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REVIEW

The lipid theory in the pathogenesis of calcific aortic stenosis

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Abstract *Aims:* Biologically active phenomena, triggered by atherogenesis and inflammation, lead to aortic valve (AV) calcification. Lipids play an important role in activating the cell signaling leading to AV bone deposition. This review, based on evidence from animal and human studies, mainly focused on the involvement of lipids and atherogenic phenomena in the pathogenesis of calcific aortic stenosis (AS).
Data synthesis: The role of elevated low density lipoproteins for the risk of both vascular atherosclerosis and AS has been elucidated. Lipid disorders act synergistically with other risk factors to increase prevalence of calcific AS. Atherosclerosis is also involved in the pathogenesis of bone demineralization, a typical hallmark of aging, which is associated with ectopic calcification at vascular and valvular levels. Animal studies have recently contributed to demonstrate that lipids play an important role in AS pathogenesis through the activation of molecular cell signalings, such as Wnt/Lrp5 and RANK/RANKL/Osteprotegerin, which induce the transition of valvular myofibroblasts toward an osteogenic phenotype with consequent valvular bone deposition. Although all these evidence strongly support the lipid theory in AS pathogenesis, lipids lowering therapies failed to demonstrate in controlled trials a significant efficacy to slow AS progression. Encouraging results from animal studies indicate that physical activity may counteract the biological processes inducing AV degeneration.
Conclusions: This review indicates a robust interplay between lipids, inflammation, and calcific AS. This new pathophysiological scenario of such an emerging valvular disease paves the way to the next challenge of cardiovascular research: “prevent and care aortic valve stenosis”.

Background

Calcific aortic stenosis (AS) is the most prevalent form of valvular heart disease in the Western world. Aortic valve (AV) sclerosis is observed in 75% of people aged more than 85 years, with severe AS reaching a 3% prevalence in the population over 75 years [1]. New insights in the pathogenesis of AS have accumulated in recent years. In fact, AS was previously thought to be the result of a passive,

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degenerative disorder of aging, related to mechanical stress. Recent in vitro and in vivo studies have instead demonstrated that atherogenesis and inflammation trigger biologically active and progressive phenomena leading to valve calcification and bone deposition [2,3]. At this regard, it has been found that lipids play an important initiating role in activating cell signaling leading to valvular calcification, and oxidized low-density lipoproteins (ox-LDLs) have been identified in calcified valves [4]. The hypothesis that lipids play a role in the development of calcific AS is further supported by the observation of diffused atherosclerotic lesions in the aortic leaflets, as well as in coronary arteries, of patients with familial hypercholesterolemia and no other traditional atherosclerotic risk factors [5]. A causative role of elevated LDLs in the risk of both vascular atherosclerosis and AS has been supported by the Cardiovascular Health Study [6] whereas an association between other studies early, pre-stenotic lesions of AV and increase (up to 50%) rate of coronary events was also reported, thus supporting a mechanistic relationship between atherosclerosis and valvular and vascular lesions [7,8]. Calcific AS is also significantly associated with increased prevalence of metabolic syndrome (MS) which is characterized by a cluster of cardiovascular risk factors including atherogenic dyslipidemia [9]. Finally, dyslipidemia also seems to be the link between bone loss and cardiovascular calcification as indicated by the observation that patients with lower bone density and osteoporosis have more severe atherosclerosis [10–12].

The aims of the present review were: i) to summarize evidence on the involvement of lipids and atherogenic phenomena in the pathogenesis of calcific AS gained from animal and human studies; ii) to identify relationships between AV disease and other pathologic conditions that recognize lipid metabolic disorders as common pathogenetic mechanisms; iii) to explore the putative molecular and cellular mechanisms leading to AV calcification and evaluate potential links with other biological phenomena that characterize vascular atherosclerosis and altered bone turnover; iv) to review the current controversies regarding the efficaciousness of lipid lowering therapies to slow AS progression; v) to report the results of experimental studies testing the efficacy of physical training in AS primary prevention.

Role of lipids in the pathogenesis of aortic stenosis: experimental and clinical evidence

Experimental studies in mice have demonstrated that increased cholesterol levels after an high cholesterol diet enhance oxidative state in the AV endothelium associated with increased levels of Ox-LDL and abundant inflammatory cell infiltrates, containing mast cells, macrophage and T lymphocytes [13]. Accordingly, himmunoistochemical studies of human stenotic AV described the presence of oxidized LDLs, T-cells and macrophages in the subendothelial layer of the fibrosa and close to calcium accumulation [4,14]. These histological evidence raised the hypothesis that the combination of extracellular oxidized lipids and matrix vesicles released from aortic valve myofibroblasts might represent nuclei for subsequent calcium deposition and calcium nodules formation. In vitro studies, showing that oxidized LDLs strongly promote mineralization when assessed in isolated AV interstitial cells [15], further supported the role of lipids oxidation as a crucial step in the pathogenesis of AV calcification. It has also been demonstrated that the small dense LDLs, that have greater ability to infiltrate tissues and are prone to the oxidation process, were the only lipid fraction associated with the accumulation of ox-LDL in the AV are the small, dense LDL that [16]. In this vein, the high proportion of small, dense LDLs in patients with MS [9] might explain the faster progression rate of AS in this group of patients [9]. The clinical association between LDLs and AS has been recently evaluated in 6942 patients of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium [17]. In this study, genetic elevation in LDL-C, but not in HDL-C or triglycerides, was associated with increased prevalence of AV calcium and incident AS at a follow-up of 15 years. Yet, the role of HDL in AS remains controversial, since, due to their antiatherogenic and anti-inflammatory properties, a protective role in AS pathogenesis and progression would be expected. In this regard, an high total cholesterol/HDL ratio and low serum HDL-cholesterol levels have been found to be associated with a rapid rate of AS progression [18]. Furthermore, the amount of valvular HDL is reduced in human stenotic AV [19]. Besides to the recognized effect on LDL-oxidation reduction, increased expression of adhesion molecules, increased nitric oxide production, and inhibition of apoptosis represent additional potential protective mechanisms of HDL on AV degeneration [20,21]. Yet, other evidence suggest that HDL might promote AS. In fact, in explanted stenotic human AV, apolipoprotein A1 of HDL has been found close to calcific nodules and contributes to the production of amyloid proteins which promote the transition of isolated valvular interstitial cells (VICs) toward an osteoblast phenotype [22]. Studies conducted on hypercholesterolemic rabbits showed that infusion of apoA-I mimetic could favorably affect AS progression in terms of valve area and leaflets thickness [23]. It has been hypothesized that HDLs might be retained and modified in AV, thus promoting lipid retention and contributing to trigger mineralization by being transformed into amyloid substance.

AS and altered bone turnover: the role of lipids

It has been shown that mature lamellar bone formation occurring in calcified human AV presents similarities to osteoblastogenesis during skeletal bone formation [3]. Histological and immunohistochemical studies have demonstrated that stenotic AV expresses osteopontin, bone sialoprotein, osteocalcin, alkaline phosphatase, and the osteoblast-specific transcription factor core-binding factor alpha 1 (Cbfa1) [3]. Skeletal bone osteogenesis involves the differentiation of mesenchymal cells into preosteoblasts and osteoblasts with consequent synthesis

and deposition of bone matrix proteins [24,25]. Similarly, in the AV the calcification process starts with the transition of VICs to a myofibroblast and osteoblast phenotypes and deposition of mineralized extracellular matrix [3,26–28]. Noteworthy, there are evidence that bone deposition within the AV is inversely correlated with bone mineralization [29]. This phenomenon has been observed in osteoporosis, in patients with chronic kidney disease (CKD) [30] and in less frequent bone disorders such as Paget's disease [31]. The relationship between ectopic calcification and reduced bone mineral density is commonly defined as "bone paradox" [32,33]. This phenomenon has been largely demonstrated for vascular calcification and several evidence indicate that it could be also involved in the progression of AS [34,35]. In a recent experimental study conducted in apoE$^{-/-}$ mice, a murine model of atherosclerosis, an association between AV calcification, arterial calcification and bone mineral loss has been demonstrated [36]. The authors also demonstrated that a systemic proinflammatory status contributed simultaneously to bone demineralization and cardiovascular calcification. Evidence from this study add new insights in the pathogenesis of calcific AS, indicating that inflammation and atherosclerosis could represent both the initiating process and the link between valvular calcification and altered bone metabolism [36]. Interestingly, the described association between atherosclerosis, inflammation and ectopic calcification/bone demineralization seems to be enhanced in the presence of CKD. Hjortnaes and coll [35], described increased osteogenic activity in the femurs and greater arterial and aortic valve osteogenic signal intensity in apoE$^{-/-}$ mice with CKD when compared to animals with normal renal function. These findings are in line with the clinical evidence of bone demineralization and arterial calcifications in patients with CKD [37].

Role of lipids in the activation of molecular pathways involved in AV calcification

The lipoprotein-associated phospholipase A2/LDL/ lysophosphatidylcholine axis

It is widely accepted that enhanced endothelial oxidative stress promoted by nitric oxide synthase uncoupling, reactive oxygen species accumulation, and production of highly reactive lipid-derived oxidized species represent crucial phenomena leading to AV degeneration and mineralization [38]. Importantly, ox-LDL are converted into lysophosphatidylcholine (LPC) by the lipoprotein associated phospholipase A2 (Lp-PLA2) that is upregulated in calcified AV [39]. In vitro studies indicate that LPC is a strong promoter of mineralization in isolated aortic VICs through a cyclic adenosine monophosphate (cAMP)/protein kinase A pathway. Lp-PLA2 is produced within the AV by macrophages and/or is transported in the aortic valve by LDL, particularly by small, dense LDL. Lipoprotein (a) represents a vector for oxidized phospholipids transport into the AV and its plasma levels have been found to be associated with increased risk of AS [40,41]. Another molecule involved in the process of lipid retention/ modification in AV is the phospholipid transfer protein (PLTP) which is overexpressed in AS valve tissues [42]. It has also been observed that stimulation of Toll-like receptor (TLR) 2 promotes the expression of PLTP by VICs [42]. PLTP binds to HDLs, thus decreasing their ability to perform reverse cholesterol transport. Stimulation of TLR-2 and the consequent PLTP overexpression may also be promoted by biglycan, a proteoglycan, which binds to LDL particles and induces the retention of lipids in the valve.

Renin-angiotensin system activation in AS

Activation of the renin-angiotensin system (RAS) is also implicated in AS pathogenesis and RAS inhibition slowers AS progression [43]. In explanted human stenotic AV, angiotensin-converting enzyme (ACE) is expressed and colocalized with angiotensin II [43]. Notably, ACE can be transported in the aortic valve by LDL, thus promoting local production of angiotensin II and triggering the process of tissue fibrosis which represents an hallmark of AV remodeling. An intriguing recent hypothesis is that the presence of ectopic visceral fat might contribute to activate the RAS system in AV [44], and preliminary data from our group indicate a strong association between thickness and inflammatory profile of epicardial fat, the visceral fat depot of the heart, and AS [45].

Wnt/Lrp5 signaling pathway

The upregulation of the Wnt/lipoprotein receptor-related protein 5 (Lrp5) signaling pathway is considered a relevant molecular phenomenon that can trigger calcification [36,46]. Lipids and other cardiovascular risk factors induce oxidative stress [38,47] in the AV endothelium which in turn activates the secretion of cytokines and growth factors activating cell signaling. Wnt3 secretion from valvular endothelium and the activation of Wnt canonical pathway through the Lrp5 are largely dependent on the abnormal oxidative stress environment promoted by atherosclerosis [47]. Lrp5 is a member of the family of structurally closely related cell surface LDLRs (receptors involved in the LDL metabolism) that have diverse biological functions in different organs, tissues and cell types [48,49]. The activation of the canonical Wnt pathway is critical in osteoblastogenesis [50]. In the bone, Wnt protein forms a complex with a frizzled protein and Lrp5/Lrp6, the so called Lrp5/Wnt/Frizzled complex [51]. The activation of this signal leads to an accumulation of β catenin, which controls the expression of a number of genes including Cbfa1, a key regulator of genes involved in osteoblastic differentiation. In the AV, Wnt is secreted from endothelial cells into the subendothelial space and binds to his receptor on the myofibroblast extracellular membrane forming the complex Lrp5/Wnt3/Frizzled, which triggers the phenotypic transition of these cells toward osteoblasts [28]. Figure 1 highlights the molecular mechanisms, at bone and AV levels, probably involved in the "bone paradox" phenomenon.

It has been demonstrated that bone matrix protein expression in the AV is regulated by the Lrp5 pathway in the presence of hypercholesterolemia. Development of AS

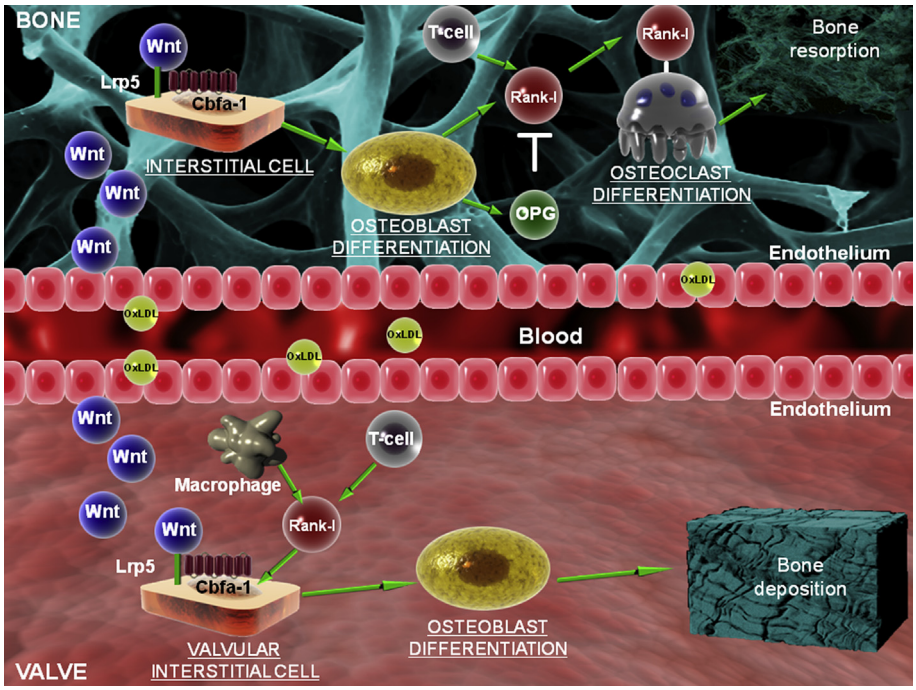

Figure 1 Cell signaling events involved in both bone remodeling and calcific AS. Bone: the activation of Wnt/Lrp5 pathway leads to the differentiation of interstitial cells into mature osteoblasts. Osteoblasts production of OPG and RANK-L modulates osteoclasts activity. In particular, binding of RANK-L to RANK receptor produces osteoclast differentiation and consequent bone resorption. Importantly, T-cell recruitment significantly contributes to RANKL production and bone resorption. Valve: Wnt/Lrp5 pathway and RANK-L, produced by recruited inflammatory cells, both regulate myofibroblast differentiation into osteoblast-like cells with consequent bone deposition.

was tested in eNOS$^{-/-}$ mice feeded with a high cholesterol diet [13]. Cholesterol treated eNOS mice developed severe AS associated with an increase of Wnt3a, Lrp5, Cbfa1 levels in the valve [13]. These evidence suggest that the osteoblast differentiation process may be mediated by the Wnt/Lrp5 pathway and that hypercholesterolemia and oxidative stress may play a main role in initiating this event.

Osteoprotegerin (OPG)/RANKL/RANK

OPG/RANKL/RANK represents another signaling pathway potentially involved in AV calcification. The role of this signaling in the regulation of bone mass and bone turnover has been widely described. RANKL/RANK and osteoprotegerin are regulators of osteoclast development and function [52]. RANKL is a cytokine, member of the TNF ligand superfamily, produced by activated T lymphocytes and osteoblasts [52]. RANKL binds to its receptor RANK, which is expressed on osteoclasts and provides the signal to drive their differentiation from progenitor cells to mature osteoclasts [52].

Osteoprotegerin (OPG) is a cytokine member of the TNFα superfamily and is produced by osteoblasts. It is as a decoy receptor for RANKL, thus inhibiting osteoclastogenesis [53].

OPG/RANKL/RANK pathway is considered to be involved in vascular and AV calcification [54]. In addition, opposite regulation of this pathway in bone and vasculature may explain, at least in part, the calcification paradox [32] (Fig. 1). It has been demonstrated that the osteoblastic transition of AV myofibroblasts may be promoted by RANKL, produced by lymphocytes and macrophages [55]. In calcified AV, RANKL expression is highly increased while OPG expression is not detectable with the net result of a decrease of the calcification inhibition potentially associated with OPG [55]. Furthermore, in cultured AV myofibroblasts, exogenous RANKL accelerates the transition toward an osteogenic phenotype. The antagonistic role of OPG on the pro-osteogenic stimuli provided by RANKL is also well established. Exogenous OPG attenuates impairment of AV function in stenosis-prone hypercholesterolemic Ldlr$^{-/-}$ Apob$^{100/100}$ mice [56], with significant reduction of valve calcification. It has been demonstrated that OPG may also decrease monocyte infiltration into hypercholesterolemic valves by significantly reducing expression of monocyte chemo-attractant protein-1 in valve tissue [56]. In presence of oxidized lipids, T-lymphocytes enhance RANKL production both in AV and skeletal bone [57,58].

Comprehensively, these data point to common molecular pathways that characterizes vascular and valvular atherosclerosis as well as bone resorption.

Statin therapy in AS: lights and shadows

In the last years, several studies explored the effects of targeted medical therapies in slowing AS progression. Several experimental studies demonstrated that statins can block the signaling pathways responsible for bone deposition in the valve leaflets [37,59]. In particular, recent in vitro studies from Rajamannan et al. [13] showed that

atorvastatin attenuates lipids-associated oxidative stress in AV endothelial cells and increases eNOS functional enzymatic activity. Furthermore, statins reduced Wnt secretion from valve endothelium and the consequent activation of LRP5 pathway [13]. In humans, atorvastatin at a dose of 20 mg per day, reduced plasma levels of OPG, osteopontin and soluble RANK in patients with aortic sclerosis and mild AS [60]. A study conducted on a large study population of 4105 patients with aortic sclerosis, who were treated with ACE-Is/ARBs or statins and followed for a mean of 1078 $\pm$ 615 days, demonstrated that statin in the early phase of AV degeneration significantly delayed AS progression [61] Finally, in the randomized RAAVE study, patients with asymptomatic moderate to severe AS and elevated cholesterol levels were treated with rosuvastatin and followed-up for a mean of 18 months. In this study, statins significantly slowed the primary end points of hemodynamic AS progression as measured by peak velocity, aortic valve area, peak gradient, and mean gradient. In addition, secondary end points, including CRP levels, IL-6, sCD40L, and serum LDL levels, were all reduced significantly in the rosuvastatin-treated patients with moderate AS [62]. Despite the favorable effects of statins observed in other studies [63,64], including also patients with severe AS and independently of cholesterol levels, conflicting, and predominantly negative results, have been reported in prospective controlled trials that tested statins in patients with moderate to severe AS. In the Scottish Aortic Stenosis Lipid Lowering Therapy Impact on Regression (SALTIRE), high dose atorvastatin was not able to induce regression or reduce the progression of valve disease [65]. The two largest and most recent trials, evaluating the effects of lipid lowering therapy in AS, were the Simvastatin and Ezetimibe in Aortic Stenosis (SEAS) trial [66] and the Aortic Stenosis Progression Observation Measuring Effects on Rosuvastatin (ASTRONOMER) trial [67]. Both studies failed to demonstrate a significant effect of statins on AS progression. The discrepancy between the conspicuous benefits demonstrated by statin in experimental and patient-based studies and the neutral results reported in controlled trials remains to be clarified. It is likely that differences in study designs, enrollment criteria, statin medication, timing of therapy, and duration of follow-up can explain conflicting results. In addition, it should be pointed out that although statins substantially reduce LDL-cholesterol they have neutral or modest effect on circulating small, dense LDL that is the only lipid fraction associated with the accumulation of tissue ox-LDL. Finally, the role of LDL in AS pathogenesis could be mostly relevant only in the initial stages of the disease and in the early calcification and mineralization phase, thus explaining the favorable results of statin therapy in patients with aortic sclerosis and mild AS.

Exercise training and calcific AS: experimental observations in the setting of lipid metabolic disorders

Regular physical exercise training plays an important role in primary and secondary prevention of atherosclerotic cardiovascular diseases [68]. Exercise improves endothelial function, attenuates oxidative stress, and has a significant impact on blood lipids and lipoprotein profiles [69–72]. All these factors are known to be involved in AV degeneration and in AS development. This represented the rationale to test exercise training to prevent the progression of AV disease. In LDLR deficient mice, an experimental animal model which is largely used to mimic human atherosclerosis, a regular exercise training program prevented the development of AV sclerosis [73]. Interestingly, in this study, the cellular and molecular mechanisms by which exercise counteracted the processes of AV degeneration were: i) preservation of the integrity of valvular endothelium; ii) attenuation of oxidative stress and reduction in ox-LDL; iii) attenuation of proosteogenic signaling pathways.

Unfortunately, extrapolating the effects of training in the setting of secondary prevention, there are evidence indicating that once the process of AV calcification is started, exercise fails to prevent AS progression. In fact, a more recent experimental study, conducted in the same animal model of LDLR deficient mice, indicate that, once AV degeneration is consolidated, exercise does no longer induce the favorable molecular effects described above and has no more slowing effects on valve calcification process [74].

Future perspectives and conclusions

In this review, we have highlighted the robust interplay between lipids, inflammation and calcific AS. Although AV disease has been defined for several years as a passive age-related disease, the initiating processes leading to AV calcification and obstructive valve disease start early and generate clinical manifestation only in the more advanced stages of the disease. This probably explains the difficulties to adopt adequate preventive strategies programmes to counteract such an emerging cardiovascular condition. The better understanding of the molecular mechanisms involved in AV degeneration might significantly help to identify novel therapeutic targets. Of interest, the observation that AS shares several pathophysiologic mechanisms not only with vascular atherosclerosis but also with non cardiovascular comorbidities, such as bone disorders, could open an intriguing scenario for therapies targeting specific molecular signaling pathways, such as RANKL inhibition. In the next future, prospective controlled trials on more selected AS study populations will hopefully clarify the potential benefits of statins. In the last years, several controlled trials evaluated the efficacy of non statin lipid lowering therapies on different cardiovascular outcomes but none was designed to evaluate the effects on AS progression. While the hypothesis that raising HDLc by niacin would confer any cardiovascular benefit failed to be confirmed in the large HPS2-THRIVE trial [75], the results of ongoing trials on CETP inhibitors will contribute to clarify the role of HDL on cardiac outcomes and might suggest to design new studies having AS progression as the main outcome [76,77]. Accordingly, the favorable results of a recent trial, evaluating the effects of PCSK9 inhibition in

heterozygous familial hypercholesterolemia [78], pave the way for future evaluations in AS. Finally, an attractive prospective is represented by the introduction of exercise training in primary prevention programmes considering the effect of physical activity in attenuating cellular and molecular mechanisms leading to bone deposition in the AV.

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