

STATE-OF-THE-ART REVIEW

Focusing on the Native Matrix Proteins in Calcific Aortic Valve Stenosis



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HIGHLIGHTS

- CAVS remains without pharmaceutical therapy.
- Sustained destruction and defective remodeling of the ECM, facilitates cellular activation and osteoblastic differentiation.
- Altered mechanical function of the valve has a direct impact on ECM remodeling and osteoblastic differentiation.
- Diverse and complex phases of calcium phosphate depositions are found in the lesion.
- Calcification development is tightly related to native ECM protein alterations, where further research is needed.

SUMMARY

Calcific aortic valve stenosis (CAVS) is a widespread valvular heart disease affecting people in aging societies, primarily characterized by fibrosis, inflammation, and progressive calcification, leading to valve orifice stenosis. Understanding the factors associated with CAVS onset and progression is crucial to develop effective future pharmaceutical therapies. In CAVS, native extracellular matrix proteins modifications, play a significant role in calcification in vitro and in vivo. This work aimed to review the evidence on the alterations of structural native extracellular matrix proteins involved in calcification development during CAVS and highlight its link to deregulated biomechanical function. (J Am Coll Cardiol Basic Trans Science 2023;8:1028-1039) © 2023 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Calcific aortic valve stenosis (CAVS) affects mainly people ages >65 years, with a prevalence of 12.4% in people ages >75 years in Western countries. It develops silently over more than a decade, and 24.4% of patients remain asymptomatic at the final stage.¹ When angina, syncope, or dyspnea develop and the valve is left unreplaced, it is characterized by death rates of ≤50% by year 2.² In the congenital bicuspid valve, characterized either by 2 sinuses of Valsalva or 3 sinuses with cusp

fusion,³ degeneration, and stenosis are seen as early as the fourth decade.⁴

The human aortic valve consists of 3 semilunar cusps, with a 3-layered tissue structure: the fibrosa at the aortic side comprised mainly of collagens, the ventricularis at the ventricular side consisted of elastin and collagen fibers, and the spongiosa as the medial layer, rich in proteoglycans⁵ (Figure 1, Table 1). Each layer of the valve confers different biomechanical properties: the fibrosa bears the high

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Manuscript received November 14, 2022; revised manuscript received January 9, 2023, accepted January 10, 2023.

circumferential stress during aortic backflow, being stiffer in comparison with the ventricularis, which in turn is responsible for the flexibility, stretch, and elastic recoil.^{6,7} Collagen fibers constantly align their orientation depending on the mechanical loading.⁸ Although mostly avascular, on the cusp basis, where tissue is thicker and oxygen diffusion is expected to be inadequate to meet demands, a microcirculation system in the spongiosa has been described.⁵ The main resident cell population responsible for the maintenance of the extracellular matrix (ECM), are the valve interstitial cells (VICs), which are characterized by significant phenotypic heterogeneity; they are recognized as a specialized type of fibroblasts, although evidence exists on a small population of α -smooth muscle actin + VICs in the ventricularis.⁹⁻¹¹

The current understanding of CAVS pathobiology focuses on the osteoblastic metaplasia, where VICs are initially activated to a myofibroblast type, remodel the ECM leading to fibrosis, subsequently develop osteoblastic pathways such as RunX2, and then actively deposit calcium phosphate on the remodeled ECM. The exact causes are unknown, although lipid oxidation, apoptosis, excessive inflammation, mitochondrial dysfunction, endothelial-to-mesenchymal transition (EndMT), impaired autophagy, active participation of extracellular vesicles (EVs), and indirect evidence of DNA damage have been reported. The detailed description of CAVS pathogenesis in the

cellular and molecular level has been discussed previously.¹² In this review, we highlight findings on the pathogenetic role of the structural ECM proteins alterations per se, along with insights gained from relevant research from in-vitro experiments.

Calcification of the aortic valve is a special case of the broader ectopic soft tissue calcification entity, produced by diverse mechanisms.¹³ In arterial calcification, apart from the intima-bound pathology linked to atherosclerosis and aging, a distinct form located in the media is linked to end-stage renal disease, hypertension, or diabetes mellitus. The corresponding lesion to the end-stage renal disease-related clinical phenotype of CAVS has not been demonstrated yet, and the extent of overlap in mechanisms with degenerative CAVS is not well-understood yet.¹⁴

Mineral phase identification in the calcific aortic valves¹⁵ has revealed nanoscale to submicron-scale calcium phosphate crystals, mainly hydroxyapatite (HAP; $\text{Ca}_{10}[\text{PO}_4]_6[\text{OH}]_2$), the main inorganic phase of bone,¹⁶ along with plate-like octacalcium phosphate crystals (OCP; $\text{Ca}_8[\text{HPO}_4]_2[\text{PO}_4]_4 \cdot 5\text{H}_2\text{O}$) and canonical geometric dicalcium phosphate dehydrate ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) crystals.¹⁷ X-ray diffraction and Fourier transform infrared spectroscopy techniques have shown that at an initial mineralization state,

ABBREVIATIONS AND ACRONYMS

- AGE** = advanced glycation end product
- CAVS** = calcific aortic valve stenosis
- ECM** = extracellular matrix
- EndMT** = endothelial-to-mesenchymal transition
- EV** = extracellular vesicle
- HAP** = hydroxyapatite
- OCP** = octacalcium phosphate
- TGF** = transforming growth factor
- VEC** = valvular endothelial cell
- VIC** = valve interstitial cell

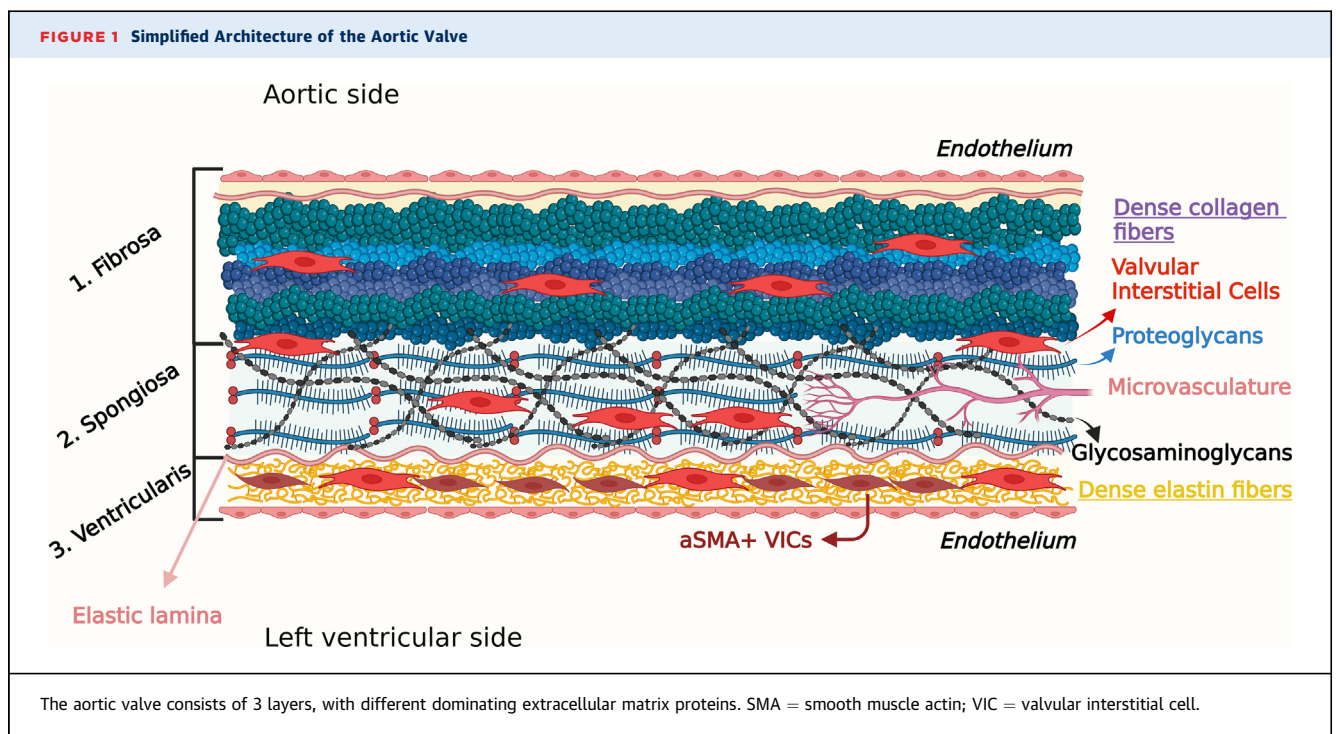


TABLE 1 Native Structural ECM Protein Alterations in CAVS

	Reference	Source
Proteins with specific mechanistic insights in CAVS		
Physiological collagens		
Collagens I, II, III, IV, V, VI, XVI, and XVIII	24,27,31,32	Protein, mRNA
New collagen isoforms in calcified valves		
Collagens VIII, X, XI, and XII	24,33-37	Protein, mRNA
Proteins with increased synthesis		
Collagens I, II, III, IV, V, and VI	27,31,33-35,37-39	Protein, mRNA
Laminins	24,34,36	Protein, mRNA
Fibronectin-1	24,32	Protein, mRNA
Periostin	31,38	Protein, mRNA
Prolargin	38	Protein
Osteonectin	24,35	Protein, mRNA
Microfibrillar-associated protein 4,5	34,35,37	Protein, mRNA
Fibulin-1, 6	35,37	Protein, mRNA
Lumican	31,32,35,38,40	Protein, mRNA
Biglycan	31,38	Protein, mRNA
Decorin	32,38	Protein
Aggrecan	24,34,35	Protein, mRNA
Versican	32	Protein
Testicans	24	Protein, mRNA
Tenascin-C	24,35	Protein, mRNA
Annexin A2	32,37,38	Protein, mRNA
Galectin-1	38	Protein
Proteins with decreased synthesis		
Tenascin-X	24,25	Protein, mRNA
Proteins without known specific mechanistic insights in CAVS		
Multimerin-2	24,34,37	Protein, mRNA
Galectin-3-binding protein	24,37	Protein, mRNA
SPARC-related modular calcium-binding protein	24,37	Protein, mRNA
Vitronectin	24,32	Protein, mRNA
Chondroadherin	24	Protein, mRNA
Myocilin	37	Protein, mRNA
Nephronectin	35	mRNA
Collagen triple helix repeat-containing protein 1	34	mRNA
Hyaluronan and proteoglycan link protein 3	34	mRNA
Keratin, type I cytoskeletal 18	34	mRNA
Angiopoietin-related protein 4	34	mRNA
Significantly altered native structural ECM proteins in CAVS, as derived from proteomics and transcriptomics analyses.		
CAVS = calcific aortic valve stenosis; ECM = extracellular matrix; mRNA.		

calcified valves, substantial incorporation of magnesium and silicon has been explored by energy-dispersive x-ray analysis of the calcium phosphate deposits.²¹ Magnesium, also detected in bio-prosthetic valves that showed slight calcification, can substitute calcium within the crystal lattice of HAP and stabilize amorphous calcium phosphate within vesicles where the calcification initiates.²¹ Furthermore, silicon seems to be involved in the initial and later calcification stages.²¹ Therefore, the mineralogical and chemical analysis of the calcified deposits could provide evidence of the calcification mechanism.

EVIDENCE ON STRUCTURAL ECM PROTEIN ALTERATIONS IN CAVS

The ECM provides structural support, harbors growth factors and cytokines, regulates cell-to-matrix communication²² and is an emerging drug target in cancer,²³ making it a putative pharmaceutical target in CAVS, as well. Structural ECM proteins interact extensively directly or mediated by receptors or secreted molecules, comprising a highly regulated network.²² In CAVS, collagen fibers are broken down enzymatically,^{12,24,25} with proteinases colocalizing in vivo with macrophages.¹² Unbiased tissue investigations reveal a wealth of synthesized proteins. Focusing on the native ECM structural proteins, we have included in **Table 1** differentially expressed proteins derived either from mass spectrometry analyses or protein-coding RNA sequences and characterized as “extracellular matrix” proteins, using Uniprot database.²⁶ New nonphysiological ECM proteins, growth factors, enzymes, and serum-derived proteins were excluded.

The majority of collagen synthesis happens in the spongiosa, with fibers found spatially disorganized.²⁷ The importance of collagen synthesis is highlighted in the rare congenital pediatric CAVS, where stenosis is tightly related to increased depositions of structurally immature collagens and a decrease in the glycosaminoglycans.²⁷ In adult CAVS, the most abundant forms are collagens I, II, III, IV, and XVIII, comprising the collagen triple helixes, and the basal membranes, collagens V and XI, regulators of fibril spacing and diameter; collagen XVII anchors cells on the basal membrane. The stability and strength of the collagen fibers is mediated by hydroxylated proline residues of the triple helix, a post-translational modification mediated by prolyl hydroxylases, along with the degree of collagen cross-linking, which is catalyzed by lysyl oxidase.^{27,28} Elastin fibrils are fragmented and disorganized and colocalize with calcification.²⁹

cardiovascular calcified deposits are poorly crystalline carbonated-substituted apatite (B-type, CO₃²⁻ group substitute PO₄³⁻ group).¹⁸ Raman microimaging analysis has shown that the calcification progresses from initial OCP-like compounds to tricalcium phosphate and finally into stable B-type HAP.¹⁹

Amorphous calcium phosphate, containing Na⁺, Mg²⁺, CO₃²⁻, and pyrophosphate (PPi), has also been identified in valve calcifications.²⁰ In biological and prosthetic heart valves, OCP structurally consisting of apatitic layers (similar to HAP) separated by layers of water molecules has been suggested as a precursor phase of biological apatite.²⁰ In heavily

Elastin fragmentation is also linked to neoangiogenesis in early CAVS, in the absence of inflammation.³⁰

Critical regulating glycoproteins are increased (Table 1). Among them, the laminins are major basal membrane components. VICs cultured on a laminin-coated substrate, demonstrated extensive calcification, mainly of dicalcium phosphate dihydrate (instead of OCP), tricalcium phosphate, or HAP, which was statistically higher than in normal samples.⁴¹ Fibronectin is necessary for the ordinary collagen fibril packing and is closely associated with calcification.⁴² It harbors binding sites for proteoglycans and integrins bridging ECM components with cells.⁴³ Periostin is essential for the normal embryonic development of the aortic valve.³⁰ It regulates collagen fibrillogenesis, acting as a polymerized scaffold,⁴⁴ and stabilization by activation of lysyl oxidase.²⁸ A lack of periostin induced extensive calcium deposits in the aortic valve cusps.⁴⁴ Periostin is required for the observed function of VICs during disease: its absence decreased aortic valve fibrosis and metalloproteinase-2,13 expression,³⁰ osteonectin regulates collagen fibril formation and cross-linking with other ECM proteins in mineralized tissues, and is upregulated in pediatric and adult CAVS.^{27,45} Fibulin, along with microfibril-associated glycoprotein, interacts with elastin fibers, forming a fine network.

Calcification is associated with proteoglycan expansion toward the fibrosa layer, particularly at the hinge region.⁴⁶ Lumican, which affects collagen fiber packing,⁴⁷ interacts directly with HAP.⁴⁷ It inhibits matrix metalloproteinases⁴⁷ and its decrease in calcified areas impairs collagen architecture,⁴⁸ leading to intracollagen calcification.²⁹ Aggrecan affects calcium phosphate binding capacity.⁴⁹ Decorin, biglycan, and versican, which also bind to collagen, are present around the calcified nodules, decreased in the nodular edge, and absent or considerably decreased in the actual nodules.^{50,51} Proteoglycans are also expected to interact with galectins and elastin fibers.⁵²

Tenascins are expressed in the basal membrane, where they interact with fibronectin, proteoglycans, and cellular integrins. Tenascin-C substantially upregulates especially in the spongiosa, around, but not within, the calcified area^{24,35} and is associated with the progression of CAVS.⁵³ It is expected to directly interact with osteopontin.⁵⁴ In contrast, tenascin-X is decreased in the disease,^{24,25,55} suggesting an impaired synthesis or a consuming process. The family of annexins are released by implicated cells into the ECM, incorporated in the EV membrane, and mediate binding to the ECM and calcium accumulation inside the EVs.⁵⁶ Galectin-3 is

increased in CAVS and induced inflammation and osteogenic turnover in VICs.⁵⁷

Protein-protein interaction networks suggest close coregulation of ECM regulating proteins with collagen II.³⁸ Similarly increased production of collagens, proteoglycans, and regulating glycoproteins is observed during development of the aortic valve.^{27,55} Overall, the above constitute an embryonic shift in the ECM synthesis. This is consistent, along with the activation of respective pathways⁴⁶ and the EndMT phenomenon.^{27,58}

IMPACT OF BIOMECHANICAL AND ECM ALTERATIONS IN CAVS MECHANISMS

The biomechanical role of the ECM is highlighted by the fact that cells typically differentiate into phenotypes responding to the mechanical forces imposed on their environment, through a constant process mediated by specialized receptors or ion channels (cellular mechanosensation) and intracellular signaling pathways (mechanotransduction), leading to respective changes in gene expression.

In CAVS, among the recognized mechanotransduction pathways, evidence exists on the activation of the transforming growth factor (TGF)- β axis,⁷ the noncanonical Wnt axis,⁵⁹ the RhoA axis,¹² the PI3K/AKT, AMPK,⁶⁰⁻⁶² the Hippo/YAP,⁶³ and the Notch axis,⁶⁴ leading to fibrosis or RunX2-mediated osteoblastic differentiation. Among mechanosensitive channels, TREK-1, Kir6.1, TRPV4, and TRPC6 are found in aortic VICs, with TRPV4 channel affecting the expression of collagen III in response to mechanical stretch⁶⁵ while inhibiting the DDR2, which directly senses collagen fibers, produces fibrosis and RunX2-mediated osteogenic calcification of the valve.⁶⁶

At the tissue level, a progressive valve stiffening impairs its mechanical function, activates VICs, and facilitates osteogenic differentiation via RhoA/ROCK pathway.^{7,12} The calcification pattern coincides with areas of high flexion stress and increased strain.⁷ The importance of valve stiffness becomes apparent as stretching of the valve alone can induce osteoblastic transformation and produce mineralized spheroids.¹² In other tissues, mechanical stress-induced myofibroblast activation implicates fibronectin, in a reversible process directly linked to the imposed mechanical forces,⁶⁷ which also directly affects matrix metalloproteinase-mediated proteolysis⁶⁸ and directly shapes the collagen-fibronectin interactions.⁴² In contrast, myofibroblast activation of VICs, results in greater ECM stiffness, as they contract and add mechanical forces upon the remodeling tissue.⁶⁹

The role of biomechanics becomes apparent in the case of the bicuspid valve, which is linked to mutations to several genes, especially of the Notch family.⁶⁴ In the bicuspid valve, similar increases in collagen, proteoglycan, and elastin content are seen, with a possible overexpression of osteonectin and laminins, and less collagen XI and metalloproteinases as compared with tricuspid degenerative CAVS.⁷⁰ There is a maldistribution of the mechanical load during diastole, and stenosis appears earlier in the cases of anatomical asymmetry among cusps.⁷¹ The fusion area is modelled to increase the stiffness of the valve,⁷² and the blood flow dynamics on the fibrosa are disturbed resulting in endothelial dysfunction.⁶⁴

Superficial blood flow dynamics play an important role in CAVS. Increased oscillatory forces induce endothelial cell activation, leucocyte infiltration, and the overexpression of critical pathways such as TGF- β , nuclear factor- $\kappa\beta$, and Notch axes.¹² Altered hemodynamics affect in a different way the fibrosa and the ventricularis, owing to inherent characteristics of the respective layer's valvular endothelial cells (VECs), which are developed in different flow conditions. In a VEC-VIC co-culture, the ECM synthesis depends on the exposure to the physiological flow pattern on the fibrosa and the ventricularis, respectively.¹² Similarly, changes in flow conditions alter the production of matrix degradation enzymes; thus, the total ECM content of the valve is influenced directly by blood flow conditions. Increased shear stress imposed on a VIC-VEC co-culture produced di- and octa-calcium phosphates (Figure 2).⁷³

Moreover, the mechanical properties of the ECM are directly related to EndMT, a fundamental mechanism in CAVS, which precedes osteoblastic transformation.⁶ In particular, VECs exposed to a stiff matrix demonstrated increased EndMT under TGF- β 1 stimulation, compared with a soft matrix.⁷⁴ Cyclic strain imposed *ex vivo* in a different direction to the cells alignment resulted in severe EndMT, via TGF- β or Wnt pathways, depending on the external force imposed.^{75,76} Glycosaminoglycans facilitate EndMT in *ex vivo* models of calcific aortic valve disease, via TGF- β 1 and ERK1/2 pathway (a member of MAPK family).^{6,74} Moreover, the mechanosensitive integrin-linked kinase, was downregulated in CAVS, and its silencing induced EndMT and RunX2-mediated osteoblastic differentiation *in vitro*,⁷⁵ while a similar role is proposed for the midkine pathway, which can be activated by several mechanosensitive and ECM-related receptors.⁷⁶

Glycosaminoglycan expansion facilitates lipid oxidation; they help more lipid particles to be trapped in the ECM, providing more substrate for oxidation reactions.⁷⁷ Biglycan colocalized with phospholipid

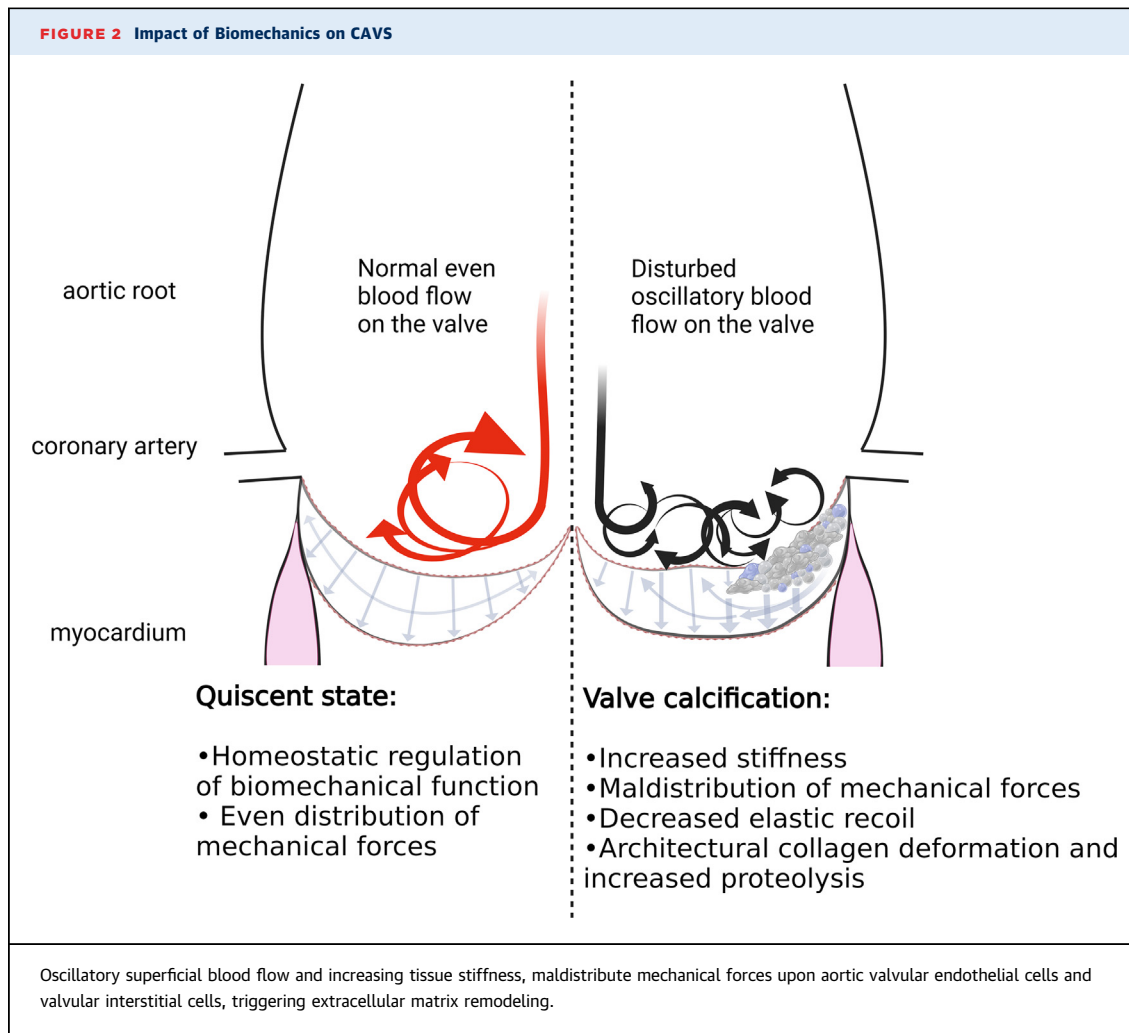
transfer protein and Toll-like receptor-2 receptor, suggesting an increased cellular absorption of the trapped lipid particles.⁷⁸ Furthermore, ECM protein fragmentation products can serve as a source of immune cell activation via the Toll-like receptors and integrins. Local macrophages can migrate into areas of collagen deformation, driven by mechanical stretching forces alone.⁷⁹ Fibronectin, biglycan, decorin, versican, and tenascin C activate Toll-like receptor-2/4,⁸⁰ whereas endostatin, a collagen XVIII fragment, is significantly increased.⁸¹ Thus, ECM proteolysis can directly increase local inflammation. Moreover, a fibrotic and disorganized ECM can bind more EVs, through interaction with fibronectin, or by providing more positively charged sites on collagen and glycosaminoglycans.⁸² A thickened ECM, may also induce hypoxia *in vivo*.⁵ Hypoxia-induced factors 1 and 2 are overexpressed in calcified aortic valves and, *in vitro*, are correlated with collagen X, local metalloproteinase activity, neoangiogenesis, and the nuclear factor- $\kappa\beta$ pathway.^{5,83,84} Matrix metalloproteinase 9 increased as O₂ saturation decreased. Hypoxia enriches the EVs released to the ECM with lysyl oxidase,⁸⁵ which might increase collagen cross-linking.

POST-TRANSLATIONAL MODIFICATIONS

There are >400 post-translational modifications identified in homeostasis and disease. Still, only a few are recognized to play a role in CAVS.

OXIDATION. Protein oxidative reactions include the removal of electrons from peptides through several diverse nonenzymatic and enzymatic reactions, initiated by reactive oxygen species, reactive nitrogen species, or reactive sulfur species. They lead to a variety of unstable products that further react until a stable byproduct is formed. Excessive oxidation is well-documented regarding lipid oxidation.¹² Nitric oxide, a reactive oxygen species scavenger, affects calcification of the aortic valve through Notch signaling, as shown *in vivo* and *in vitro*.⁸⁶ Indirect evidence implicates reactive oxygen species-mediated DNA damage to the valve, with the levels of poly(ADP-ribose) polymerase having an inverse correlation to severity of stenosis.⁸⁷ In contrast, isolated structural valve proteins oxidation and their consequences in calcification are less understood.

ENZYMATIC AND NONENZYMATIC GLYCATION OF PROTEINS. Glycation, the most frequent post-translational modification of proteins, is mainly controlled enzymatically and involves 10 monosaccharides and ≥ 12 pathways.⁸⁸ The aortic valve



contains mainly sialylated and core fucosylated N-glycans, which follow specific spatial distribution in the layers of the valve. In congenital CAVS, deregulated collagen glycosylation characterizes the thickened parts of the valve.⁵⁵ Interestingly, lumican was found to have decreased glycosylation in calcified valves, suggesting a possible role in collagen disarray during calcification.

Furthermore, glycation of lysine and arginine residues of protein moieties is also produced non-enzymatically during the formation of advanced glycation end products (AGEs). Prolonged increased concentrations of sugars induce permanent glycation of proteins or lipids. Oxidation is chemically necessary; thus, AGEs are an indirect marker of oxidative stress. Both enzymatic and nonenzymatic glycation of proteins coexist *in vivo*, posing

analytical challenges in interpreting research findings on protein glycation.

Well-controlled *in vitro* studies reveal that glycated collagen fibers demonstrate microstructural deformation, possibly owing to charging changes, hydrophobic interactions, and decreased physiological enzymatic cross-linking that stabilizes and protects collagen fibrils.^{89,90} The *ex vivo* glycation of collagenous tissues increases their mass and stiffness and decreases tensile strength, relaxation ability, elasticity, and intrafibril sliding.⁹¹ Patients with diabetes mellitus demonstrate increased calcification and aortic valve stenosis rates. AGEs induce the activation of nuclear factor- κ B,⁹² the AGE products receptor is upregulated in aortic valve stenosis,⁹³ and antidiabetic medication reduced calcification. Moreover, AGE products receptor-deficient mice

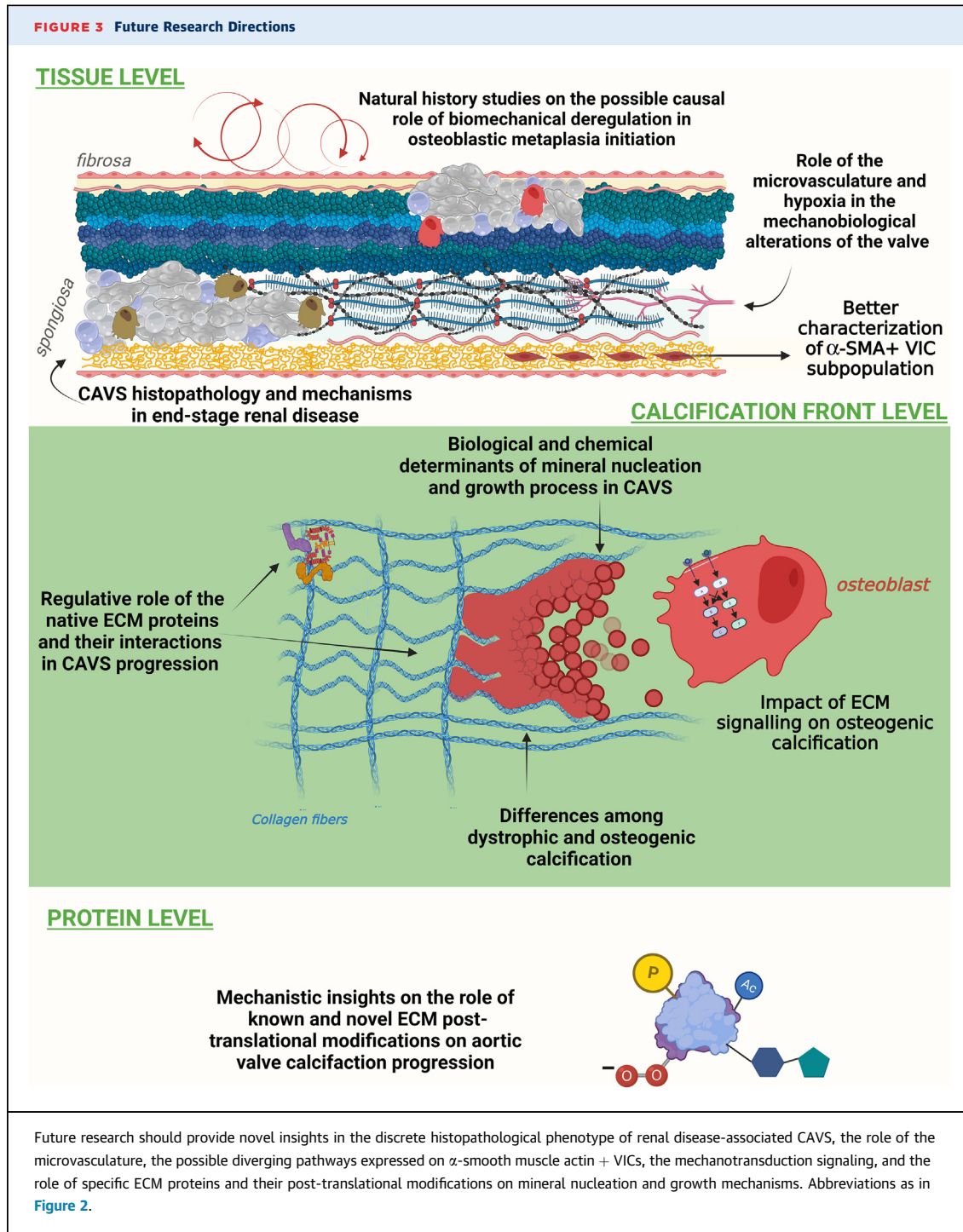
developed less aortic calcification.^{94,95} N ϵ -(Carboxymethyl)lysine and pentosidine, have been validated in CAVS.⁹⁶

CALCIFICATION DEVELOPMENT. The ECM significantly impacts the formation of calcified aortic valves since matrix can be calcified even in the absence of cells.⁹⁷ In CAVS, the presence of HAP–particles surrounding a dense, poorly crystalline apatite–has been found on cusp surfaces and embedded within lesions.⁹⁸ The apatitic mineral deposits cause further stiffening and mechanical failure of the aortic valve cusps.⁹⁹

Calcification deposits have been discriminated pathologically in the dystrophic (amorphous basophilic-stained material) and the osteogenic subtype (lamellar bone-like matrix in the presence of osteoblasts and osteoclasts).¹⁰⁰ Dystrophic calcification has been observed in damaged tissues with extensive cellular necrosis. In contrast, osteogenic calcification is linked to active osteoblast-mediated biomineralization. Osteogenic calcification has been classically identified in CAVS, but it coexists with dystrophic calcification. Dystrophic calcification is found in 83% of diseased valves and is characterized by amorphous and crystalline deposits, whereas osteogenic calcification is present in 13% of valves having dystrophic calcification and is characterized by an osteoid matrix similar to active bone formation.¹⁰¹ Dystrophic and osteogenic calcification formation depend on mechanical stress and proinflammatory factors. Dystrophic calcification affects more often the aortic valves¹⁰² and possibly precedes the osteogenic form long before the rapid phase of valve stenosis. In vitro models of valvular calcification describe the formation of calcified nodules in VIC cultures with conditions mimicking the dystrophic and osteogenic calcification of aortic valve cusps.¹⁰¹ Dystrophic nodules, evident in dead cells, are formed on stiff substrates and lead themselves to the differentiation of VICs into activated myofibroblasts. In contrast, smaller than dystrophic, osteogenic nodules are formed on bone matrix secreted by VICs undergoing osteogenic differentiation.¹⁰¹ It is unclear whether these calcification processes are linked or independent.¹⁰³ Osteopontin, a highly acidic and phosphorylated ECM protein not normally expressed in the aortic valve, binds strongly to HAP and has been found in vascular dystrophic calcification regions.¹⁰⁴ This finding could be indirect evidence that these 2 processes are interconnected. Both calcified nodules contain calcium and phosphorus, with osteogenic nodules having abundant calcified

spheres on the surface, in contrast with dystrophic nodules having negligible or no surface calcification.¹⁰¹ Furthermore, aggregations of elongated cells have been observed around the dystrophic nodules, whereas living cells occurred within osteogenic nodules.¹⁰¹

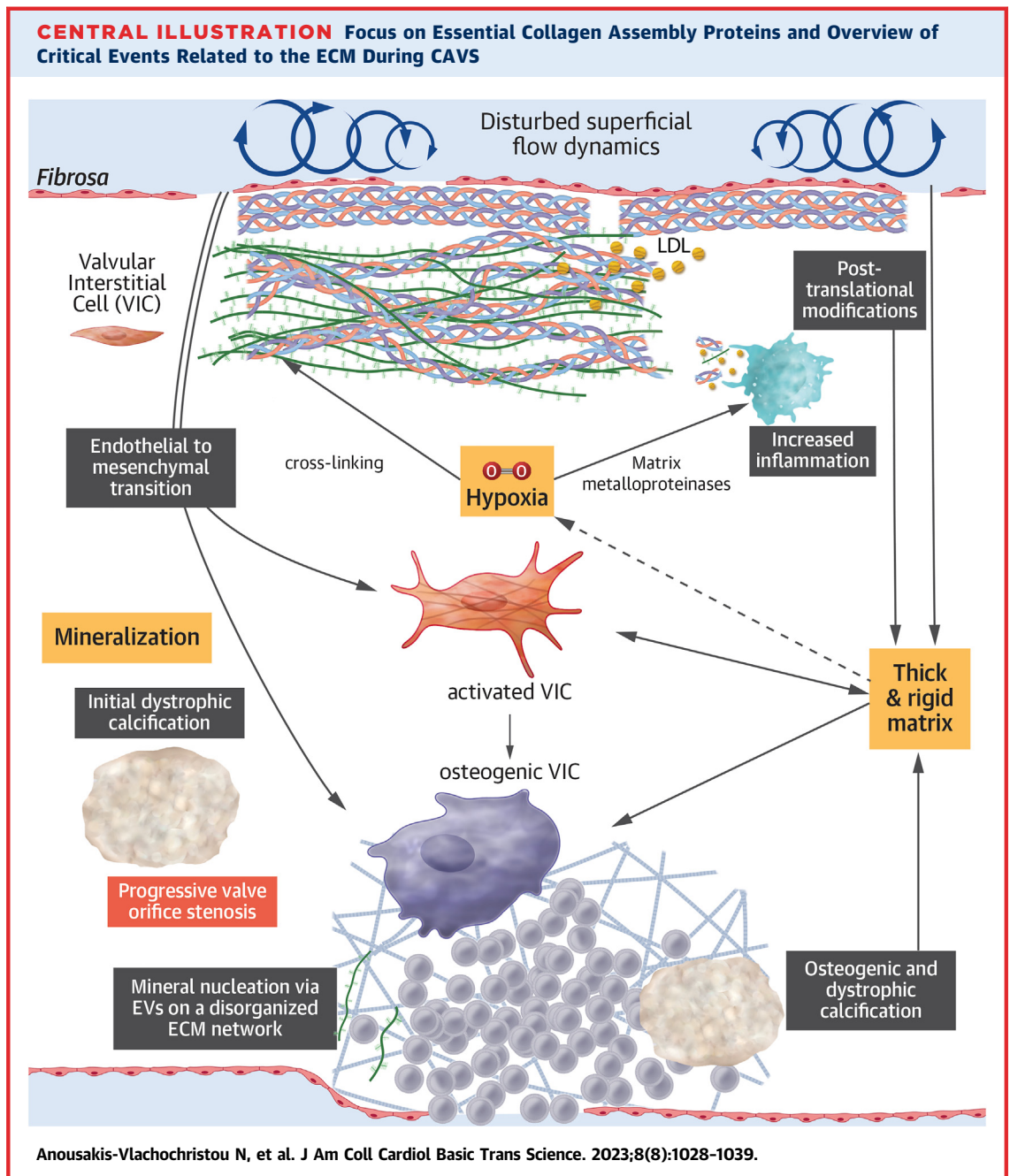
The calcification nucleation and growth processes significantly depend on the local chemical and biological microenvironments. The existence of calcified spherical nodules could denote a particle-based crystallization pathway. However, more evidence should be provided to prove this, along with the amorphous or crystalline state of the observed material.¹⁰⁵ Calcium salt deposition and nucleation is facilitated through EVs, which actively promote mineralization, providing locally high calcium concentrations and enzymatically liberating free phosphate ions.⁸² Furthermore, calcification can simultaneously be promoted through passive deposition when constantly present natural inhibitors of calcification are reduced, such as PPI and matrix Gla protein. Degenerative CAVS patient-derived VICs demonstrated lowered levels of adenosine triphosphate, a PPI donor, owing to mitochondrial dysfunction,¹⁰⁶ whereas the induction of adenosine triphosphate and PPI decreases calcification of porcine valves and VICs *ex vivo*.^{107,108} Matrix Gla protein inhibits HAP crystal growth and is reduced in the serum of patients with degenerative CAVS, correlating with stenosis severity.¹⁰⁹ Moreover, The DNA damage response product, poly(ADP-ribose) colocalizes with calcification in both physiological (bone) and pathological ECM calcification (vascular and valvular), and mediates crystal nucleation.^{87,110} To date, damaged DNA fragments have not been associated causally with calcification deposits. Recently, DNA-based biomaterials have been used as biomimetics for growing hierarchically mineralized structures, meaning that DNA can act as a scaffold for mineralization.^{105,111} Under physiological conditions, collagen functions as a template, which directs the nucleation and growth of ~2-nm-thick calcium phosphate crystals like the ones found in bone. During this process, charged macromolecules stabilizing transient amorphous calcium phosphate precursor phases facilitate its infiltration to the collagen.¹¹² Interestingly, in Jurkat cells, oxidative stress, which is also present in CAVS, resulted in the exocytosis of EVs with DNA or DNA-binding proteins bound to their membrane, which mediated the EVs binding to fibronectin,¹¹³ suggesting a putative mechanism of active calcification propagation through ordinal EV buildup on the ECM template.



FUTURE RESEARCH DIRECTIONS

Collectively, the presented research data delineate the role of ECM alterations in critical aspects of the pathobiology recognized in CAVS, linking changes in the local mechanobiology with osteogenic

differentiation and excessive calcification of the valve cusps (Figure 3). Data describe a vicious cycle between significant mechanobiological alterations and ECM remodeling with VIC activation and osteoblastic differentiation. The possibility that aging and comorbidities lead to the gradual mechanical



degradation of the valve, with chronically persistent ECM remodeling as a maladaptive reparative mechanism, which in turn acts as a causal factor for osteoblastic metaplasia, needs to be evaluated.

Further investigation is required for a better understanding of disease progression and provide possible treatment options. The **Central Illustration** summarizes essential research areas that should be developed. Further investigation is required to

identify ECM proteins that regulate collagen and ECM damage at early CAVS stages, and which post-translational modifications impact the stiffness alterations and calcification of the valves at a high level. Despite data on calcified spherical particles, there is no direct evidence about the calcification in multiple scales, which ECM proteins participate during this process, and what their specific role would be. Furthermore, there is no clear evidence

of whether dystrophic and osteogenic calcification are part of the same process. The histopathology of CAVS at a final stage of renal disease has not been identified yet, in contrast with arterial calcification. More evidence should be provided on the native matrix proteins at the earlier stages of the CAVS, and their interactions during disease. This work could lead to pathophysiological hypothesis generation and ultimately the design of new therapeutics for decreasing the rate of disease progression.

ACKNOWLEDGMENTS The authors thank Antigoni Miliou, PhD, for comments and reading of the final manuscript. Figures were created using BioRender.com. Ethical approval was not obtained, as per review paper.

FUNDING SUPPORT AND AUTHOR DISCLOSURES

Drs Anousakis-Vlachochristou, Athanasiadou, and Toutouzas acknowledge the European Union (E.U.), and Greek funds through the Operational Program “Human Resources Development, Education and Lifelong Learning” (NSRF 2014-2020), under the call “Supporting Researchers with an Emphasis on Young Researchers – Cycle B” (MIS: 5047949), Athens, Greece, for funding. Dr Carneiro has reported that she has no relationships relevant to the contents of this paper to disclose.

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KEY WORDS Biomineralization, calcific aortic valve stenosis, calcification, extracellular matrix proteins, mechanobiology