3**.

in clinical trials assessing the efficacy of novel, potentially disease-modifying therapies. Indeed, ^{18}F-fluoride PET has the potential to provide both mechanistic insights and a far more rapid readout of efficacy than CT calcium scoring or echocardiographic parameters.

POTENTIAL NOVEL DISEASE-MODIFYING THERAPIES

As our understanding of the pathophysiology of aortic stenosis has improved, the key role that calcification plays in driving disease progression has led us away from targeting inflammation and lipid deposition and toward therapies capable of directly halting valve calcification (86). How might this be achieved? The close association between disorders of skeletal bone metabolism and increased calcification in the vasculature offers a potential starting point. A growing body of pre-clinical and clinical data indicates that treatments for osteoporosis, such as bisphosphonates and denosumab, can reduce vascular calcification and that these agents hold considerable promise as novel therapies for aortic stenosis (87).

BISPHOSPHONATES. Bisphosphonates are inhibitors of osteoclast-mediated bone resorption, are well tolerated in elderly patients, and have been widely used for the treatment of osteoporosis (88). Interestingly, bisphosphonates also have important cardiovascular effects, demonstrating a consistent reduction in calcification of the vasculature and the aortic valve (87,89,90). This, in part, appears to be a consequence of their inhibition of bone resorption, which results in reduced release of calcium and phosphate into the circulation and, therefore, in the reduced systemic availability of these procalcific substances (**Figure 2B**) (87). However, bisphosphonates also appear to exert direct anticalcific effects on the aortic valve tissue itself. They reduce the production of IL-1β, IL-6, and TNF-α (key inflammatory cytokines implicated in the early stages of aortic stenosis [91]) and inhibit the secretion of matrix

metalloproteinases 2 and 9, which remodel the valve as aortic stenosis progresses (**Figure 1**) (92,93). Moreover, nitrogen-containing bisphosphonates act as inorganic pyrophosphate analogs (48), which, as discussed, have powerful anticalcific properties in the vasculature. Finally, bisphosphonates attenuate the differentiation of aortic valve myofibroblasts into cells with an osteogenic phenotype (94), the key step in triggering the propagation phase of aortic stenosis (86). In combination, these data offer support for bisphosphonates as a treatment strategy for aortic stenosis that is increasingly being supported by observational clinical data. A recent analysis of 3,710 women in the MESA (Multi-Ethnic Study of Atherosclerosis) indicated that bisphosphonate use was associated with less valvular and vascular calcification in older women (users vs. nonusers: aortic valve ring calcium 38% vs. 59%; $p < 0.0001$) (95). Other studies appear to support these findings with a direct beneficial effect of these drugs on echocardiographic measures of aortic stenosis progression (96–98), as well as reducing valvular calcification in patients with renal failure and amongst those with bioprosthetic valves (87,99). Although encouraging, such retrospective, observational studies are prone to bias, cannot assess cause-and-effect, have provided conflicting results (100), and are confounded by the underlying effects of the osteoporosis for which these agents were prescribed. Indeed, the true effect of bisphosphonates in aortic stenosis will only become clear within the context of a randomized controlled trial (101).

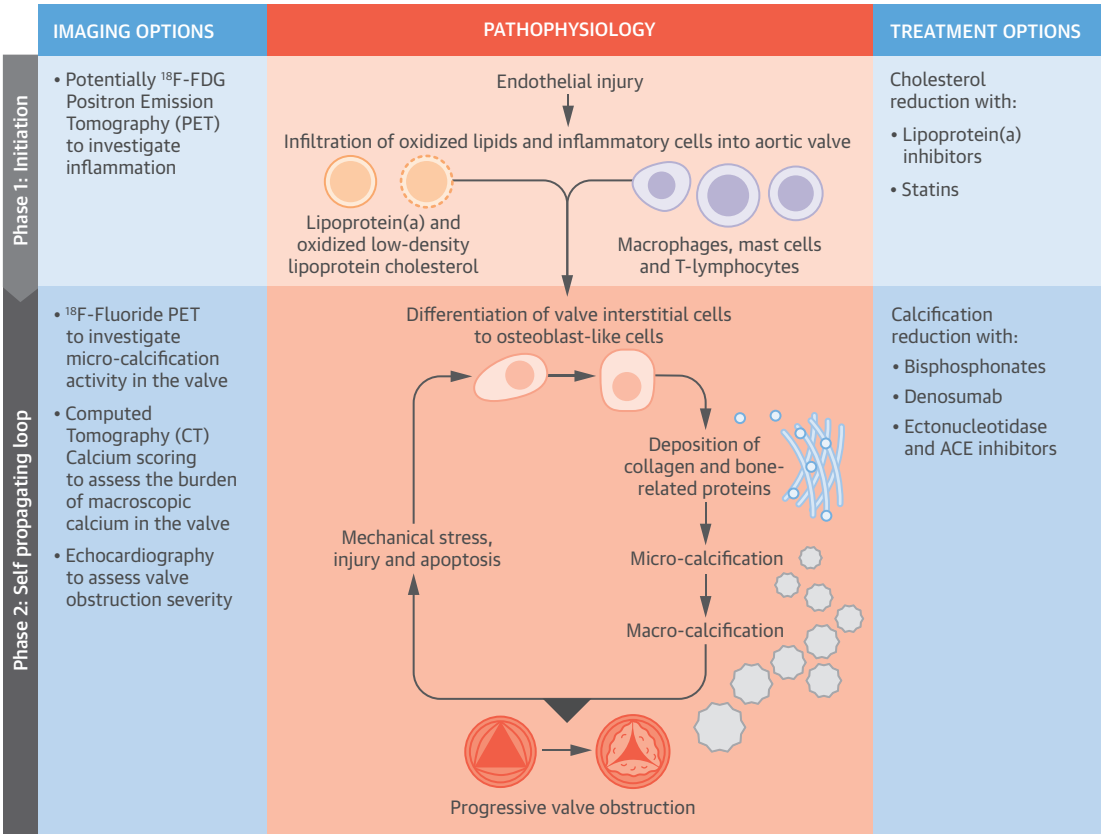
DENOSUMAB. As discussed, the OPG/RANK/RANKL axis appears to play a pivotal role in aortic valve calcification and may provide an explanation for the link between osteoporosis and increased vascular calcification. It, therefore, represents an attractive therapeutic target for reducing vascular calcification (**Figure 2C**). Denosumab is a human monoclonal antibody to RANKL that prevents its binding to RANK, thereby recapitulating the actions of OPG. In a trial of 7,868 post-menopausal women with osteoporosis, denosumab increased bone mineral density and reduced vertebral fracture rates by 68% over a 3-year period (102). Importantly, denosumab was extremely well tolerated, with very few adverse side effects and no major excess of adverse events. Given the central regulatory role of the OPG/RANK/RANKL system in vascular and aortic valve calcification, denosumab also holds considerable promise as a novel treatment for aortic stenosis. Again, this is supported by preclinical data, with denosumab halving the aortic calcification observed in a murine model of osteoporosis (103). Interestingly, in the same study, this reduction was closely associated with inhibited bone resorption from the skeleton, indicating that the cardiovascular effects of denosumab are, like bisphosphonates, in part related to reduced calcium and phosphate release from bone into the circulation.

FUTURE PERSPECTIVES

Bisphosphonates and denosumab hold promise as novel treatments for aortic stenosis and are currently being investigated as part of an ongoing randomized control trial (NCT02132026) (104). However, even if these prove ineffective, we believe that future treatments should still be directed at the propagation phase and at breaking the self-perpetuating cycle of valvular injury, osteogenic differentiation, and calcium deposition. As discussed, a rapidly expanding list of signaling pathways and molecular processes governing the pathogenesis of aortic stenosis have been elucidated, uncovering many additional targets at different phases of the disease; these are discussed in the following paragraphs. In addition, further investigation is warranted to assess whether potentially pro-calcific drugs, including calcium supplements and coumarins, should be avoided in patients with aortic stenosis.

Ultimately, many of the procalcific pathways in the valve appear to converge on the up-regulation of osteogenic differentiation factors (e.g., BMP-2, Wnt-β-catenin) that establish osteoblast-like function within the valve. These factors therefore provide an attractive therapeutic strategy, although, given the overlap in factors governing calcification in the bone and the valve, the major challenge will be to slow aortic stenosis progression without compromising bone health. One potential approach would be to target the upstream cytokines that activate BMP, such as using inhibitors of IL-6 or TNF-α (as already used in rheumatoid arthritis). However, once again, it remains unclear whether targeting inflammation will be effective in the propagation phase once the procalcific processes have become established. Targeting ectonucleotidases may be more effective, given their apparently central role in establishing the positive feedback loop by which calcium begets calcium. Ectonucleotidase inhibitors have already been tested in the warfarin rat model and have been shown to prevent the development of calcific aortic valve disease (105). Interest also surrounds $P2Y_2$ receptor antagonists as a means of reducing VIC apoptosis and the calcification that this induces. Therapeutic administration of fetuin-A, or a mimetic of MGP, could

CENTRAL ILLUSTRATION **The Pathogenesis of Aortic Stenosis in 2 Stages**



Pawade, T.A. et al. J Am Coll Cardiol. 2015; 66(5):561–77.

Initiation phase: similar to the early stages of atherosclerosis, endothelial injury facilitates the infiltration of oxidized lipids and inflammatory cells into the valve and the release of proinflammatory mediators. These trigger the very early stages of valve calcification so that the incidence of aortic stenosis closely relates to traditional cardiovascular risk factors for atherosclerosis. Propagation phase: these proinflammatory processes subsequently induce VICs to undergo osteogenic differentiation. The VICs first lay down a collagen matrix and other bone-related proteins before producing calcium (7,8). Calcification of the valve induces compliance mismatch, resulting in increased mechanical stress, injury, and apoptosis (9), which triggers further calcification (10). Hence, a self-perpetuating cycle of calcification, valve injury, and osteogenic activation is established that drives the propagation phase of the disease. As a consequence, disease progression in aortic stenosis more closely relates to procalcific factors, rather than lipid infiltration or inflammation. The different stages of calcification in the valve can be imaged using ^{18}F-Fluoride PET (newly developing microcalcification) and CT calcium scoring (macroscopic calcific deposits). Given the central role of calcification in the propagation phase of aortic stenosis, it is perhaps unsurprising that these imaging techniques provide important information with respect to prognosis and disease progression. Moreover, calcification represents an important potential therapeutic target, with the use of drugs, such as denosumab and bisphosphonates, to interrupt the vicious cycle of calcification that drives progressive narrowing of the valve.
ACE = angiotensin-converting enzyme; CT = computed tomography; FDG = fluorodeoxyglucose; PET = positron emission tomography; VIC = valvular interstitial cell.

simultaneously target multiple pathways thought to drive valvular calcification.

The ability of lipoprotein(a)-lowering therapies to modify aortic stenosis disease progression is likely to form the basis of a future clinical trial. Given the failure of the statin trials, it will be of great interest to determine whether a more targeted lipid intervention will have greater success in reducing disease progression in the propagation phase (106). On the basis of the apparent contribution of the RAS to the initiation of aortic stenosis, it is also not unreasonable to consider ACE inhibitors, or even selective AT_1 receptor antagonists or novel renin inhibitors, as novel treatments. Indeed, these agents are also likely to have a beneficial effect with respect to hypertension and left ventricular remodeling in aortic stenosis,

given the role that the RAS system also plays in driving myocardial hypertrophy, fibrosis, and the transition to heart failure.

CONCLUSIONS

Aortic stenosis is a common condition that is set to become an increasing health care burden. We lack effective medical therapies capable of slowing its relentless progression toward major surgery or adverse events. Recent insights into the pathophysiology of aortic stenosis have indicated that although lipid and inflammation may be important in establishing the disease (initiation phase), it is the self-perpetuating processes of calcification that are predominantly responsible for driving disease progression (propagation phase) (**Central Illustration**). On this basis, imaging modalities capable of quantifying aortic valve calcification will be best placed to predict its natural history, whereas novel anticalcific therapies hold major promise as methods of treatment. Randomized controlled trials of such agents, perhaps using imaging endpoints such as CT calcium scoring and ^{18}F-fluoride PET activity, are now required to establish their early efficacy.

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