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The interplay of collagen, macrophages, and microcalcification in atherosclerotic plaque cap rupture mechanics

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Abstract

The rupture of an atherosclerotic plaque cap overlying a lipid pool and/or necrotic core can lead to thrombotic cardiovascular events. In essence, the rupture of the plaque cap is a mechanical event, which occurs when the local stress exceeds the local tissue strength. However, due to inter- and intra-cap heterogeneity, the resulting ultimate cap strength varies, causing proper assessment of the plaque at risk of rupture to be lacking. Important players involved in tissue strength include the load-bearing collagenous matrix, macrophages, as major promoters of extracellular matrix degradation, and microcalcifications, deposits that can exacerbate local stress, increasing tissue propensity for rupture. This review summarizes the role of these components individually in tissue mechanics, along with the interplay between them. We argue that to be able to improve risk assessment, a better understanding of the effect of these individual components, as well as their reciprocal relationships on cap mechanics, is required. Finally, we discuss potential future steps, including a holistic multidisciplinary approach, multifactorial 3D in vitro model systems, and advancements in imaging techniques. The obtained knowledge will ultimately serve as input to help diagnose, prevent, and treat atherosclerotic cap rupture.

Keywords Atherosclerosis · Tissue mechanics · Collagen · Macrophages · Microcalcifications

Introduction—atherosclerotic plaque cap rupture risk

Failure of an atherosclerotic plaque is the culprit of many, often disabling or lethal, cardiovascular events, including myocardial infarction and stroke [15, 99, 144]. These acute events are associated with vascular biomechanics, and the cellular composition and matrix architecture of a plaque. The most common cause of failure of the atherosclerotic plaque is rupture, accounting for approximately 60% of events. Plaque erosion (35% of events) and calcified nodules (up to 5% of events) can also cause thrombi, however less frequently [249]. The probability of rupture is dependent on the stage of the atherosclerotic lesion. Virmani and colleagues proposed a lesion classification scheme, based on histopathological evaluation, starting with intimal thickening and progressing to intimal xanthoma, pathological intimal thickening, fibroatheroma and finally the vulnerable thin-cap fibroatheroma [268]. This last phenotype is considered as the main lesion with potential for rupture and is, therefore, named vulnerable plaque. These vulnerable plaques are characterized by a thin collagenous cap (i.e., the fibrous

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cap), which is often inflamed, calcified, and depleted from smooth muscle cells (SMCs) [17, 18, 81, 119, 252]. This cap is overlying a large lipid and/or necrotic core which is accompanied by inflammation and intraplaque hemorrhage [72]. Although great advancements in clinical imaging have been made to accurately image those features associated with vulnerability [58, 66], like cap thickness [108], the size of the lipid and/or necrotic core [181], inflammatory cells [85], calcifications [254], and intraplaque hemorrhages [51], phenotypic identification of the vulnerable phenotype is still found to have suboptimal predictive power for future clinical events [68, 176, 259]. Traditionally, a cap thickness of less than 65 μm is accepted to define a rupture-prone coronary plaque, while for carotid plaques, this threshold is set to 200 μm [36, 201]. However, clinical studies have shown that physical activity can trigger atherosclerotic plaque rupture, even in coronary plaques with a thickness of 70–140 μm [127]. Furthermore, carotid plaques have also been shown to rupture at thicknesses up to 500 μm [201]. As such, guidelines to identify vulnerable plaques continue to be revised, with various phenotypic features of atherosclerotic plaques playing a role in creating a rupture-prone phenotype.

Years of extensive research have exposed various biochemical processes that precede and initiate plaque failure, as excellently reviewed by others [17, 20, 89, 152]. Rupture itself is a mechanical event that generally occurs at the cap when local stress levels exceed local tissue strength, which underlines the importance of understanding cap mechanics to prevent and foresee plaque failure [9, 204]. The stress distribution within the cap depends on the cap geometry, the mechanical properties of the cap constituents, and the external loading conditions (e.g., blood pressure). Nevertheless, due to the inter- and intra-cap heterogeneity, the stress distributions, as well as ultimate strength values, and failure mechanisms are substantially different. A wide range in ultimate tensile cap strength values have been reported (158 kPa [234] to 870 kPa [111]). Importantly, geometry, composition, and resulting cap stress and strength will change in a spatiotemporal manner due to the iterative interplay between the cellular populations and environmental cues, including matrix properties and mechanical loading [96, 195, 207, 260].

To improve risk assessment, it is a requisite to highlight the importance of cap mechanics in plaque biology and cap failure. This review would like to create awareness for this topic by evaluating current knowledge on the role of cap constituents in plaque cap mechanical (de)stabilization. We hereby focus on the end-stage, vulnerable thin cap, and assess the role of three components present in this phase. The first constituent included is collagen, which is the main load-bearing component of the cap [101] and often scarcely renewed in vulnerable caps due to SMC depletion and potential senescence [17, 18, 81, 252]. Second, macrophages

are discussed as major cell population correlated with an enhanced rupture risk [225] by directly initiating extracellular matrix (ECM) degradation via the secretion of degrading compounds like MMPs [175]. Third, the role of microcalcifications is emphasized, as being the most abundant type of calcification present in the vulnerable plaque cap [116]. Microcalcifications are included in this review due to their increasingly named role as local stress concentrators, possibly being a cause of cap rupture [42, 52, 116, 257]. We will elaborate on the contribution of these three constituents to cap mechanics directly, their biological interplay and discuss future work that could enhance our biomechanical understanding to better identify the cap at risk of rupture.

Cap components: macrophages, collagen, and microcalcification

Macrophages

Atherosclerosis is a lipoprotein-induced chronic inflammatory disorder [263]. Upon endothelial dysfunction, low-density lipoprotein (LDL) accumulate within the intima and oxidize, after which monocytes are recruited [49, 62, 147]. Inside the vessel intima, monocytes mature into macrophages, behaving as either more pro-inflammatory (i.e., M1) or anti-inflammatory (i.e., M2), depending on their activated intracellular signaling pathways and the cellular microenvironment [26, 231]. Although lesional macrophages are heterogeneous and can differentiate into various phenotypes, it is generally accepted that the M1/M2 ratio is high in vulnerable caps [76], with more pro-inflammatory-like macrophages in the shoulder regions and lipid core, while fibrous mid-cap sections generally contain both phenotypes [76, 228]. Many of the macrophages within the plaque can phagocytose lipids [146] and remove apoptotic cells and other cellular debris [217]. However, due to their inability to digest oxidized LDL, these macrophages become oversaturated with lipids and transform into foam cells, contributing to formation of a lipid pool and/or necrotic core [143].

In line with plaque heterogeneity, the macrophages that reside in the plaque are considered to be highly versatile and plastic [24, 54, 110, 149]. The generally proposed M1/M2 macrophage diversity is oversimplified, and various other phenotypes have been proposed to exist (i.e., M2a, M2b, and M2c). Recently, athero-specific subsets (M4, M(Hb), and Mhem) were observed in human plaques [24, 32, 54, 110, 149, 228, 262], but although they are considered to be either proatherogenic (M4) or atheroprotective ((M(Hb) and Mhem) [26], how these subsets affect their nearby microenvironment and alter cap mechanical properties is still largely unknown.

Of note, besides macrophages, various other immune cells play a role in the initiation and progression of atherosclerosis. In thin fibrous caps specifically, large numbers of macrophages and T cells were found to correlate with an enhanced rupture risk [225]. Herein macrophages are known as the principal cell actively secreting degrading compounds, including matrix metalloproteinases (MMPs) [182], whereas T cells predominantly have an instructing role stimulating macrophages to release MMPs [174]. Therefore, the macrophage is chosen as the focus of this review.

Collagen

Soluble factors produced by inflammatory cells that reside within the plaque induce the migration and proliferation of vascular smooth muscle cells (SMCs) toward the intima. It is generally assumed that they undergo a transition from a quiescent, contractile phenotype to a proliferative, synthetic phenotype. SMCs synthesize ECM molecules, including collagen, elastin, as well as glycosaminoglycans (GAGs) and proteoglycans. These ECM components induce thickening of the intima layer and contribute to the formation of a fibrous plaque cap [144]. The composition of ECM components changes during atherogenesis. In advanced plaques, collagen is the major constituent of the ECM accounting for up to 60% of the total protein content [14, 82, 114, 203, 218], while in healthy arteries, the majority of protein is elastin [82]. Moreover, the ECM of fibrous caps of advanced plaques is subjected to aging as a consequence of reduced SMC proliferation and increased SMC death and senescence, while the growing core extends outwards, resulting in further thinning of the fibrous cap [17, 18, 81, 252]. The main collagen types in the fibrous cap of advanced plaques are the load-bearing fibrillar collagens type I and III [14, 114, 193, 218]. Both types are diffusely (co)distributed in plaques, with their local content and relative proportions varying both within and between plaques [114, 165]. The tensile strength and torsional stability of these collagens primarily regulate cap structural integrity [14, 114, 218]. Other collagen types regularly detected in atherosclerotic lesions include collagen type IV, V, VI, and VIII [114, 193, 250], with varying primary locations and functions.

Intermolecular covalent cross-links are formed between the collagen molecules, catalyzed by enzymes such as lysyl oxidase (LOX) [151, 161], and proteoglycan-rich matrix is deposited between fibrils that together affect collagen mechanical performance [230]. Furthermore, collagens in atherosclerotic plaques are exposed to extracellular glycation- or oxidation-induced cross-linking, also called advanced glycation end products (AGEs)-related cross-linking, impairing functional interaction of collagen with cells and stiffening the matrix [87, 210, 242]. AGEs in human plaques have been found predominantly in inflammatory

atheromatous lesions that are often classified as rupture-prone plaques [92].

Microcalcification

It is currently widely accepted that vascular calcification is an active, cell-mediated process [40, 139], instead of a passive accumulation of calcium. Crystalline hydroxyapatite (HAP) is the main component of calcifications [166, 171]. In addition, precursors of HAP, such as amorphous calcium phosphate, octacalcium phosphate, and dicalcium phosphate dehydrate, have been reported in atherosclerotic lesions [88, 133, 191, 205, 270].

Multiple mechanisms for the formation of calcification have been suggested. One of these is the release of SMC- or macrophage-derived [115] calcifying extracellular vesicles (EVs), where calcium crystals nucleate on the EV surface and mature over time into HAP [105, 191]. Another potential pathway is osteochondrogenic trans-differentiation of vascular SMCs. Several lines of evidence showed that atherosclerotic calcification shares features with bone formation [148, 183, 219]. Apoptosis of vascular SMCs and macrophages also seems to play a substantial role in the onset of calcification, as calcium deposits are often located near or in the necrotic core and in close proximity to apoptotic cells [110, 164]. Moreover, loss of calcification inhibitors is known to influence calcification formation. All these mechanisms have been extensively reviewed [31, 40, 124, 134, 183, 209].

Different categories of calcifications can be observed in the cap, categorized by their size. Macrocalcifications can be divided into three types: speckled, spotty calcifications (~50 μm), sheet-like fragments (> 2 mm) and diffuse segments of calcifications (> 5 mm) [15]. Various studies have shown that macrocalcifications can lower overall stresses in the cap, due to their load-bearing capacity [150, 264]. Where macrocalcifications are, thus, often seen as a cap-stabilizing component, computational studies show that microcalcifications (< 50 μm) might act as local stress concentrators. Microcalcifications are distributed heterogeneously throughout the necrotic core, where they are believed to be not mechanically relevant, and in the cap [156], where they can contribute to rupture [42, 52, 116, 257]. Since it has been shown with high-resolution μCT that microcalcifications are the most abundant type of calcification in the vulnerable plaque cap [116], we have chosen to focus on the role of microcalcifications on cap mechanics in this review.

Cap components: role in cap mechanics

Where the impact of mechanics is extensively studied in atherogenesis [95], its role in cap rupture is evident but less well understood. Plaques are exposed to various mechanical

forces (Fig. 1A) that can induce peculiar mechanical behaviors that vary with plaque geometry, location, and composition [9, 101]. Thin caps of vulnerable plaques must possess sufficient strength to endure the force-driven stresses to which they are exposed (Fig. 1B). The most influential stress experienced by the cap is the circumferential stress induced by the blood pressure, acting perpendicular to the arterial wall, leading to deformations within the cap. The effect of blood-flow-induced shear stress on cap deformation is believed to not be substantial as circumferential stresses are orders of magnitude higher. Their influence on cap rupture can, however, not be neglected as shear stress is believed to affect cap erosion as well as endothelial function and thereby cap biological composition [84, 160]. Axial tensile stress, as a consequence of among others hemodynamic loading,

differences in vessel geometry and plaque morphology, is believed to predominantly affect the rupture events upstream of the plaque where these stresses are highest [223].

As cap constituent material properties affect the stress distribution throughout the cap and determine the strength of the cap, this section specifically elaborates on the variations in collagenous matrix properties, macrophages, and microcalcification seen within human atherosclerotic plaque caps (Fig. 1C) and the impact thereof on cap mechanics and stability (Fig. 1D). Cap mechanics and stability are hereby described on the mesoscale by terms such as tissue stiffness and strength as defined in Table 1. For an in-depth description of the complexity of plaque biomechanics, nano- and micro-scale mechanics of the collagenous matrix, as well as the effect of collagenous matrix properties on SMC-driven

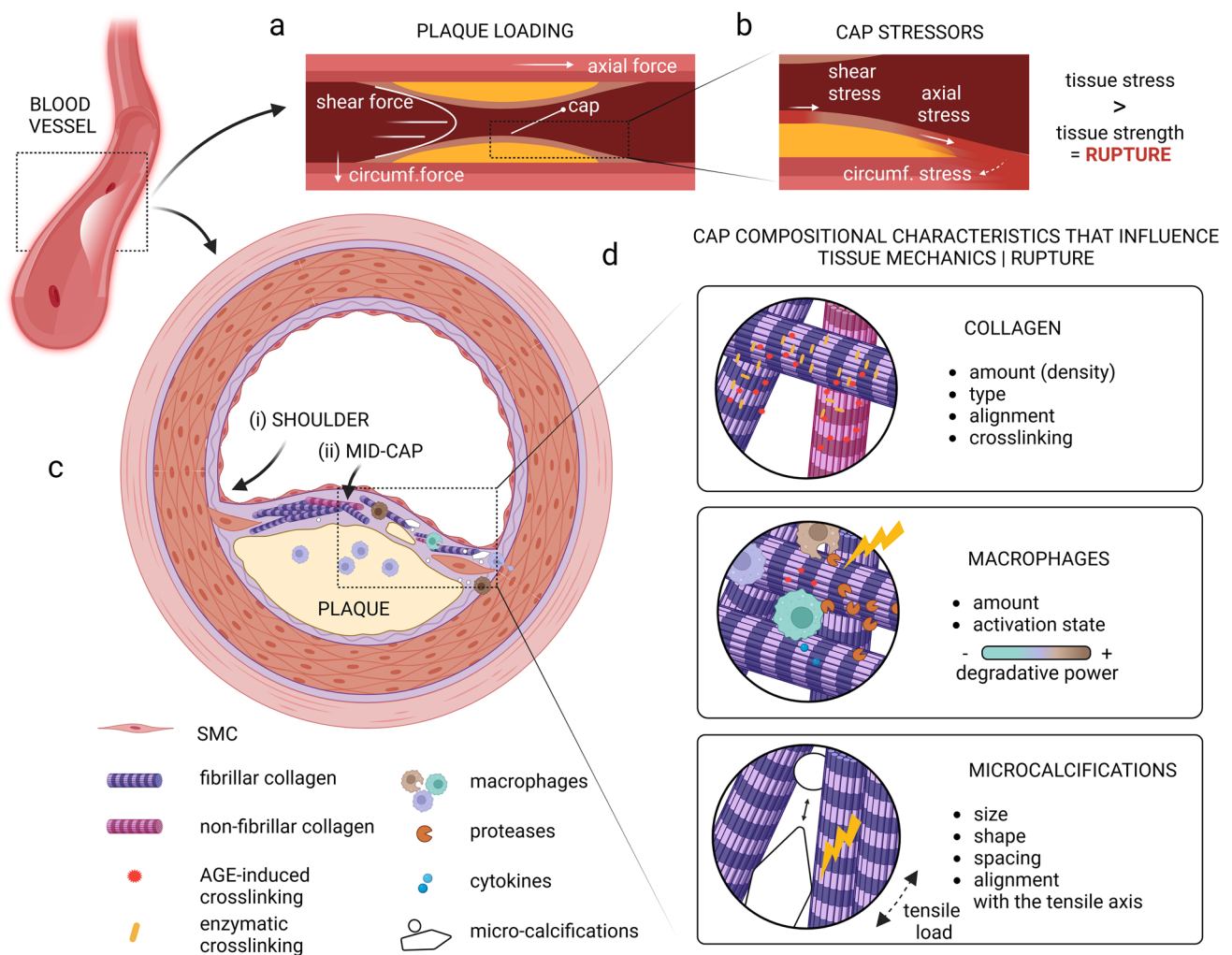


Fig. 1 Graphical representation of a vulnerable plaque and cap with its most important mechanical stressors, cap components, and their influence on tissue mechanics. **A** A simplified graphical representation of a longitudinal section of an atherosclerotic plaque with the primary forces acting on it. **B** Zoom-in of the cap region with the primary stressors locally initiating tissue stress. **C** Graphic of a trans-

verse section of a vulnerable plaque with a fibrous cap, showing the shoulder (i) and mid-cap (ii) regions. **D** The cap compositional characteristics which influence tissue mechanics, being collagen, macrophages, and microcalcifications, and their effect through various individual characteristics on cap mechanics and consequent cap rupture. Created with BioRender.com

Table 1 Mechanics terminology

Descriptor	Description*
Strength	The maximum capacity to withstand a load, expressed as a stress threshold value
Ultimate tensile strength/stress	The maximum stress that can be withstand while being exposed to tensile loads
Stress	Force per unit area
Peak circumferential stress	The highest stress value in the circumferential direction (the direction that undergoes (highly likely) the largest deformation in vivo)
Strain	Deformation measure
Uniaxial extension	Stretch in only one direction
Stiffness	Measure of the material's resistance to elastic deformation
Elasticity	The ability to return to its original shape after undergoing deformation
Elastic (Young's) modulus	Measure of the material's resistance to elastic deformation, assuming a linear response
Tensile testing	A test wherein a sample is subjected to a controlled tensile load, often until failure

*Various definitions possible, however aimed at simple definitions for non-expert in the field

matrix formation and degradation, we refer the reader to the following literature [9, 39, 80, 84, 193, 203, 223, 255, 265].

Role of collagen on cap mechanics

The plaque cap resistance to rupture largely depends on the load-bearing collagenous network [8, 100, 103]. This aspect in turn depends upon the balance between collagen synthesis and degradation, the latter being significantly affected by macrophages that secrete proteases [145] as discussed in “[Interplay between macrophages and collagen](#)”. Although increasing collagen amount has been correlated with increments in stiffness and strength for human plaques and their fibrous caps [43, 59, 135], correlations were not always convincing. This can be explained by the knowledge that not solely amount but the cumulative effect of nano- to macro-scale collagen properties determine its mechanical behavior [80]. Variations in molecular structure and intracellular and extracellular processing can reflect in the collagen type, alignment as well as its cross-linking. This section focuses on those studies that explored the effect of these properties on cap mechanics.

The primary contributors to the mechanical strength of tissue in the cap are the fibrillar collagens [14, 230]. Since collagen type I is believed to be profibrotic and modulate tensile strength, and collagen type III is associated with tissue elasticity [193, 222], the relative presence of both types is expected to affect mesoscale cap mechanical behavior. To assess this impact, Burleigh et al. compared the collagen type I and III proportion with the ultimate tensile strength for ulcerated and non-ulcerated human aortic plaque caps and adjacent intima. Where the strength of the collagen in the caps appeared to be lower when compared to the collagen of adjacent intima, the proportion of collagen type I to III was not related to the ultimate tensile strength [37]. Although not directly related to atherosclerosis, several

studies in tendons did demonstrate an impact of varying proportions of collagen type on tissue stiffness. These studies showed that the quantity of collagen type III is inversely correlated to the elastic modulus [12, 34]. These variances in tissue stiffness are believed to be related to the differences in protein structure, and fiber and fibril diameter [3, 23]. The presence of fibrillar collagen type V, as well as the non-fibrillar collagen types IV, VI, and VIII in atherosclerotic plaques potentially influences the tissue mechanical stiffness and strength via affecting either collagen type I fiber diameter (type V) [3], SMC activation (type IV) [14] or the interaction with other ECM components (type VI/VIII) [21, 193], though their direct effect on plaque tissue mechanics remains to be explored.

In addition to collagen type, several studies have demonstrated that less dispersed alignment of collagen fibers into the load direction correlates with greater tissue strength and/or stiffness for both healthy arteries [214] as well as atherosclerotic plaque caps [111]. However, where collagen fibers in healthy arteries have a distinct oriented collagen network, intra- and inter-plaque fiber orientation vary substantially [8]. Within human coronary plaques, shoulder regions (Fig. 1A) were defined by Douglas et al. as more dispersed and less aligned when compared to the mid-cap [64], while Tornifoglio et al. showed that the rate of disorganization and microstructural arrangements varied considerably between human plaque caps [238]. Mechanically, the more disorganized samples appeared to be weaker in comparison to the samples with a more predominant circumferential orientation, when exposed to uniaxial tensile testing in the circumferential direction [238]. These findings suggest that collagen fiber alignment might be a relevant parameter for rupture risk assessment.

Finally, cross-linking of collagen is known to impact local and global mechanical properties. Enzymatic cross-linking of collagen, a process controlled by enzymes such as LOX,

is believed to be essential for collagen fibril structure and integrity, which directly influences the tissue strength [198, 224]. In human plaques, LOX presence was associated with a more stable plaque phenotype and LOX expression correlated negatively with markers of immune activation and the incidence of myocardial infarction (MI) [185], suggesting a protective mechanism. Mechanically, LOX deficiency has been shown to cause serious collagen network weakening [154] and, although related to human heart valves, enzymatic cross-links were shown to play a dominant role over collagen content affecting valve biomechanical tissue behavior [13].

As the cross-linking of collagen reduces its sensitivity to proteolytic degradation [159], one could suggest that cross-linking enhances cap mechanical strength. However, a reduced sensitivity to proteolytic degradation also contributes to impaired matrix remodeling, as tissues with a slow turnover are more susceptible for irreversible AGE-driven cross-linking [79]. AGEs are related to enhanced tissue stiffness [69, 224], due to limited sliding between fibers and fibrils [79].

In conclusion, it is the sum of all collagen properties, such as type, orientation, and cross-linking, that determines its mechanical strength and stiffness.

Role of macrophages on cap mechanics

Macrophages play an important role in the onset and progression of atherosclerosis, as well as the process of plaque cap rupture [11]. More pro-inflammatory macrophages are present in the shoulder region [228], where 65% of ruptures occur [153]. One of the first studies which demonstrated that caps of ruptured plaques have higher amounts of macrophages compared with intact caps was performed by Lendon et al. [136]. In this study, strips of plaque cap tissue underwent uniaxial extension and ruptured caps were compared to intact caps. Higher macrophage accumulation was associated with a lower ultimate tensile stress [136], supporting the hypothesis that macrophage infiltration increases rupture risk. By combining immunostaining of macrophages and the onset of events in patients, a positive correlation was found between macrophage infiltration and the occurrence of cerebral ischemic events [104] and acute coronary symptoms [163]. Similarly, the analysis of cellular characteristics in rupture sites of thrombosed arteries extracted from MI patients revealed macrophages to be the dominant cell type at the site of rupture, suggesting an active inflammatory reaction [251]. Kolodgie et al. demonstrated extensive apoptosis of macrophages at the rupture site by staining lesions of sudden coronary death, suggesting a potential destabilizing role of apoptotic macrophages in plaque caps [120]. However, it should be noted that analysis of caps post-rupture

cannot be used to conclude that macrophages were the primary cause of rupture.

To be able to assess the role of macrophages on local tissue mechanical properties, intravascular elastography was performed [122, 212], and local high-strain spots were correlated with the presence of macrophages [122]. Uniaxial tensile tests with notched fibrous cap tissues were performed to characterize rupture behavior, where caps with higher macrophage density were found to rupture at lower stresses [59]. Potential correlations between macrophage presence and rupture behavior could be explained by the indirect role that macrophages play in the degradation of the matrix, which will be elaborated on in “Interplay between macrophages and collagen”.

The experimental results discussed above were validated by computational modeling using a patient-specific set of 3D computational models [232]. To be able to reflect inflammation in the model, material stiffness in the cap was reduced to reflect cap weakening due to inflammatory processes. This weakening led to large cap strain conditions when combined with a thin cap and hypertension. Furthermore, the lower stiffness led to lower cap stress [232].

Macrophages and inflammatory processes, thus, seem to affect cap mechanics and rupture. However, the number of experimental studies is limited.

Role of microcalcification on cap mechanics

Microcalcifications are prevalent in atherosclerotic caps and non-uniformly distributed in the shoulder and mid-cap regions [157]. These calcified deposits are believed, based on ex vivo histopathological observations combined with computational simulations, to influence cap rupture [204].

Microcalcifications can amplify the stresses in the cap [25, 41, 200, 245, 246] and transfer the peak cap stress (PCS) to their location in the cap [257]. Moreover, microcalcifications have the most detrimental effect depending on their location [257]. Specifically in regions of high stress [245] or thin caps [245, 246], microcalcifications can induce high mechanical stress to promote cap rupture.

Aside from location, morphological characteristics of the microcalcification, such as the size and shape, can influence the fibrous cap stress. A critical microcalcification diameter is described by Kelly-Arnold et al. from 5 to 65 μm . Outside this range, microcalcifications are thought to be less harmful [116, 155]. This critical size is, however, also dependent on their localization, as microcalcifications located in a thin cap were shown to compromise the mechanical stability of the cap the most [56]. It is currently, however, not known in what way the ratio of the microcalcification size and cap thickness influence each other. Distinct from the spherical microcalcifications, elongated microcalcifications substantially increase the cap stress [41, 52, 245]. In

addition, higher volume fraction of microcalcifications also increases the fibrous cap stresses [257], and interparticle spacing has been shown to be a principal determinant of rupture risk [155]. Cardoso et al. showed that local stress levels are increased by a factor of five for closely spaced microcalcifications [41, 116, 157]. In the fibrous cap, most microcalcifications are situated together in clusters [109], which could, thus, increase the stress accumulation even more than a single microcalcification. Furthermore, their alignment with the tensile axis can significantly increase cap stress [41, 52, 116]. Notably, these findings are almost all based on numerical studies.

Experimentally, the effect of micro-beads, representing microcalcifications with varying diameters and concentrations, within a silicone-based material has been investigated [55]. Larger beads (diameter > 80 μm) reduced the ultimate tensile stress significantly, while smaller beads (diameter 6 μm) only were of effect in thin (100 μm) experimental samples. Furthermore, higher concentrations of particles correlated with lower stresses at rupture [55], endorsing the results from numerical models.

Microcalcifications are, thus, assumed to act as local stress amplifiers in the fibrous atherosclerotic cap, with microcalcification size, shape, spacing, and alignment with the tensile axis as key determinants in the resulting stress.

The interplay between collagen, macrophages, and microcalcification

While collagen, macrophages, and microcalcifications each distinctively affect cap mechanics, as discussed in “[Cap components: role in cap mechanics](#)”, they cannot be considered as independent contributors. Their continuous interplay affects plaque cap development and ultimate rupture risk (Fig. 2). This section sets forth on the current knowledge regarding their interplay. Other cellular players that are generally accepted to be involved in fibrous cap destabilization, like T cells and SMCs, are occasionally mentioned. Figure 2 provides an overview of the most important reviews describing their interplay with the three compositional parameters elaborated on in this review.

Interplay between macrophages and collagen

Regarding collagen-dependent cap strength, macrophages play a dual role, presenting a matrix-degrading or a matrix-preserving role. Each role is accompanied by the secretion of a specific set of active compounds that cumulatively affect cap (in)stability. This section focusses on how macrophages degrade or preserve their surrounding collagens, by secreting (i) proteases, (ii) cytokines, (iii) ROS, and (iv) ECM

components, and ends with the reciprocal effect of the collagenous matrix itself on macrophage biology.

The most underlined contribution of the macrophage to plaque rupture involves the secretion of proteases, including metalloproteases (MMPs), cysteine (e.g., cathepsins), and serine proteases [63, 126, 175, 247]. There is compelling evidence that human atherosclerotic plaques are associated with the presence of macrophages and proteases, and this presence was linked to thin fibrous caps, and even cap rupture [1, 63, 229]. While an excess of degrading proteolytic enzymes over their inhibitors contributes to collagen remodeling and cap destabilization [175, 266], it is worth noting that the type and amount of protease and their inhibitors secreted by macrophages can vary based on their differentiation state and the stimuli they perceive [175, 186].

The load-bearing fibrillar collagens type I and III are resistant to most proteases and can only be cleaved by certain MMP collagenases, including MMP-1, -8, -13, and -14 (MT1-MMP) [70, 71] that can be secreted by macrophages in copious amounts [173, 175]. These collagenases cleave collagen I–III into fragments that quickly denature into gelatin, which can be further degraded by MMP-2 and -9 [4].

Although MMP-2 and -9 are classified as gelatinases, they also possess collagenolytic activities that affect fibrillar collagens [7, 22, 189]. Of the cathepsin family, cathepsin K has been identified as the most potent cathepsin affecting tissue strength, by cleaving proteoglycan-GAG interfibrillar bridges as well as fibrillar collagens [73, 142, 187].

Of note, proteases not only actively degrade collagen; MMP-2, -4, and -9 are known stimulators of SMC proliferation and migration [172], suggesting a possible matrix-preserving and stimulatory role for these macrophage-secreted proteases.

Besides proteases, macrophages secrete various active compounds. Frequently observed ones to be present in vulnerable atherosclerotic plaques include the inflammatory cytokines IFN- γ [30], TNF- α [188], as well as ROS [179] that all have been described to increase protease secretion and activity [199, 229, 266], contributing to collagen degradation. Moreover, they have been shown to also destabilize the collagenous matrix by either being involved in SMC apoptosis, inhibiting SMC proliferation, reducing collagen production or inhibiting LOX expression in vascular human or animal SMCs [10, 33, 93, 184, 226], increasing plaque vulnerability.

Cytokines that are generally thought to be on the matrix-preserving side include platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF β). PDGF has been found within macrophages in all phases of atherosclerotic plaque development [206] and is known for its mitogenic potential stimulating SMC migration and proliferation, inducing intimal growth [159]. TGF- β is secreted by the more anti-inflammatory activated macrophages [26]

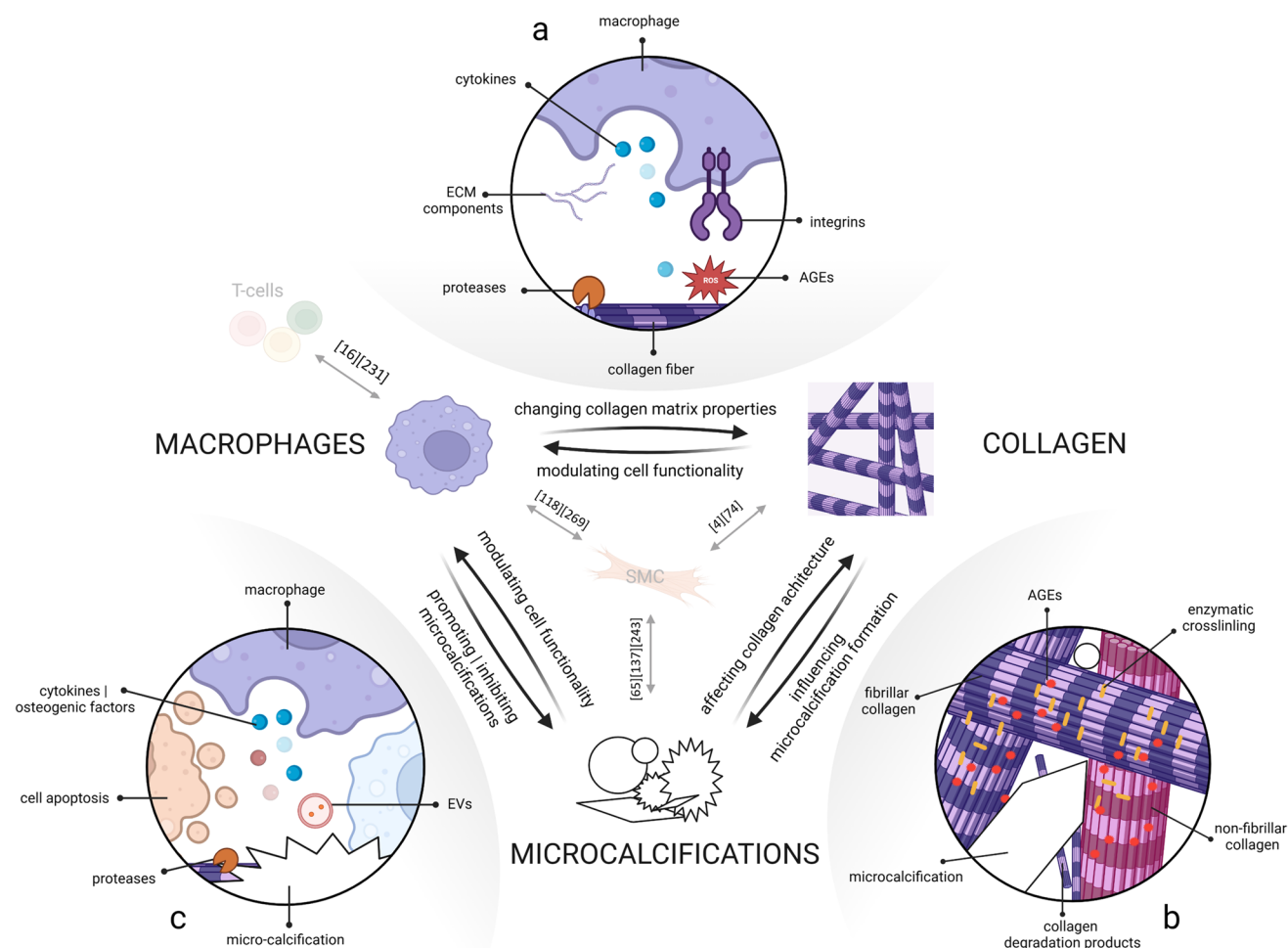


Fig. 2 The interplay between the main cap components: collagen, macrophages, and microcalcifications and essential reviews discussing the influence of SMC's and T cells on these components [16, 65, 74, 118, 137, 231, 243, 269]. **A** Macrophages influence the collagenous matrix by secretion of cytokines, ECM components, AGEs, and proteases. Furthermore, integrins detect matrix properties and regulate processes of macrophage biology, by which the collagenous matrix properties, thus, modulate cell functionality. **B** Collagen serves as a scaffold for microcalcification formation and collagen

type, cross-linking and degradation can affect the formation of microcalcifications. Furthermore, microcalcifications themselves affect collagen architecture. **C** Macrophages release cytokines, osteogenic factors, proteases, and EVs that can alter calcification formation. In addition, macrophage apoptosis contributes to microcalcification formation. Microcalcifications, in turn, modulate macrophage functionality by promoting release of pro- or anti-inflammatory factors. Created with BioRender.com

and has a well-established role in ECM synthesis, enhancing collagen deposition [208, 237]. While both factors have been correlated to tissue stabilization, their protective role regarding cap rupture must be interpreted with caution as some studies show correlations to cellular apoptosis [202], calcification [220], and cap rupture [28].

Macrophages are furthermore a source of AGEs, that are formed in conditions of hyperglycemia or oxidative stress, which are seen in aging, diabetes, and settings of inflammation and hypoxia [242]. AGEs have been shown to promote vascular damage and accelerate atherosclerotic plaque progression, partly through a direct mechanism of altering ECM molecules, including collagen. AGE products are known to

form protein adducts or cross-links, and accumulate for years, contributing to an irreversible, highly cross-linked collagenous matrix and a stiff fibrous cap [79, 121, 242]. It was discovered that macrophages can synthesize ECM components, including collagen, themselves [123, 215, 256]. Studies showed that macrophages express all collagens and collagen-related mRNAs and were able to secrete collagen type VI and VIII considerably in vitro [215, 256] and in human atherosclerotic plaque conditions [256].

Finally, the collagenous matrix itself plays an important role in regulating various processes of macrophage biology, including monocyte-to-macrophage differentiation, lipid uptake [258], metabolism [244], polarization [131],

migration mode [86] as well as their ability to secrete proteases [138, 258]. It has been shown *in vitro* that macrophages increase their MMP-9 secretion when cultured on monomeric type I collagen, in comparison to when they were exposed to the polymerized variant [138]. Lastly, AGEs are known to affect cells via its interaction with the key receptor for AGE (RAGE), which is present in almost all cells that reside in a plaque, including macrophages, and induces the subsequent secretion of pro-inflammatory compounds, including ROS [242].

In conclusion, macrophages secrete a plethora of multifaceted active compounds that cumulatively determine collagenous matrix properties and rupture risk. In turn, macrophage biology is affected by collagen. Therefore, macrophage and collagen continuous crosstalk can contribute to changes in the local microenvironment that promote plaque rupture.

Interplay between collagen and microcalcification

Collagen plays a complex and multifactorial role in microcalcification formation, and many of the underlying mechanisms are currently being studied. Collagen characteristics that are named to influence microcalcification formation include (i) amount, (ii) integrity, (iii) type, (iv) alignment, and (v) cross-linking.

Hutcheson et al. found an inverse relationship between collagen amount and calcification, with microcalcifications forming in regions of collagen fiber degradation [105], as disrupted or fragmented collagen fibers may serve as nucleation points for microcalcification formation [5]. By aggregation of EVs in between the collagen fibers, calcifying structures were formed [105]. Collagen was shown to act as a scaffold for microcalcification formation, where collagen alignment directly influences microcalcification shape, by modulating calcification EV aggregation to gain an elongated morphology [105]. Thus, local collagen organization can influence calcification development and create a multitude of calcification-fibrous tissue interactions, with varying effects on tissue mechanics [15]. Calcifying EVs locate in proximity to collagen fibrils [113, 213] and interestingly, EVs isolated from vascular SMCs were only capable of inducing calcification along collagen type I fibrils [47]. Type I collagen was also shown to promote calcification of vascular SMCs, by stimulating their differentiation into osteoblast-like cells [102, 112, 177] or increasing mineralization parameters such as calcium incorporation and mineral formation [255]. Type IV collagen, on the contrary, inhibited these processes [255]. The overall matrix stiffness is also a known contributor to vascular calcification [177]. Matrix stiffness can be detected by matrix-binding cell surface receptors such as discoidin domain receptor tyrosine kinases (DDRs) and integrins, present on vascular SMC

[4]. DDR-1 has been shown to act as a mechanical sensor altering matrix deposition [125, 255], activating MMPs [29], promoting the secretion of EVs [125, 255] and regulating osteogenic differentiation of SMC [178], ultimate processes that contribute to calcification. Matrix stiffening, as a result of AGE-dependent cross-linking, was found to hinder collagenase-mediated degradation, subsequently promoting SMC differentiation into adapting the osteogenic phenotype [235]. In addition, LOX-dependent cross-linking was linked to vascular calcification [112].

Taken together, various collagen characteristics affect microcalcification formation, but there is a paucity of information on reciprocal effect of microcalcifications on collagen properties. In view of tissue mechanics, one could speculate that microcalcifications can affect collagen remodeling processes, while microcalcification-induced stress accumulations might locally affect collagen integrity. Furthermore, microcalcifications can merge to form macrocalcifications, causing altered interactions with the collagenous matrix. Advanced macrocalcifications can be also formed when collagen fibers themselves calcify [15, 98], but osteoid metaplasia resembling bone structure is relatively rare event. Gijzen et al. showed that four distinct fiber patterns around macrocalcifications can be recognized: attached, pushed-aside, encircling, and random [83]. Only in the first pattern, the fiber structure was still visible inside the calcifications, indicating that this type of calcification might develop due to fiber mineralization, while the others are formed due to the agglomeration of EVs in between the collagen fibers [83].

Interplay between macrophages and microcalcification

The hypothesis that macrophages and microcalcifications are involved in a complex interaction first originated from studies where arterial inflammation was found to be correlated with the localization of calcifications [2, 35, 44, 169]. Microcalcifications were shown to induce M1 macrophage polarization [162], in comparison to macrocalcification, that was associated to a M2 phenotype instead [130, 132, 227].

The reciprocal effect of inflammation on microcalcification formation has been described at various interconnected levels: (i) the release of calcifying EVs; (ii) through the production of proteases; (iii) through the release of cytokines and osteogenic factors; and finally (iv) through the induction of apoptosis.

First, the release of calcifying EVs plays a role in the formation of microcalcifications. Cells present in the atherosclerotic cap, such as vascular SMCs and macrophages, secrete EVs that are loaded with mineralization inducing factors, including tissue non-specific alkaline phosphatase (TNAP), thus serving as a nucleating point for calcification [105, 106]. Hutcheson et al. demonstrated that aggregation

of multiple calcifying EVs causes the formation of microcalcifications in atherosclerotic lesions [105]. In addition, it has been demonstrated that macrophage-derived EVs, enriched with S100A9 and Annexin V, accelerate microcalcification formation in chronic kidney disease and diabetes mellitus [115, 171]. Taken together, vascular cell-derived EVs can be seen as active contributors and building blocks of microcalcifications.

The second pathway by which macrophages affect calcification is through collagen degradation, as discussed above, resulting in nucleation loci for calcification [5, 141, 170]. Various MMPs, mainly produced by activated macrophages, have been associated with calcification. MMP-9 and MMP-10 enhance both inflammatory and calcifying processes [48, 197], and MMP-1 levels are found to be higher in calcified plaques compared to non-calcified lesions [194].

Besides these proteases, there are also factors released by macrophages such as cytokine TNF- α , that trigger osteoblastic activity in vascular SMCs, consequently promoting calcification [6, 107, 219]. Not only do macrophages trigger osteoblastic activity in vascular SMCs, they also release osteogenic factors directly. In the tumor microenvironment, macrophages have been shown to tune microcalcification formation by the release of osteogenic factors (e.g., BMP-2 [253]) and inhibiting factors (e.g., osteopontin [50, 180]). In line with the secretion of osteogenic factors, several studies suggested that macrophages themselves can contribute to an osteoblast-like or osteoclast-like phenotype, aggravating or resorbing vascular calcification [38, 75, 140].

Apoptosis of macrophages (and macrophage-induced apoptosis of SMCs [33]) might be yet another pathway resulting in the onset of calcification. Various lines of research have suggested that calcification initiates within cell debris, and that necrosis and apoptosis might, therefore, induce calcification by serving as nucleation sites [53, 67, 196]. Microcalcifications often form around the necrotic core [209], within a dense population of macrophages and apoptotic debris [120]. When phagocytic clearance of apoptotic bodies (i.e., efferocytosis) is compromised, apoptosis will transition into secondary necrosis, increasing the amount of calcification [236].

Not only do inflammatory processes affect calcification, but vice versa in a procalcified environment with elevated phosphate levels, macrophages shift to a M2 phenotype [248]. Within this *in vitro* study, macrophages showed anti-calcifying activity, which may protect the tissue from further calcification [248]. M2 macrophages phagocytize necrotic fragments and apoptotic cells [117], limiting nucleation sites for calcification. Macrophages also encapsulate, internalize, and resorb deposits of calcium [129, 168, 190] such as HAP particles or related precursors that have been shown to distinctly induce pro- [46, 168, 190], anti- [267] or ‘hybrid’ macrophage phenotypes *in vitro*, a process that depends

on microcalcification size and shape [132, 167, 227]. Whether these observations can be translated to the plaque itself remain to be examined. Deep phenotyping studies of plaque macrophages in proximity to calcification would help explaining the interaction between calcification and macrophages. Recent histological evaluation of human carotid plaques showed that CD163 and CD86 positive cells correlated with macrocalcifications, while plaques with microcalcifications had a high presence of M1 macrophages [162]. On the contrary, another study showed that CD163 + M(Hb) macrophages can restrain vascular calcification *in vitro* and demonstrated an inverse correlation between CD163 + macrophages and vascular calcification in human atherosclerotic plaques [211].

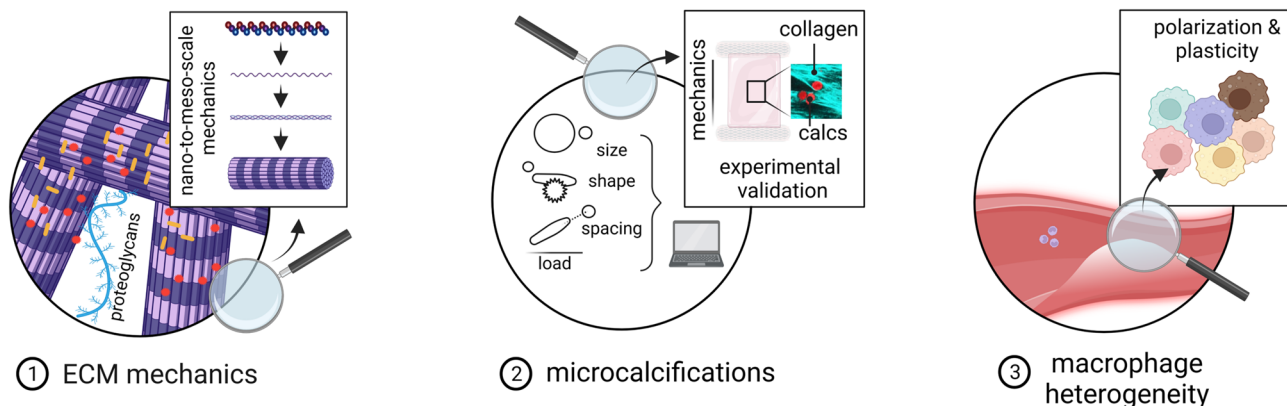
Overall, these observations insinuate that microcalcifications are not merely a passive result of persistent inflammation but also initiate a feed-back loop controlling inflammation, and consequently contribute to the disease progression and cap rupture risk. Nevertheless, many reciprocal relationships between macrophages and calcification remain to be exposed.

Outlook

Throughout this review, we emphasized that the presence, characteristics, and iterative interplay of collagen, microcalcification, and macrophages have clear implications for tissue mechanics and consequently the risk of rupture. We believe that enhanced mechanistic insight into how biological processes change cap mechanical properties is a prerequisite for identifying the cap at risk of rupture.

In view of cap mechanics and the interplay between collagen, macrophages, and microcalcification, it can be concluded that much is still largely unknown (Fig. 3). Regarding collagen, emerging studies focus on the mechanical properties of collagen using a bottom-up approach studying nanoscale collagen mechanics [80], to be able to link this to mesoscale. In addition, increasing attention is being paid to the differences in global and local mechanical behavior [57, 241]. With respect to microcalcification, in-depth histological or micro-CT analyses of patient material, assessing microcalcification clustering, particle shape and density, are lacking. In addition, there is limited knowledge about microcalcification surface topography, which might greatly influence macrophage functionality [132] and consequently the collagenous matrix. Concerning macrophage heterogeneity, more athero-specific subsets are found to exist, mainly due to advances in single cell analysis [60], that provides a plethora of compounds affecting plaque properties and preventing rupture risk. The generally accepted statement that primarily pro-inflammatory macrophages are involved in rupture is obsolete as more atheroprotective subsets have

WHAT IS STILL UNKNOWN?



WHAT DO WE NEED?

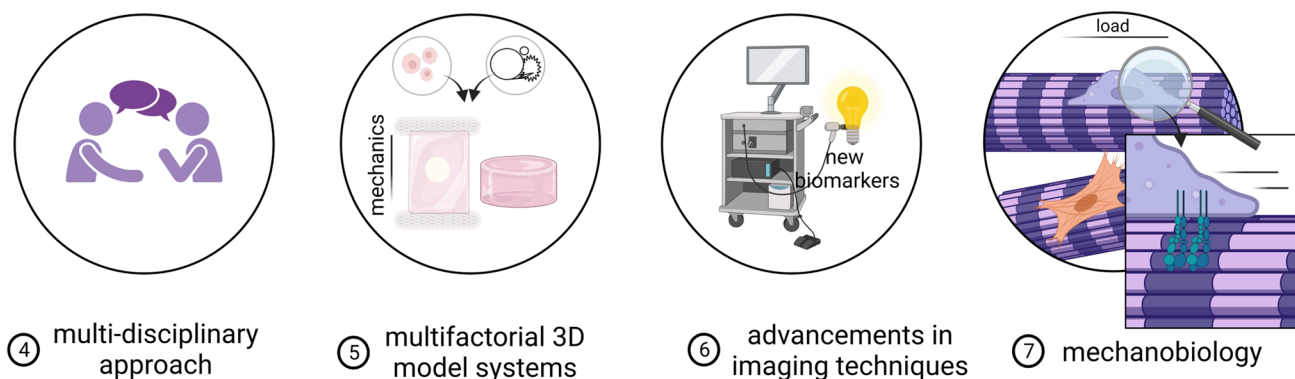


Fig. 3 Overview of the knowledge that is currently still lacking and what is needed to improve cap rupture prediction. (1) For the understanding of ECM mechanics, collagen properties need to be assessed from nano-to-meso-scale as well as more in-depth analysis of other ECM components. (2) Microcalcifications need to be structurally investigated in experimental studies regarding size, shape, and spacing. (3) A better understanding of macrophage heterogeneity

is needed. (4) A multidisciplinary approach is desired combining fields such as vascular biology and engineering. (5) Multifactorial 3D model systems are required to study more complex processes. (6) To be able to stratify findings to patients, advancements in imaging techniques are mandated. (7) Mechanobiology, the effect of cardiovascular mechanics on cellular behavior, should be the topic of research. Created with BioRender.com

been correlated with plaque vulnerability [19, 211]. A better distinction regarding macrophage phenotype and function should be made in relation to the environment to better understand macrophage-driven cap weakening. Furthermore, the impact of other ECM components (e.g., elastin, GAGs) [221, 230], or immune cells (non-macrophages) [61] needs to be assessed.

To fill the knowledge gaps, a holistic multidisciplinary approach is needed where the fields of immunopathology, vascular biology, biomechanics, tissue engineering, and biomedical engineering are combined. Many of the experimental studies discussed utilize animal, 2D in vitro or numerical models. Although informative and required, tissue content and mechanics are known to substantially diverge between animals and humans [97] and direct translation of 2D in vitro or numerical work to human cap mechanics is complex. To improve translatability and to validate

computational approaches, more sophisticated 3D in vitro platforms are required that increasingly resemble pathophysiology, wherein human cells are exposed to various environmental factors simultaneously in a controlled manner (i.e., a multifactorial 3D in vitro model) [128]. Examples of such models include the collagen hydrogel that was utilized by Hutcheson et al. to mimic structural features of the atherosclerotic plaque to study microcalcification formation [106]. In addition, Mallone et al. bioengineered atherosclerotic plaques to investigate etiopathogenesis [158]. Our group recently made use of tissue engineering concepts to scrutinize relationships between tissue composition, the presence of microcalcifications, and mesoscale mechanical properties [109, 261]. Similar model systems can also be used to study patient variation [45, 121], as differences in age, sex, and co-morbidity are likely to affect tissue mechanics [91, 121]. Moreover, computational approaches combining models that

describe blood flow and plaque deformation simultaneously could potentially provide new insights in the role of biomechanical factors in atherosclerosis [216].

To ultimately confirm findings and stratify them to patients, advanced imaging techniques are needed. Non-invasive imaging modalities including CT and MRI are currently the most widely used diagnostic tools in the clinic. However, invasive imaging techniques such as optical coherence tomography (OCT) and intravascular ultrasound (IVUS) can achieve higher resolution, and fusing these invasive techniques provides even further advancements for biomechanical modeling [90]. Recent hybrid imaging systems, such as PET–CT or PET–MRI can facilitate the detection of microcalcification and inflammation, to be able to create patient-specific modeling approaches and evaluate local mechanical properties [233]. Furthermore, specific tracers or diffusion tensor imaging can be used to visualize cap components, such as collagen type, maturity [192], and alignment [8, 239, 240].

Where this review elaborates on how cap compositional parameters affect mechanics, it is important to also obtain better mechanistic insight in how cell functionality and matrix properties are determined by the physical cues in their environment (i.e., the mechanobiology). It was shown that hemodynamic loading can determine macrophage phenotype [94] (add ref) and matrix content [27], among others by changing collagen's susceptibility to degradation [77, 78]. It is, thus, likely that hemodynamic loading also influences other cap components such as microcalcifications, possibly altering their shape, size or topography.

Taken together, the creation of more complex, multifactorial 3D in vitro model systems as well as advancements in imaging techniques are required to better assess the impact of individual cap components as well as their reciprocal interplay on cap strength and vice versa. Obtained knowledge will ultimately serve as input to help in the diagnosis, prevention, treatment, and design of new therapies against atherosclerotic cap rupture.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard The manuscript does not contain clinical studies or patient data.

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