

REVIEW ARTICLE

Cellular Mechanisms of Valvular Thickening in Early and Intermediate Calcific Aortic Valve Disease

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Abstract: Background: Calcific aortic valve disease is common in an aging population. It is an active atheroinflammatory process that has an initial pathophysiology and similar risk factors as atherosclerosis. However, the ultimate disease phenotypes are markedly different. While coronary heart disease results in rupture-prone plaques, calcific aortic valve disease leads to heavily calcified and ossified valves. Both are initiated by the retention of low-density lipoprotein particles in the subendothelial matrix leading to sterile inflammation. In calcific aortic valve disease, the process towards calcification and ossification is preceded by valvular thickening, which can cause the first clinical symptoms. This is attributable to the accumulation of lipids, inflammatory cells and subsequently disturbances in the valvular extracellular matrix. Fibrosis is also increased but the innermost extracellular matrix layer is simultaneously loosened. Ultimately, the pathological changes in the valve cause massive calcification and bone formation - the main reasons for the loss of valvular function and the subsequent myocardial pathology.

Conclusion: Calcification may be irreversible, and no drug treatments have been found to be effective, thus it is imperative to emphasize lifestyle prevention of the disease. Here we review the mechanisms underpinning the early stages of the disease.

Keywords: Aortic valve, aortic stenosis, calcification, atherosclerosis, inflammation, disease.

1. INTRODUCTION

Calcific Aortic Valve Disease (CAVD) is an active inflammatory disease characterized by several hallmarks of atherosclerosis. The main difference is the ultimate phenotype – CAVD does not lead to vulnerable plaques but rather, heavily calcified and ossified aortic valves. As the accumulation of calcium and bone seems to be irreversible, early prevention is of paramount importance. Due to the active nature of the disease, it seems plausible that there would be a causal chain of events leading to the final disease phenotype. It will be necessary to clarify the early stages of the disease if we are to prevent the progression. In this review, we summarize the mechanisms of early valvular thickening – the stage in which CAVD may become symptomatic.

Cellular and subcellular events of early CAVD take place in valves consisting of a three-layered extracellular matrix (ECM) (Fig. 1). Each layer has its own characteristic main structural protein [1]. The main layers are termed ventricularis, spongiosa and fibrosa with their corresponding

main components being elastin, glycosaminoglycans and collagen. While these layers are rather stable, some studies in porcine models have indicated that the ultrastructural organization may change with aging [2]. The three layers also have somewhat different functional roles. In the ventricularis, elastin provides the necessary stretching ability, but it also serves as a scaffold to maintain valvular collagen in place [3, 4]. The spongiosa layer distributes hemodynamic stress across the valve leaflets, acting as a shock- and vibration-absorbing cushion [5, 6]. Finally, the fibrosa has a highly dynamic pressure-dependent circumferential collagen structure, and it is the main load-carrying layer in the valve [7].

The ECM of aortic valve cusps consists of a heterogeneous class of mesenchymal cells, called valve interstitial cells (VICs, reviewed in [8, 9]). VICs have several functions, including the maintenance of valve ECM as well as contributing to certain physical characteristics of the valve cusps, and their responses to injury. VICs and other mesenchymal cells share many similarities and display non-specific features of both smooth muscle cells and myofibroblasts. Valvular VICs seem to have a distinct surface antigen expression profile and an ability to respond to vasoactive agents and in these respects differ from the mesenchymal cells present in pericardium and skin [10]. Five different phenotypes of VICs have been postulated [11]: embryonic progenitor endothe-

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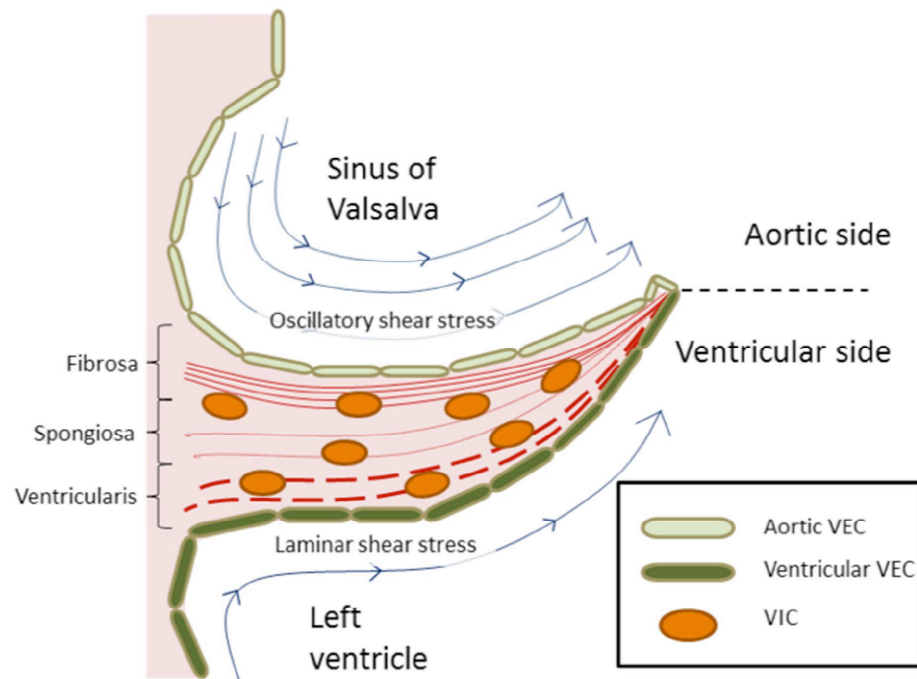


Fig. (1). A schematic cross-section depicting the anatomy and histology of a single valvular cusp with relevant shear forces (arrows). As the blood is pumped through the valve, it creates a turbulent, oscillating shear stress within the Sinus of Valsalva on the aortic side of the valve. This is different from the laminar shear that affects the ventricular side of the valve cusp. In addition, the slightly different types of Valvular Endothelial Cells (VECs) are highlighted on the figure. Inside the valve, a population of valvular interstitial cells (VICs) are shown along with the three differing layers of extracellular matrix. The fibrosa layer is located closest to the aortic surface and ventricularis in the ventricular side. The Spongiosa layer is located between these two layers.

lial/mesenchymal cells, quiescent VICs (qVICs), progenitor VICs (pVICs), activated VICs (aVICs) and osteoblastic VICs (obVICs).

2. COMPARING THE EARLY INITIATION OF ATHEROSCLEROSIS AND CAVD

In the first seminal human study investigating the early stages of CAVD, autopsy samples were histochemically characterized in order to examine the pathophysiological features associated with this disease [12]. Several hallmarks of atherosclerosis were seen already in non-stenotic yet thickened valves. The basement membrane below the endothelium was found to be disorganized and the tissue had become infiltrated by neutral lipids (mainly triglycerides, as revealed by Oil Red O staining). Thickening of the valve was found to be due to increased ECM of the fibrosa layer. These areas also contained macrophages, foam cells and T lymphocytes, whereas only scattered macrophages were found in the control valves. Small calcifications were also found in the early thickened regions.

More recently, newly formed lesions have also been found to contain apolipoproteins B (apoB), (a) and E [13]. In atherosclerosis, the attachment of apoB-containing low-density lipoprotein (LDL) -particles in the intima-layer of coronary arteries is both sufficient and essential to trigger the initiation of atherosclerosis [14]. A significant discovery supporting this concept is that if the electrical interactions between apoB primary structure and proteoglycan are removed, transgenic mice become highly resistant to atherosclerosis despite significantly elevated serum LDL levels

[15]. Because of their similar histologies, it is reasonable to propose that an analogous process initiates CAVD.

Retained apoB containing particles are predisposed to undergo a variety of oxidative modifications. Oxidized LDL (oxLDL) particles are strong triggers evoking an inflammatory response and the valves in the early stages of CAVD also exhibit oxLDL [16]. Certain proteoglycans such as decorin [17] and biglycan [18] colocalize with oxLDL, indicating that they can bind these particles within the valvular ECM. Recent studies have also shown that isolated LDL particles from aortic valves have larger diameters and are oxidatively modified, demonstrating that they aggregate and become modified within the valves, very likely contributing to the early inflammatory signals [19]. In conclusion, the data suggest that the early events in both atherosclerosis and CAVD are very similar (Fig. 2).

Localization of coronary plaques and the effects of hemodynamics also provide important clues to the similarities between early CAVD and atherosclerosis. Plaques are mostly located in the branching regions and arterial bends, where local shear stress is lower [20, 21]. This causes endothelial cells to express an inflammatory response which includes the induction of several vascular adhesion molecules (vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and E-Selectin), ECM breakdown, increased uptake and permeability of LDL particles, apoptosis, widening of intercellular junctions (which allows inflammatory cells to infiltrate through the endothelium), oxidative stress, increased thrombogenicity and impaired vasodilatation.

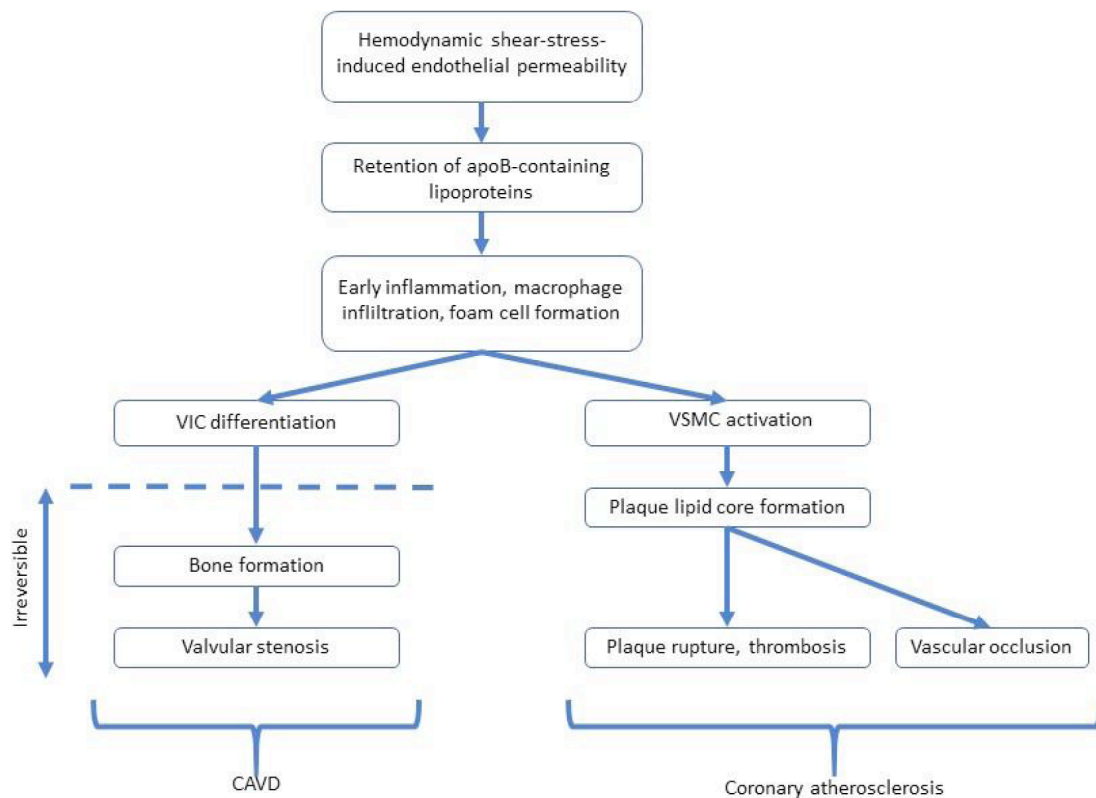


Fig. (2). Schematic flowchart highlighting the similarities and differences of calcific aortic valve disease (CAVD) and atherosclerosis. Both pathologies share similar initiating events but following foam cell accumulation, the different cellular environment (VICs in aortic valves, SMCs in coronary arteries) causes the downstream processes to diverge. While atherosclerosis leads to plaque formation and thrombosis or occlusion, CAVD enters an irreversible process of ossification. Abbreviations: VIC, valvular interstitial cell; VSMC, vascular smooth muscle cell.

Many of these processes remain to be explored in CAVD but several independent lines of evidence suggest that local hemodynamics also affect valvular tissue in a similar, pro-atherogenic fashion: 1) The aortic side of the valves is subject to turbulent and transiently reduced shear stress (reviewed in [22]). This is also the side where early lesions are invariably found [12]; 2) The bicuspid aortic valve (BAV) causes hemodynamically different conditions characterized by larger differences in shear stress compared to the more typical tricuspid valve (reviewed in [23]). BAV usually leads to a faster progression of CAVD and earlier clinical manifestation by about two decades [24, 25]; and 3) In tricuspid valves, the right- and left-coronary cusps are adjacent to the ostia leading to the coronary arteries. This causes these cusps to be also subjected to more laminar shear stress. The non-coronary cusp has a different hemodynamic environment and is usually affected most by early valvular thickening [26].

3. INFLAMMATION IN EARLY ATHEROSCLEROSIS AND CAVD

It has become widely recognized that in atherosclerosis, LDL retention in the arterial intima is the initiating event for plaque development. The subsequent inflammatory response is likely intended to clear the ectopic cholesterol from the arterial wall. If the macrophage-derived foam cells are successful, the initial fatty streaks are removed. However, if the exposure to pro-atherosclerotic factors persists, inflammation

becomes chronic, leading to the formation of an atheroma. In addition to macrophages, other cells of the immune system, including T-cells, mast cells and B cells are also present in atherosclerotic lesions (this process has been thoroughly reviewed [27-31]).

In early CAVD, many of these cells are also encountered e.g., macrophages and foam cells [12], T lymphocytes [12, 32], mast cells [33] and B cells [34]. Macrophages are localized close to lipid depositions [16] whereas T lymphocytes are found near both lipids [16] and calcifications [32]. Mast cells are more evenly distributed throughout the valves although with a slight preference for calcific nodules [33] and finally B cells are situated close to the macrophages [34].

In addition to diverse cell types, several molecular components of an inflammatory response have also been found in CAVD. For example, the levels of vascular cell adhesion molecule 1, intercellular adhesion molecule 1 and E-selectin are upregulated in diseased valves compared to controls [35, 36]. Mechanistic studies have elucidated some of the inflammatory pathways in more detail. Increased expression of interleukin-1 β has been detected in the leukocytes present in stenotic human aortic valves [37]. VICs were exposed to interleukin-1 β , resulting in increased production of matrix metalloproteinases (MMPs) -1 and -2. Their presence has also been confirmed immunohistochemically in diseased valves. In addition, tumor necrosis factor- α has been detected in valvular macrophages and shown to upregulate the ex-

pression of MMPs in VICs [38]. These studies emphasize that not only is inflammation clearly present in early CAVD but that it also contributes to ECM remodeling early on in the disease spectrum.

4. ACTIVE EXTRACELLULAR MATRIX REMODELING

During the intermediate stage of CAVD, there is more pronounced valvular thickening. Histologically, this is characterized, in part by an initial disorganization and then a loosening of the spongiosa layer of valvular ECM [39]. In clinical terms, the valvular thickening contributes to the loss of function, creating an increased myocardial strain. In addition, thickening also promotes CAVD progression. ECM integrity appears to be critical for the physiological function of the VIC because its disruption causes apoptosis and upregulation of several disease-related markers, such as α -smooth muscle actin, alkaline phosphatase, and osteocalcin [40]. Paradoxically, the overall amount of collagen in the valves decreases with CAVD progression, despite upregulated type I collagen production [41]. This indicates that CAVD also possesses a significant component of active ECM degradation, which is also evident from the increased expression of MMPs. Concordant degradation and synthesis of ECM are general features of an active remodeling in developmental processes and several diseases (for a general review, see [42]). In the following chapters, these simultaneous processes are examined separately.

5. ACTIVATION OF THE LOCAL RENIN-ANGIOTENSIN-SYSTEM AND OTHER PRO-FIBROTIC FACTORS

The renin-angiotensin-system (RAS, or renin-angiotensin-aldosterone-system, RAAS) is known to be involved in many illnesses and systemic physiologic processes. Certain parts of RAS are found locally within many tissues which means that angiotensin II (Ang II) is also an intracrine signaling molecule (reviewed in [43-45]). 'Local RAS' has significant roles in fibrosis and inflammation and has therefore been extensively researched in the cardiovascular system (reviewed in [46-51]).

Components of the local RAS are expressed in healthy and diseased aortic valves. For example, cultured VICs are able to produce angiotensinogen, and angiotensin-converting enzyme (ACE) *de novo* [52]. ACE has also been found to physically interact with LDL particles in the plasma and with apoB proteins in CAVD, which suggests that should LDL be retained in the valve, it may also carry ACE along with it. Ang II has also been found to colocalize with apoB and ACE, which implies that the latter is enzymatically active [53]. Degranulated mast cells are also a source for Ang II in aortic valves. They can secrete chymase, which is another peptidase able to cleave Ang I into Ang II [33].

Expression of type I angiotensin receptor (AT1R) on VICs has also been reported [53]. This may have pathological significance as Ang II exerts its pro-fibrotic effect *via* AT1R. Cardiac myofibroblasts displayed upregulation of the production of LDL-binding biglycan when cultured with Ang II [54, 55], which could make it feasible to postulate that a similar response could also occur in VICs. The synthe-

sis of type I collagen is significantly increased around calcific nodules [41]. This elevated synthesis of type I collagen and biglycan can be hypothesized to be significant contributors to the fibrotic phenotype of intermediate CAVD. Support for the clinical features of this putative Ang II-mediated valvular fibrosis emerged from a bioreactor-study, in which porcine aortic valves became significantly less flexible upon incubation in Ang II-containing media [56].

Novel components of RAS have been discovered in recent years. One of these is an ACE homologue, ACE2, which is able to cleave Ang I into a distinct nonapeptide, Ang(1-9) [57]. This is also an alternative substrate for ACE which turns Ang(1-9) into Ang(1-7) that has a specific receptor called Mas [58]. Compared to AT1R, Mas seems to have opposing downstream effects [59], much like the angiotensin type 2 receptor (AT2R) (reviewed in [60]). Together, AT2R, Mas and ang(1-7) can be considered to be part of a "compensatory arm" of RAS which counterbalances the vasoconstrictive and pro-fibrotic effects of Ang II and AT1R (reviewed in [61-63]). In calcific aortic valves, Mas and AT2R are downregulated, which is consistent with the proposal that the Ang II- and AT1R-mediated pro-fibrotic local RAS is the dominating arm in this disease [64].

Another somewhat novel RAS component is the (pro)renin receptor [65]. It binds renin and prorenin and it also mediates a pro-fibrotic response (reviewed in [66]). The study by Peltonen *et al.* (2011) suggested that the (pro)renin receptor is expressed in neovessels of diseased valves. However, in overall valve tissue, its mRNA was not significantly downregulated. If confirmed in subsequent studies, this would imply that while the receptor has pro-fibrotic effects in certain cells, its total contribution to CAVD may be time and location-dependent.

Many non-RAS components also promote active fibrosis. If VICs are cultured in the presence of transforming growth factor beta-1, they begin to express a pro-fibrotic phenotype [67]. Both endothelin-1, a fibrosis-inducing factor [68-70] and its receptor are upregulated in CAVD [71]. Further mechanistic studies will be required to confirm the role of transforming growth factor beta-1 and endothelin-1 in CAVD.

Downregulation of anti-fibrotic factors may be another way for fibrosis to become dominant in CAVD. C-type atrial natriuretic peptide (CNP) is one of these anti-fibrotic factors; it has been shown to inhibit fibrosis *in vitro* [72] as well as after experimental myocardial infarction *in vivo* [73]. The expressions of CNP and its receptors are downregulated in CAVD [74], but more detailed research of the contribution of CNP for CAVD development is needed.

6. EXTRACELLULAR MATRIX DEGRADATION

Loss of the collagen content and loosening of the spongiosa layer of the valvular ECM in CAVD are directly opposing processes to fibrosis. This must be mediated by specific ECM-degrading enzymes. One of the most studied is the MMPs which have various functions in cardiovascular diseases [75, 76]. In CAVD, increased expression of MMPs - 1, 2, -3 and -9 has been reported [38, 39, 77, 78]. In order to maintain their physiological functions, the activities of the

MMPs are counterbalanced by the presence of specific tissue inhibitors (tissue inhibitor of metalloproteinases, TIMPs). Although it may seem paradoxical, it appears that expressions of TIMP-1 and -2 are significantly increased in stenotic valves [38, 78, 79]. However, there seems to be a significant overproduction of MMPs with respect to the TIMPs in diseased valves [78]. This suggests that while TIMPs may be able to inhibit some level of ECM degradation, an overwhelming persistent inflammation will eventually cause tissue degradation to become prevalent. Indeed, in milder stages of the disease, TIMP expression tends to be dominant, which is also a feature seen in atherosclerosis [80].

In CAVD, MMPs may originate from VICs, since they appear to be capable of expressing MMP-2 (and TIMP-1 and -2), in primary cell culture conditions [39]. After stimulation by pro-inflammatory TNF- α , the VICs upregulate MMP-1 production while TIMP-1 expression remains unchanged, supporting the imbalance hypothesis [38]. In addition to the VICs, macrophages/monocytes are also able to express a variety of MMPs [81]. These data also strongly implicate inflammation as a direct causal driver of ECM degradation.

Another ECM-modulating pathway, similar to the MMP-TIMP-system, involves a family of cysteine proteases called cathepsins and their tissue inhibitors. Similar to the MMP-TIMP-system, cathepsins and their inhibitors seem to be significant contributors to many cardiovascular diseases, from atherosclerosis to aneurysms [82-84]. In CAVD, a significant upregulation has been reported in the levels of cathepsins S, K and V as well as in their inhibitor, cystatin C [85]. Cathepsin V was detected in close proximity to valvular neovessels, where it may be able to degrade the ECM to make way for new blood vessels. While more studies are needed, it appears that similar to the situation with the MMP-TIMP-system, also with cathepsins and their inhibitors, the ultimate disease phenotype is caused by their degradative properties overcoming their inhibition.

7. ROLE OF Lp(a) (LIPOPROTEIN A) IN EARLY LESION DEVELOPMENT

Epidemiological studies have highlighted that elevated serum levels of lp(a) are a strong risk factor for CAVD [86-89]. Strong evidence for causality has been implied in a Mendelian Randomization study [90]. It has also been shown that the oxidized phospholipids carried by lp(a) (OxPL) are associated with a faster progression of CAVD [91].

In more mechanistic experimental studies, OxPLs on lp(a) particles have been postulated to be the drivers of pathogenesis. Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is enriched in lp(a) [92] and it has the ability to hydrolyze OxPLs into lysophosphatidylcholine (LPC) which has been reported to activate mineralization in VICs [93]. Lp(a) particles also possess another enzyme, autotaxin (ATX), which hydrolyzes LPC into Lysophosphatidic Acid (LPA). LPA is also a potent promoter of calcification [94]. ATX was also found to be expressed by VICs, indicating that although serving as an important additional source, lp(a) particles are not required for ATX-mediated calcification pathway. While macroscopically larger deposits of calcium emerge in the later stages of CAVD, it should be noted that these lp(a)-dependent primary mechanisms are likely present

already during disease initiation. This emphasizes the need for early prevention.

SUMMARY & CONCLUSION

Several early hallmarks of CAVD are very similar to those encountered in atherosclerosis; LDL retention, infiltration of inflammatory cells and subsequent ECM remodeling. Valvular thickening, which may cause many of the first clinical symptoms, is ultimately the result of an accumulation of foam cells into the valve as well as loosening of the spongiosa layer of valvular ECM. The valve's mechanical properties are also compromised by the increased fibrosis occurring in other areas. All of these are active processes that precede much of the irreversible calcification. Lifestyle interventions should always be the first line of prevention of cardiovascular disease. In the case of CAVD, this is the only feasible approach, since no drug treatments have been found to be effective. These interventions are best targeted towards classical atherosclerotic risk factors.

LIST OF ABBREVIATIONS

Ang	=	Angiotensin
apoB	=	Apolipoprotein B
AT1R	=	Type 1 Angiotensin Receptor
CAVD	=	Calcific Aortic Valve Disease
ECM	=	Extracellular Matrix
LDL	=	Low-density Lipoprotein
MMP	=	Matrix metalloproteinase
oxLDL	=	Oxidized LDL
RAS	=	Renin-Angiotensin System
TIMP	=	Tissue Inhibitor of Matrix Metalloproteinase
VIC	=	Valve Interstitial Cell

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CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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