Extracellular matrix: paving the way to the newest trends in atherosclerosis

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INTRODUCTION

The extracellular matrix (ECM) is involved in all aspects of vascular pathobiology. The major constituents of vascular ECMs are collagens, elastic fibres, proteoglycans, hyaluronan and a variety of glycoproteins, all interconnected in a complex active three-dimensional (3D) matrix network. This network regulates the biomechanical properties of blood vessels and the phenotype of the cells that reside in them, such as endothelial cells, vascular smooth muscle cells (VSMCs), adventitial fibroblasts and infiltrating immune cells from the circulation. Though many cell types mentioned can synthesize ECM macromolecules, VSMCs are considered the most prominent [1].

The initial event of atherogenesis occurs upon focal endothelial cell injury in artery branches or curvatures with nonlaminar blood flow, attracting circulating monocytes and T cells. Monocytes moving into the subendothelial intima differentiate into macrophages that secrete cytokines and exacerbate the inflammatory environment, as well as endocytose LDL particles, becoming lipid-engorged foam cells. Simultaneously, medial SMCs migrate into the intima where they proliferate and secrete collagen fibres that form a stabilizing fibrous cap. Conversely, formation of a large lipid-rich necrotic core destabilizes the lesion, potentially resulting in calcification. As a result, the atheroma becomes larger, potentially leading to plaque rupture and thrombosis.

Keywords
atherosclerosis, calcification, collagen, elastin, extracellular matrix

Purpose of review
The extracellular matrix (ECM) is critical for all aspects of vascular pathobiology. In vascular disease the balance of its structural components is shifted. In atherosclerotic plaques there is in fact a dynamic battle between stabilizing and proinflammatory responses. This review explores the most recent strides that have been made to detail the active role of the ECM – and its main binding partners – in driving atherosclerotic plaque development and destabilization.

Recent findings
Proteoglycans-glycosaminoglycans (PGs-GAGs) synthesis and remodelling, as well as elastin synthesis, cross-linking, degradation and its elastokines potentially affect disease progression, providing multiple steps for potential therapeutic intervention and diagnostic targeted imaging. Of note, GAGs biosynthetic enzymes modulate the phenotype of vascular resident and infiltrating cells. In addition, while plaque collagen structure exerts very palpable effects on its immediate surroundings, a new role for collagen is also emerging on a more systemic level as a biomarker for cardiovascular disease as well as a target for selective drug-delivery.

Summary
The importance of studying the ECM in atherosclerosis is more and more acknowledged and various systems are being developed to visualize, target and mimic it.
rapture or erosion, in high-risk, rupture-prone, also called vulnerable plaques, inducing thrombosis, which can lead to arterial occlusion, resulting in a myocardial infarction (MI) or stroke [2] (Fig. 1).

The vascular structure and function throughout life is defined from a balanced interplay among different ECM components. Specifically, almost half of the total normal arterial ECM consists of elastic fibre proteins, with lesser amounts of collagen, proteoglycans and glycoproteins. In atherosclerosis the balance is shifted with proteoglycans being the major ECM component in the early lesion phase, while, as the lesions progress collagens take over with decreased elastic fibres, proteoglycans and glycoproteins [3] (Fig. 1).

Taking into consideration the huge size of the field, this review summarizes the most recent strides to detail the active role of the ECM in driving atherosclerotic plaque development and destabilization and the newest trends on how to use this knowledge in future clinical applications.

**PROTEOGLYCANS AND THEIR GLYCOSAMINOGLYCANS IN ATHEROSCLEROSIS**

Proteoglycans (PGs) are comprised of sulphated glycosaminoglycans (GAGs) attached covalently to a protein core. Considering that a simple protein core can be modified with numerous GAGs, chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparin and heparan sulphate (HS) or noncovalently bound hyaluronan, possibly of different types, it is clear that proteoglycans are extremely diverse molecules in the pathogenesis of atherosclerosis [1].

**KEY POINTS**

- The atherosclerotic plaque ECM is not a simply a structural scaffold but rather plays a dynamic role in atherosclerosis.
- Elastin synthesis, cross-linking, degradation and its elastokines provide several point of potential intervention to modulate and image atherosclerosis.
- Plaque collagen affects plaque stability not only through quantity, but also fibrillar direction, and may serve as a selective target for drug delivery.
- Proteoglycans GAGs biosynthetic enzymes may be plausible target to treat atherosclerosis both in its early and advanced stages.
- Incorporating ECM structure – both in-vivo and through in-vitro simulation – will provide crucial mechanistic insights into future therapeutic interventions.

**FIGURE 1.** Progression of atherosclerosis and extracellular matrix components from normal vessel to early and advanced atherosclerotic lesion. COL, collagens; EL, elastin; GP, glycoprotein; PG, proteoglycan.
High-affinity complexes with lipoproteins
The current paradigm of the atherogenesis, the so-called response-to-retention hypothesis, involves the accumulation of lipoproteins in the intima driven by modified ECM molecules. Most incriminated proteoglycans in this process are versican (CSPG), biglycan (CS/DSPG), decorin and perlecan (HSPG) [3–6]. The retention ability of proteoglycans is mainly addressed in the context of SMCs and endothelial cells. However, macrophage-derived proteoglycans notably also bind lipoproteins via both their protein core and heparan sulphate GAG chains [7**]. In the same study, the authors reported that the matrisome of macrophages is sensitive to macrophage polarization. A vital regulator of these interactions is GAG biosynthesis. For example, the chondroitin sulphate GAG biosynthesis gene (ChGn-2) impacts atherosclerosis by influencing the retention of lipoproteins by SMCs as well as macrophage [8*]. Mechanistically, this occurs through a G protein-coupled receptor transactivation of TGFBR1, thus upregulating genes associated with GAG chain elongation [9]. Overall, GAG biosynthetic enzymes may be plausible targets to treat atherosclerosis both in its early and advanced stages.

Endothelial glycocalyx
The luminal surface of endothelial cells is covered by a thin negatively charged organized network of membrane-attached PGs (syndecan-1, syndecan-2, syndecan-4 and glypic-an-1) and secreted PGs (mimecan, perlecan and biglycan), often called the endothelial glycocalyx. It provides endothelial cells protection, while serving as molecular barrier, cell adhesion modulator and mechanosensor for blood flow. HS accounts for 50–90% of the total GAG pool, while the rest is composed of CS and hyaluronan [10]. In a recent study, endothelial cells exposed to pathological shear stress increased GAG synthesis, secretion and content, compared with those exposed to physiological shear stress [11*]. The extent of atherosclerosis progression is associated with thinning of vascular endothelial glycocalyx, attributed to diminished expression of HSPGs [12*].

One of the underlying mechanisms reported involves the inactivation of AMP-activated protein kinase and subsequent activation of hyaluronidase 2 that cleaves hyaluronan from the endothelial glycocalyx. This glycocalyx impairment results in increased macrophage recruitment [13]. While the presence of hyaluronan on the endothelial glycocalyx is anti-thrombotic and anti-inflammatory, suppressing leukocyte and platelet adhesion, hyaluronan can also be proatherosclerotic through abnormal accumulation due to hyaluronan synthase upregulation [14].

Hyaluronan, through CD44, induces VSMC migration and proliferation, which in turn leads to plaque growth and increased matrix production. Finally, macrophage activity is enhanced in plaques via their hyaluronan-Toll-like receptors, promoting macrophage accumulation and Th1 cell polarization.

Another secreted glycocalyx PG, mimecan, was recently related to a vulnerable phenotype in human plaques and increased risk of future cardiovascular death. Notably, in this study, mimecan is inversely correlated with SMCs, while in vitro mimecan expression is upregulated upon inflammatory stimuli in macrophages [15].

The actions of several ECM molecules also depend on the disease stage. Cartilage oligomeric matrix protein (COMP), expressed by VSMCs, protects the vasculature in early stages of plaque development by moderating disturbed flow-induced endothelial cell activation through integrin α5β1/FAK inactivation [16**]. In contrast, COMP expression in advanced plaques is upregulated and correlates with the vulnerable plaque phenotype by modulating the macrophage phenotype [17].

Immunomodulatory properties of proteoglycans and their glycosaminoglycans
After the last decades of atherosclerosis research, proteoglycans and subsequently, their GAGs have been mainly considered in the context of SMC and endothelium biology [18,19*]. However, the last years’ research offers fresh insight on how immune cells with PGs and their GAGs can orchestrate atherosclerosis progression. Esko et al. [20] reviewed the leading role of neutrophils, endothelium and macrophage HSPGs in atherogenesis, describing direct effects on for example macrophage polarization, also corroborated by Swan et al. For example, monocyte syndecan-1 expression modulated type I interferon signalling, promoting nonclassical macrophage activation, while lack of heparan sulphate sulphation promotes classical inflammatory activation. On the other hand, macrophage syndecan-4 acts as scavenger receptor, promoting foam cell formation. Of note, the syndecan-4/L-selectin axis is reported to modulate neutrophil infiltration. In turn, neutrophils extracellular traps are formed inducing inflammatory signalling by macrophages. In support of these observations, it was reported that HS biosynthesis is fine-tuned during macrophage polarisation, depicting their resolving or proinflammatory macrophage phenotype during atherosclerosis-related inflammation [21]. Recently, it was reported that M1 macrophages produced PGs with a higher level of HS chain sulphation compared with M2 macrophages [7**]. These observations encourage further studies on the
significance of HS in the immune signature of the plaques that may lead to future improved treatment against plaque destabilization.

Proteoglycans and their glycosaminoglycans: therapeutic potential
Advances on the molecular mechanisms underlying the actions of PGs and their GAGs in atherosclerosis, have lately emerged through different targeting approaches. The rationale to develop peptidomimetics to mimic the protective role or block detrimental actions of ECM molecules for the plaque was described by Lv et al. [16**] for COMP. In addition, several studies have employed CD44-hyaluronan interactions for targeting atherosclerosis, designing hyaluronan-coated polymeric micelles targeting CD44 overexpressing macrophages [22,23]. Further research is needed to characterise proteoglycan remodelling by degrading enzymes for example proteoglycanases, heparinase, at different stages of the atherosclerosis, introducing novel targets or biomarkers.

ELASTIN IN ATHEROSCLEROSIS
Elastin is an essential protein to provide elasticity and resilience to arteries, maintaining constant adaptation to haemodynamic changes during the cardiac cycle. This has been elegantly reinforced in mice models by Stoka et al. [24]. With a half-life of several decades, elastic fibres are extremely stable over time and very resistant to chemical physical attacks such as pH changes, heat and stretching [25]. After synthesis, elastin cross-linking by lysyl oxidase family members (LOX and LOXL-1) is also a process that can affect several stages of atherosclerosis, as brilliantly reviewed by Martinez-Gonzalez et al. [26]. Taken together, the elastic fibres are more than inert physical scaffolds, involved in dynamic signalling between cells and their environment.

Elastin in mineralization induction
The calcium-binding capacity of elastin is due to the presence of mineralization nucleation sites, in particular sulphydryl and carboxyl groups even in the absence of cells. Boraldi et al. [27*] have recently shown that mineral deposition is favoured on hydrolysed fibrillar structures. Exposure of multiple charged sites increase the adsorption of \( \text{Ca}^{2+} \), attracting phosphate and increasing the local ion concentration past the point of supersaturation. Even small damages on the surface of elastin fibrillar structures are sufficient to increase the mineralization process and act in synergy with peptides released from the whole elastin molecule, as inducers of an osteogenic response in VSMCs, actively modulating gene expression.

Ectopic calcification through targeted gene analysis was recently studied by Dayekh and Mequanint [28*]. They showed that partially soluble elastin upregulated VSMC osteogenic genes accompanied by downregulation of smooth muscle myosin heavy chain (Myh11), a late-stage SMC differentiation marker, suggesting a skewing towards a synthetic phenotype: two important features of vascular calcification. Another recently described mediator – specifically in stiffness-dependent VSMC calcification – is a positive feedback loop between the collagen receptor discoidin domain receptor-1 activity and actomyosin contractility [29*].

The proatherogenic effects of elastokines
Peptides obtained by degradation of elastin are known as elastokines, elastin-derived or elastin-related peptides, many of which are listed in a recent review by Heinz [30]. The elastokine-binding receptor – elastin receptor complex (ERC), first described in the 1980s – has three components: first, the splice variant of \( \beta \)-galactosidase; second, cathepsin A; and third, neuraminidase (Neu1). Binding of elastokines to ERC activates Neu1, which then modulates the CD36 sialylation, in turn regulating oxLDL uptake [31,32]. This newly discovered interaction between CD36 and the Neu1 component of ERC further contributes to the proatherogenic effects of the elastokines. Furthermore, it emphasizes the elastokine effects as modulators of macrophage functions during atherogenesis, namely phagocytosis, cytokine release, migration and reactive oxygen species production. They promote angiogenesis, induce proteases expression, modulate the vascular tone affecting NO production and relaxation of endothelial cells; contractility and proliferation of vascular SMC as well as chemotaxis and cytokine release from immune cells. Significantly, Gayral et al. [33] already in 2014 showed that elastokines injected in mice led to an increase in atherosclerotic plaque size through the immune Neu1-PI3Kgamma pathway. Moreover, Neu1 is involved in the mediation of elastokine effects in insulin resistance and thrombosis [34,35]. Actually, elastokines through lactosylceramide/p-ERK1–2 signalling pathway are also involved in adipocyte dedifferentiation, which can be relevant in the metabolic syndrome subject who often suffer from atherosclerosis too [31].

A very recent study by Bocquet et al. [36*] focused on the inhibition of neuraminidases such as Neu1 to modulate atherosclerosis, by testing oseltamivir phosphate (a widely used anti influenza sialidase inhibitor) in Ldl\(^{-/-}\) mice on high fat diet.
They showed a decrease in plasma LDL and aortic elastin fragmentation, but unfortunately without effect on plaque size, and in concert with increased liver inflammation and fibrosis.

**Elastin as an imaging target**

Another interesting use of the ECM composition is the possibility to target molecules for diagnostic purposes, particularly if the targeted molecules are specific for characteristics of the rupture-prone plaques so that patients at high risk can be timely identified before symptoms occur. Dysfunctional elastogenesis occurs during atheroma formation, leading to the formation of tropoelastin in plaques which fail to cross-link into functional elastic fibres [37]. This has been implemented in imaging attempts from several groups on the last decade, culminating with the in-vivo detection and quantification of different MRI markers in a single scan now proven feasible using simultaneous assessment of plaque-burden and inflammatory activity by dual-probe molecular MRI of progressive atherosclerosis [38,39]. A 3T MRI scanner with the novel superparamagnetic iron oxide nanoparticle contrast agent and an elastin-targeted gadolinium agent enabled the visualisation of plaque burden in mice. No significant difference between the uptake of either contrast agent between stable versus vulnerable plaques was described, although it should be noted that the concept of vulnerable plaque in mice, is in itself highly controversial [40*].

Recent advances of atomic force microscopy have shown, with unprecedented resolution, a new view on the stiffness process and elasticity impairment at the molecular level, in mice. Such precise quantification of fibre elasticity may enable tracking of fibre remodelling with improved stringency to predict plaque rupture in the future [41*].

**COLLAGEN STRUCTURE AND PLAQUE INTEGRITY**

The common feature of collagens – the most abundant proteins in mammals – is the rigid triple helical structure (Gly-X-Y repeats) intermingled with more flexible, nontriple helical regions. The resulting aggregates determine the biomechanical properties of tissues, such as the tensile strength of arteries. Of the 28 types described so far, collagen type I and III are the main forms expressed in atherosclerotic plaques [42].

The artery wall depends on the various mechanical parameters of each layer for proper function and contractility. Recently, the visualisation of such structures have become increasingly feasible, exemplified by Johnston et al. [43**] who – using the small angle light scattering technique – found that collagen fibre alignment in the circumferential direction in the plaque cap plays the most dominant role for determining plaque structural stability. These plaques exhibited superior load bearing (i.e. higher stresses and lower strains) before failure, while plaques with caps with predominately axial fibres displayed the opposite trend, suggesting a possible therapeutic utilisation to identify rupture-prone plaques. Significantly, the study also indicates that collagen content alone does not correlate with ultimate tensile stress and strain [43**]. In a murine model (Apoe-/-), similar changes in aortic adventitial collagen organization was associated with aging, with a skewing towards longitudinal – as opposed to circumferential – orientation in mice given normal chow, curiously enough with the opposite effect in mice on a high-fat diet [44*].

**Collagen as a cardiovascular disease marker**

While plaque collagen structure exerts very palpable effects on its immediate surroundings, a new role for collagen or its fragments is also emerging as an overall cardiovascular disease (CVD) indicator on a systemic level. Circulating collagen type VI alpha 2 chain (COL6A) was recently put forth by Liu et al. [45*] as an independent predictor – and thus potential noninvasive marker – of plaque erosion in patients undergoing optical coherence tomography during ST-segment elevated MI. Increased baseline levels of plasma endostatin – a type XVIII collagen cleavage product – has been associated with increased risk of mortality and severe disability following ischemic stroke in a large Chinese population. Moreover, adding plasma endostatin to a basic model constructed with conventional factors significantly improved risk stratification in this population [46*]. The collagen advanced glycation end product glucuronidine/LW-1 found in skin, rather than in the circulation, was also associated with established coronary heart disease and calcified plaque volume in diabetic patients [47*].

**Collagen: therapeutic potential**

Adding yet another layer to the role of collagen in CVD, is its selective targeting as an exciting strategy for drug-delivery. To this end, Kassam et al. has developed a collagen-targeted peptide amphiphile-based nanofibre for the prevention of neointimal hyperplasia after arterial injury. Of benefit are differing pharmacokinetics, including rapid elimination clearance, compared with traditional small molecules, which may potentially achieve a
therapeutic effect with comparatively lower plasma concentrations [48]. Peptide probes have also been devised for selective detection of denatured collagen by incorporating nonimino acids at the X and Y positions of the characteristic helical (Gly-X-Y)ₙ amino acid collagen sequence pattern [49]. Moreover, nanoparticles recognizing collagen type IV, developed by Molinaro et al. [50] for selective drug delivery to regions of endothelial cell sloughing and collagen type IV-rich basement membrane exposure was confirmed to successfully target in vivo experimentally eroded murine endothelium.

Plaque collagen is a widely used indicator of atherosclerotic plaque stability, and effects inferred by a multitude of elements have been described recently. One especially interesting family of such factors is the microRNAs (miRNA/miR), manipulations of which in murine models demonstrate a clear role in atherogenesis with promising therapeutic potential. Promoting or inhibiting a variety of miRs has been reported to infer plaque stabilization (miR-9, miR-335-5p, miR-520a-3p, 14q32 miR-494 [51,52]), attenuated plaque growth (miR-9, miR-495–3p, miR-520c-3p, miR-21, miR-520a-3p [51,52,53]), and destabilization (miR-208w [58]).

CONCLUSION

The importance of studying the ECM is increasingly acknowledged and various systems are under development both to track – such as the utilization of ECM-derived biomarkers – and to mimic it. An example of the latter, a simple, pumpless, close-loop, easy-to-replicate and miniaturized flow device introduced by Hosseini et al. [59], concurrently exposes 3D engineered vascular smooth muscle tissues to high-velocity pulsatile versus low-velocity disturbed flow conditions, manipulating elastin and collagen type I assembly. They showed that gene expression of matrix metalloproteinase and their inhibitors, rather than that of collagens and elastin, seemed to be flow-dependent and were also able to halt the effect of low-velocity flow on the ECM remodelling through treatment with doxycycline, an MMP inhibitor. Elastin-like polymers produced...
by recombinant techniques, also called elastin-like recombinomers seem particularly promising to manufacture an elastic fibrous scaffold, that can receive different cell adhesion sequences and be a potential tool in regenerative medicine [60]. Therefore, these types of platforms can be exploited for drug efficacy studies by providing crucial mechanistic insights into therapeutic interventions with effects on the ECM: an approach we are likely to see included more and more often as we look towards future intervention studies in the field of vascular pathobiology.

Overall, future investigations will need to address how tissue specific and temporal differences in ECM impact atherosclerosis. Of importance in the line with personalized treatment is the notion that individual unique changes to enzymes that affect GAGs sulphation and elongation may reflect differences in susceptibility to atherogenesis or may pinpoint a vulnerable plaque (Fig. 2).

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Conflicts of interest
There are no conflicts of interest.

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