The burden of calcific aortic stenosis: what's behind?

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Abstract. – In Western countries, calcific aortic valve stenosis (CAS) is widely common, representing the third cause of death among cardiovascular diseases (CVD). The burden of CAS is high, with an increasing prevalence rate related to age. An efficient medical treatment, according to guidelines, lacks to prevent the development and to reduce the progression of CAS. In this context, due to the aging population and the lack of effective medical management, the prevalence is expected to double-triple within the next decades. In our review, we aim to provide an overview of the underlying mechanisms of pathogenesis and the current state of the art regarding pathophysiological insights and novel potential therapeutic targets.

Key Words:
    Calcific aortic stenosis, Atherosclerosis, Aortic valve interstitial cell, Lipid-lowering agents, Antiresorptive agents, Inflammation.

Introduction

The global burden of calcific aortic valve disease (CAVD) is increasing, representing the most common valvular heart disease in the aging population. It was estimated that in 2017 more than 12 million people were affected, and it was responsible for more than 100,000 deaths[1]. The prevalence rate of calcific aortic stenosis (CAS) ranges from 1.7% in people over 65 years to more than 6% in people aged 85[2-4], having an increasing impact both for the public health and healthcare resources[4]. CAS is a chronic progressive disease strictly age-related and characterized by fibro-calcific remodeling of aortic leaflets, which results in an obstruction of the left ventricular outflow, myocardial hypertrophy, and, once symptoms appear, risk of heart failure and sudden death. No medical treatments to prevent the development or to reduce the progression of CAS are currently recommended by both American Heart Association/ American College of Cardiology (AHA/ACC) and European Society of Cardiology and the European Association for Cardio-Thoracic Surgery (ESC/EACTS) guidelines[5,6]; the only option available for the treatment of severe and symptomatic CAS still remains aortic valve replacement, even with minimal invasive approach, or trans-catheter implantation[7-11]. Patients with severe CAS in the absence of surgical or percutaneous intervention have a poor prognosis with a high rate of re-hospitalization and risk of mortality[4]. The onset of symptoms changes dramatically the natural history of the disease, with a mortality rate of approximately 50% after two years without any intervention[12]. Risk factors such as age, male sex, smoking, hypertension, dyslipidemia, high levels of Lipoprotein (a) (Lp(a)), diabetes mellitus, and obesity are evidenced in CAS and atherosclerosis leading to consider them as a diversified expression of the same disease[13-15]. In addition, the bicuspid aortic valve (BAV), a congenital malformation of the aortic valve, with related abnormal hemodynamic stress and genetic factors, is a powerful risk factor[16].

Aortic Valve Anatomy and Features

To better understand the pathophysiological insights of CAS is essential to describe the anatomic and histological features of the aortic valve (AV). The AV is an avascular structure, which modulates the unidirectional blood flow from the left ventricle to the aorta, opening during the systole and closing during the diastolic phase of the cardiac cycle. The AV is normally composed of three cusps named according to their relationship with the coronary artery ostia (the left coronary, the

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right, and the non-coronary cusp) and a fibrous annulus in continuity with the anterior leaflet of the mitral valve and the membranous septum. The AV consists of connective tissue stratified into three layers: the fibrosa, the spongiosa, and the ventricularis layer. The connective tissue is composed of extracellular matrix components (fibronectin, lamin, collagen, and elastin fibers, etc.) and predominantly two cellular populations: the aortic valve interstitial cells (AVICs) and the aortic valve endothelial cells (AVECs)[17,18]. The AVICs in healthy patients are described as "quiescent" sharing phenotypic similarities with fibroblast and can be activated by different stimuli in the osteogenic and myofibroblastic phenotype. The AVECs are located over the surface of the leaflets as a physical barrier. They have been shown to possess mechanosensitive properties and contribute to extracellular matrix homeostasis by communicating with the underlying AVICs[18]. Additionally, extra valvular cells may be involved in the homeostasis of the extracellular matrix, especially in the elderly, as suggested by experimental data based on animal models[19].

Pathophysiological Insights

CAS is a complex age-related multifactorial disease still incompletely understood. Predisposing risk factors such as age, male sex, smoking, hypertension, dyslipidemia, high levels Lp(a), diabetes mellitus, and obesity are determinants to increase susceptibility and speed up the progression[20]. Moreover, in younger patients, BAV represents the most common etiology of CAS[21].

BAV is the commonest congenital heart disease, with a worldwide incidence ranging up to 2%. Its natural history is characterized by valvular (insufficiency, stenosis, endocarditis) and vascular complications (dilatation, aneurysm, dissection)[22]. In BAV disease AV forms two instead of three leaflets and, according to the number of raphes, can be classified into three types (Figure 1)[23]. It is genetically determined in most cases. The inheritance appears to be autosomal dominant with incomplete penetrance. However, some studies[24] suggested the existence of an X-linked form, as indicated by the high prevalence of this pathology in patients with Turner syndrome. Although a single gene defect has not yet been identified, NOTCH1, GATA gene mutations, and endothelial nitric oxide synthase abnormalities were found[22]. Genetic factors in association with abnormal shear stress in these patients lead to earlier leaflets degeneration and consequent earlier need for surgery compared to tricuspid aortic valve patients[16,22].

It is, therefore, evident that CAS and vascular atherosclerosis share the same risk factors and, consequently, the early molecular pathogenic mechanisms. Different research groups have already investigated the association between atherosclerosis and CAS, finding that about 50% of patients with CAS have a concomitant coronary artery disease or vascular disease[25,26]. Both are chronic inflammatory diseases, whereas common risk factors activate and promote a self-maintenance inflammatory state leading respectively to the formation and progression of the valvular fibro-calcific remodeling and the atheromatous plaques[13]. As in atherosclerosis, mechanical stress is a key determinant of endothelial damage, leading to the infiltration and accumulation of lipids beneath the endothelium of the valve. Progressive endothelial injury and lipid oxidization activate the inflammatory response and exacerbate oxidative stress. This pro-inflammatory state promotes the activation of AVICs in CAS and the phenotypic switch of vascular smooth muscle cells in atherosclerosis, leading to extracellular matrix remodelling[27]. However, discrepancies exist, as recently reported by Lee et al[28] who showed that AV calcification progression was associated only with the progression of calcified atherosclerotic plaque but not with non-calcified plaque. Additionally, ultrastructural differences were found between leaflet calcification and atherosclerotic plaque showing in atherosclerosis the unique massive accumulation of lipids and the pronounced neovascularization[27].

For many years the pathogenesis of CAS was considered only a passive process characterized by dystrophic calcification and remodeling of valve leaflets due to cells aging and death with consecutive calcification of their degradation products[29]. New evidence suggests that CAS is a complex active process still incompletely understood in which several pathways drive the progression of the disease[15,30]. Based on this thesis, two different phases of pathogenesis can be identified: the initiation and the propagation phase (Figure 2). In the initiation phase, CAS pathogenesis is like atherosclerosis: mechanical/shear stress is at the basis of endothelial damage, allowing the infiltration and accumulation of lipids in the endothelium of the valve. The oxidation of lipoproteins activates the inflammatory response, exacerbating oxidative stress, which promotes the

phenotypic change in AVICs from a quiescent to an osteogenic and myofibroblastic one[14].

The AVICs activation starts the propagation phase leading to leaflets calcification and fibro remodeling due to activated and self-maintained calcific and inflammatory signaling pathways[15].

The Initiation Phase

Endothelial damage represents the *primum movens* of CAS pathogenesis, allowing cell infiltration and lipid accumulation through mechanic-sensitive signaling pathways. Dysfunctional endothelium by altered paracrine signaling leads to up-regulation of cell adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (V-CAM-1), which promote the invasion of macrophages and T cells in the valvular fibrosa. The immune cells infiltration results in pro-fibrotic and pro-inflammatory markers activation, such as extracellular protease, cytokines, and growth factors[27,31]. Interestingly macrophages demonstrated a crucial role in extracellular matrix remodeling and degradation[13]. Histopathologic studies[32,33] on early calcific valve lesions showed lipoproteins deposition and diffuse distribution of T cells both in the bicuspid and tricuspid aortic valves. On the contrary, on normal aortic valves, the absence of these cells has been reported. Moreover, neo-vascularization and inflammatory infiltrates are reported as histological features of the CAS valve, in addition to intra-leaflet hemorrhages and iron accumulation[27,34-36]. Laguna-Fernandez et al[36] found that the iron accumulated can be uptake by AVICs and potentially contributes to their activation and extracellular matrix remodeling and calcification. Emerging evidence highlighted the involvement of the NF-κB pathway through toll-like receptors and NOD-like receptor signaling pathways[37,38]. The immune system activation leads to the production and release of several cytokines. Interestingly Urban et al[39] found in their work that patients with CAS had higher levels of pro-inflammatory cytokines than both controls and patients with aortic regurgitation. Transforming growth factor β1 (TGF-β1), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) are reported as the predominant inflammatory cytokines involved in vascular calcification[40]. They contribute to a self-maintained inflammatory state by increasing the local production of matrix metalloproteinases, modulating apoptosis, cell proliferation, mi-

gration, and differentiation. Additionally, they can promote the endothelial-mesenchymal transition of AVECs into AVICs and, subsequently, their activation in the osteogenic phenotype[41]. AVICs (myo)fibroblast phenotype differentiation can be stimulated directly by TGF-β1 and leads to extracellular matrix rearrangement, collagen deposition, etc[42]. Interestingly, Chakrabarti et al[43], in a study on animal model, found that the inhibition of the TGFβ1-dependent SMAD3 signaling pathway reduces significantly the AV calcification. Inflammatory cytokines from different stimuli increase oxidative stress, which promotes the formation of oxidized low-density lipoproteins (Ox-LDLs) and phospholipids (Ox-PL). Oxidized lipids trigger and add further stimuli to endothelial dysfunction and inflammation by up-regulating cell adhesion molecules and inducing the activation of the Toll-like receptors and NF-κB (nuclear factor κB) pathway[44]. In addition to inflammatory stimuli, biomechanical stress, and valve injury can activate an autocrine signal which leads to AVICs activation and differentiation into the osteoblast-like and myofibroblast-like phenotype[45]. In a recent pre-clinical study, Rogers et al[46] found that the retinoid acid may be involved in AVICs osteogenic differentiation and potentially represent a novel therapeutic target.

The Propagation Phase

When the inflammatory state is established, and the AVICs are activated from prolonged different stimuli, CAS disease progresses into the propagation phase. The AVICs switch phenotype into myofibroblast- and osteoblast-like cells may represent the critical step in the pathogenesis and progression of CAS, leading to valve calcification and remodeling. At the beginning of pathogenesis, the inflammatory pathways seem to drive the progression of the disease. On the contrary, in the later stages, the calcific pathways such as the RANK (receptor activator of nuclear factor kappa B)/RANKL (RANK ligand)/OPG (osteoprotegerin), the Wnt (wingless and Int-1)/β-catenin, and the NOTCH signaling pathways seem to become predominant[44]. The NOTCH1 signaling pathway is involved in the repression of Runx2 a transcriptional regulator of osteoblast cell fate. Inactivating NOTCH1 mutations was reported as a predisposing risk factor and faster progression for CAS and also may contribute to the develop-
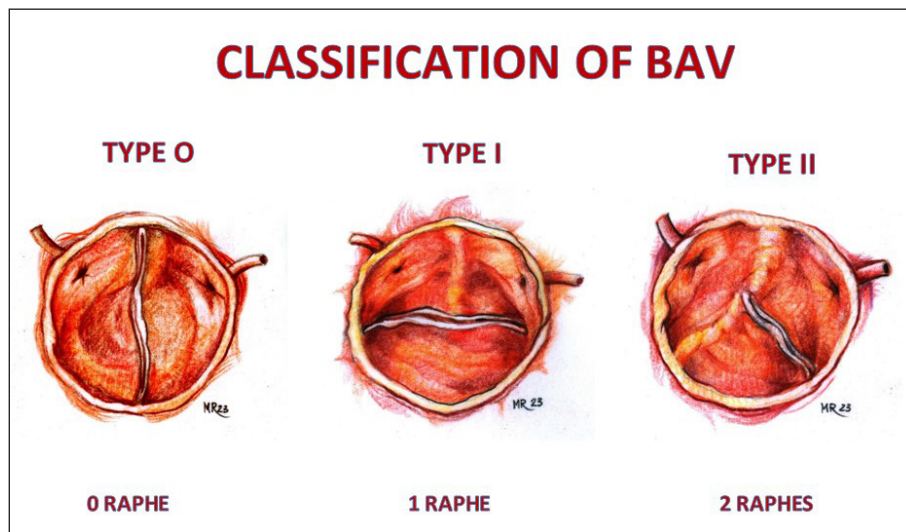
Figure 1. Representation of BAV morphologies, according to Sievers classification[23]. BAV type 0 or "true" BAV is characterized by the absence of raphe; on the contrary, type I and type II are characterized by the presence, respectively, of one and two raphes. BAV: bicuspid aortic valve.

ment of BAV[47,48]. Moreover, NOTCH1 appears to be involved in the activation of bone morphogenetic protein (BMP)-2, essential for osteoblastic differentiation[49]. The RANK/RANKL/OPG pathway can promote AVICs switch phenotype and subsequently matrix calcification and calcific nodules deposition. Not only the differentiation but also the apoptosis of AVICs can enhance and perpetuate the disease through dystrophic calcification[30].

### Dyslipidemia and Lipid-Lowering Therapy

The pivotal role of dyslipidemia in triggering and promoting AV calcification is well recognized by several studies[50,51]. In patients with homozygous familial hypercholesterolemia is described an increased incidence and progression of valve degeneration. Elevated low-density lipoproteins (LDL) and reduced high-density lipoproteins (HDL) levels were associated with a higher risk of incidence of CAS[14,52]. Several immunohistochemical studies[33,52,53] demonstrated the presence of various apolipoproteins, including apoE, apoA1, apo(a), and ApoB in association with high levels of oxidized phospholipid (OxPL)[33], which concentration directly correlated with the degree of inflammation and fibro-calcific remodeling[52,53]. Visceral obesity and metabolic syndrome are important risk factors for CAS leading to an inflam-

matory state and enhancing oxidative stress[54,55]. Interestingly, these conditions increased the risk of CAS not only due to hypercholesterolemia and the consequent increase in LDL levels but also to the reduction in adiponectin and HDL serum levels[20,55]. In particular, adiponectin is a peptide hormone with anti-inflammatory and anti-atheromatous properties, produced by the adipocyte, which is greatly reduced in obese patients (BMI $\geq$ 30) and in patients with metabolic syndrome[56]. Serum levels of adiponectin have been recognized as a risk factor for atherosclerosis and a potential novel therapeutic target for CAS and its progression[57,58].

The potential of Statin therapy in CAS disease was largely described in literature[14,59-62]. Statins are competitive inhibitors of HMG-Coa (3-hydroxy-3methylglutaryl-coenzyme) reductase, a key enzyme in sterol biosynthesis, and are a milestone of cardiovascular disease prevention. They have been shown to possess notable pleiotropic effects. In addition to lowering lipid levels, statins reduce the expression of inflammatory cytokines decreasing oxidative stress and inflammation as well as improving endothelial function[63]. Despite the strong correlation of CAS with altered lipid metabolism, chronic inflammation, and essential factors of atherosclerosis, none of the randomized clinical trials showed significant benefits of statin therapy[64-66] regarding both clinical presentation and CAS disease progression, with the exception of a non-randomized study[67].

Nevertheless, a limitation of all these studies[63-67] may be the introduction of statin therapy in the late phases of the disease when fibrosis and structural changes of the leaflets are already in an advanced step and when the pro-inflammatory state was established and AVICs activated in a self-feeding circuit. Differences age-related in patients with CAS were shown by Owens et al[68]; they found that elevated LDL was a risk factor for CAS only in participants younger than 65 years. In addition, results from the PROGRESSA study[69] and the EPIC-Norfolk prospective study[70] evidenced that, especially in younger patients, there was a significant association between high apoB/apoA-I ratio (ApoB is the main component of LDL and ApoA-I of HDL) and the hemodynamic progression rate of CAS; conversely, in elderly patients, this association was less evident, probably due to the predominant role of age-related factors such as osteoporosis, disorders of the calcium-phosphorus metabolism and the other side statins effects such as osteogenic properties, increased levels of Lp(a) and increased resistance to insulin. Likewise, dyslipidemia may have a pivotal role in the early stage (initiation phase) and has a minor impact when the activation of AVICs and the pro-inflammatory state are established (propagation phase). Lp(a) is a lipoprotein composed of apoB100 of LDL covalently attached to apo(a). It is genetically determined and represents a random and independent risk factor for CAS[70,71] and atherosclerotic cardiovascular disease[72,73]. As reported in recent reviews[73,74], elevated Lp(a), as a major carrier of OXlp, promote a pro-inflammatory state by stimulating activation of monocytes and macrophages, pro-inflammatory cytokines (e.g., IL1β, IL-6, IL-8, and TNF-α) and mediators both involved in the development and progression of CAS and atherosclerosis. Elevated Lp(a) levels and the corresponding associated genotypes (rs10455872, rs3798220, kringle IV type 2 repeat polymorphism) were also correlated with an increased risk of aortic stenosis in the general population and a tripled risk for Lp(a) > 90 mg/dl[75]. Due to its potential as a novel therapeutic target, great interest emerged in the scientific community regarding agents that can potentially act to decrease Lp(a) levels. Lp(a) levels are not significantly modified by statin treatment, but the use of new therapeutic agents such as Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) inhibitors and antisense oligonucleotides (ASOs) gain a huge interest **(Supplementary Table I)**. Emerging evidence showed that patients with a loss-of-
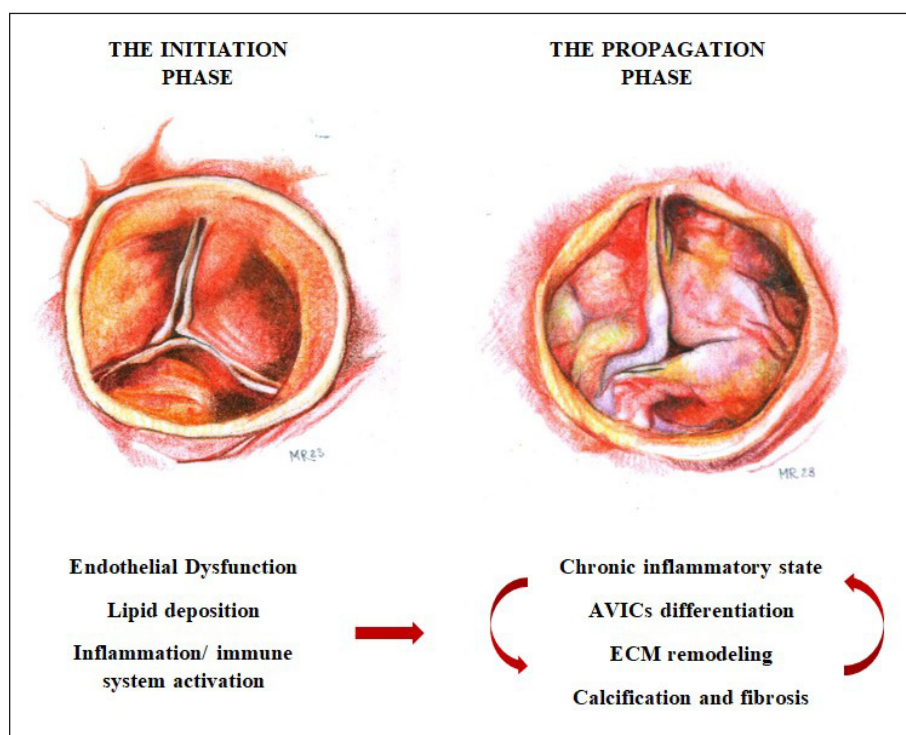
Figure 2. Schematic representation of CAS pathogenesis and its related two phases: the initiation and the propagation phase. CAS: calcific aortic stenosis, ECM: extracellular matrix components.

function mutation of PCSK9 have reduced levels of Lp(a), LDL cholesterol and lower risk of CAS and cardiovascular diseases[76,77]. Indeed this protein reduces the hepatocyte receptors that remove LDL cholesterol (LDL-C) from the blood; its inhibition leads to a reduction in the degradation of the receptor with a consequent reduction in blood concentrations of LDL-C[78]. Newsworthy, the use of monoclonal antibodies against PCSK9, Evolocumab, and Alirocumab, seems to significantly reduce Lp(a) values and potentially the incidence of cardiovascular disease and adverse events[72,79,80]. Promising results from clinical trials[81,82] showed the effectiveness of PCSK9 inhibitors as lipid-lowering therapy, especially in association with statin. The FOURIER (Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Patients With Elevated Risk) trial[83] evaluated the effectiveness of Evolocumab, a monoclonal antibody against PCSK9, compared with placebo in patients with dyslipidemia who were receiving statin therapy to reduce the cardiovascular death, myocardial infarction, and stroke (ClinicalTrials.gov Identifier: NCT01764633). A significant reduction in all cardiovascular events was found. The combination of Evolocumab with statin therapy lowered the LDL-C levels by 60% compared to statin therapy alone. A secondary analysis of the FOURIER trial highlighted the potential of Evolocumab to reduce the risk of CAS progression and its adverse events[84]. In the ODYSSEY Outcomes trial[82] (Evaluation of Cardiovascular Outcomes After an Acute Coronary Syndrome During Treatment With Alirocumab) in patients with previous acute coronary syndrome, the combination of Alirocumab with statin therapy significantly reduce the risk of cardiovascular events (ClinicalTrials.gov Identifier: NCT01663402). An interesting ongoing clinical trial[85] is investigating the effectiveness of monoclonal antibodies against PCSK9 in association with statins in preventing and delaying the progression of CAS (ClinicalTrials.gov Identifier: NCT04968509). Emerging evidence highlights the potential of synthetic ASOs as a pharmaceutical intervention to directly decrease Lp(a) levels in patients with cardiovascular disease or CAS[86,87]. Indeed the antisense oligonucleotides IONIS-APO(a)Rx and the IONIS-APO(a)LRx, completed, respectively, the trial phase 2 and phase 1[86]. Randomized, double-blind, placebo-controlled trials (ClinicalTrials.gov Identifier: NCT02160899; ClinicalTrials.gov Identifier: NCT02414594, ClinicalTrials.gov Identifier: NCT03070782; EudraCT Number:

2012-004909-27)[86-89] were conducted to evaluate their efficacy and safety to lower Lp(a) levels. Both the two ASOs resulted tolerable and highly effective to reduce Lp(a) concentrations[88,89] (Supplementary Table I). Additionally, they seem to reduce the pro-inflammatory activation of circulating monocytes in patients with elevated Lp(a)[90]. Promising findings resulted from a clinical trial[91] investigating the effectiveness of Mipomersen, an ASOs inhibitor of apo(b) synthesis, in the management of patients at higher cardiovascular risk with severe hypercholesterolemia. Interestingly, Thomas et al[91] found that Mipomersen as an add-on therapy significantly modified LDL-C and lipoproteins levels (Supplementary Table I).

Newsworthy, some studies[92] suggest that Autotaxin, an enzyme involved in the production of extracellular lysophosphatidic acid, may promote inflammation and osteogenic transition in the AVICs resulting in a potential novel biomarker of CAS progression.

Dysregulated Mineral Metabolism and Antiresorptive Agents

Recent evidence[93-95] suggests the association between CAS and dysregulated mineral metabolism and/or osteoclast deficiency, although the underline mechanisms remain still unclear. A correlation has been observed between the incidence of CAS and disorders of bone turnover, such as low bone mineral density, as well as chronic kidney disease and Paget's disease[93,94]. In the pathophysiology of CAS, a critical step is represented by the AVICs differentiation in the osteogenic phenotype leading to increase expression of osteoblast-specific proteins such as bone sialoprotein and osteopontin[30]. Calcific signaling pathways seem to have a dominant role in the later phases of the disease, when the differentiation of AVICs drives the disease progression. The AVICs activation enables positive feedback in which calcium deposition on leaflets increases mechanical stress and consequently injury-induced activation of the Wnt/b-catenin pathway, with further osteoblast differentiation[30,95]. Interestingly it has been reported that the factors, such as inflammatory cytokines and modified lipoproteins, that in skeletal bone cells induce bone resorption in vascular mineralization appear to have the opposite effect[30,96]. Therefore, over the last few years, there has been growing interest in studying the potential of antiresorptive agents (e.g., denosumab and alendronate, etc.) on AV calcification (Supplementary

Table I). Denosumab is a human monoclonal antibody RANKL inhibitor used for the medical treatment of osteoporosis[97]. *In vitro* and observational studies[98,99] have demonstrated the potential of Denosumab as an inhibitor of AVICsto delays CAS progression. Additionally, Alendronate, a bisphosphonate that inhibits bone resorption by suppressing the activity of osteoclast, appears to slow down the progression of CAS, especially in patients with concomitant osteoporosis[100]. Despite the promising findings of *in vitro* and observational studies[98,99], in a recent double-blind, randomized controlled trial[101,] neither Denosumab nor Alendronate affected the progression of valve calcification in patients with CAS (Supplementary Table I).

Novel Therapeutic Targets

Both innate and adaptive immunities seem to be independently related to the leaflet remodeling process and the CAS progression. *In vitro* studies[102,103] have investigated the potential of anti-inflammatory agents to suppress AVICs activation and calcium deposition, such as IL-38 and IL-37, underlying the potential of anti-inflammatory therapies in the treatment and prevention of CAS as a chronic inflammatory disease. Interestingly, two randomized clinical trials are evaluating the effectiveness of colchicine in CAS progression (ClinicalTrials.gov Identifier: NCT05162742; EudraCT Number: 2021-005586-40)[104,105] (Supplementary Table I). Additionally, some authors proposed natural antioxidant agents as a potential novel therapeutic option. In the field of vascular calcification, vitamin K2 as an inhibitor of arterial calcification has been suggested to potentially slow valve calcification, showing significant changes in observational studies in the levels of calcification agents, although no effect has been proved in elderly men treated with vitamin K2 and vitamin D supplementation[106]. Currently, another ongoing trial[107] is evaluating its efficacy on AV calcification (Supplementary Table I). Newsworthy, pre-clinical studies[108] showed that non-vitamin K antagonist oral anticoagulants (NOACs) inhibit AVICs activation and subsequently may potentially reduce aortic valve calcification.

Conclusions

CAS is a complex multifactorial disease where different molecular agents are involved and dom-

inate the progression of the disease according to the stage of pathogenesis. In the initiation phase, like atherosclerosis, endothelial injury, dyslipidemia, and inflammation trigger the activation of the AVICs in the osteogenic and fibroblastic phenotype. In the propagation phase, when the inflammatory state is established, and the AVICs are activated, a self-feeding mechanism is triggered: the AVICs activated induce calcific deposition and remodeling of the matrix through the activation of calcium pathways and the immune system, on the other hand, the immune system adds further stimulus both directly and indirectly by stimulating the activity of AVICs and enabling the rearrangement of the extracellular matrix components.

The complexity of the molecular mechanisms underlying the onset and progression of valve remodeling reveals the difficulty of identifying a therapeutic target that is effective in the various stages of the disease and, therefore, an effective medical treatment. A different therapeutic management for the prevention and treatment of CAS may be considered according to the different stages of the disease. In support of this thesis, the promising results of retrospective studies in delaying the onset and progression of CAS in patients treated for long-term with medical therapy recommended for atherosclerosis (statin therapy, etc.) didn't show effectiveness when introduced in the late phases of the disease, when the propagation phase speed out the progression and a self-maintained vicious circle was established.

To date, CAS still remains a complex, still not fully understood disease, as demonstrated by the controversial results of potential medical therapies in animal model studies and clinical trials.

Further methodological studies are needed to extend our knowledge on the pathogenesis of age-related CAS and on the effectiveness of the treatments used to prevent and control this pathology; maybe a tailored therapy characterized by drugs association could be suggested since several different pathways have been shown to be implicated in the progression of valve degeneration.

Authors' Contributions

All authors contributed significantly to this work and read and approved the final version of the manuscript. Ernesto Greco performed the conception and design of the study and revised it critically, Silvia Romiti performed the design of the study and wrote the manuscript, Mattia Vinciguerra, Mizar D'Abramo, Noemi Bruno, Carlo Gaudio and Fabio Miraldi revised it critically for important intellectual content.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article [and/or its supplementary material].

Informed Consent

Informed consent is not applicable to this study.

Ethics Approval

Ethics approval is not applicable to this study.

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