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REVIEW

Imaging

Vulnerable plaque imaging: updates on new pathobiological mechanisms

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Early identification of vulnerable, rupture-prone atherosclerotic plaques with the optimal goal of cardiovascular event prevention is a field of vigorous research. Despite the advances in imaging modalities and the *in vivo* identification of many characteristics of vulnerability, few of these plaques actually rupture and even fewer lead to clinical events, questioning the predictive value of the above techniques in clinical practice. Factors causing the higher local vulnerability of the culprit plaque within a prothrombotic environment of widespread inflammation are generally unknown. Newly recognized local features, including microcalcifications and biomechanical factors, seem to contribute. In this review article, we target on new mechanisms, implicated in vulnerable plaque formation and rupture, analysing their potential clinical value.

Keywords Vulnerable plaque • Microcalcifications • MiRNAs • Inflammation • Endothelial shear stress

Introduction

Atherosclerosis has one of the longest incubation periods among the human diseases. Although stable atherosclerotic disease has a clinically silent course, its thrombotic complications, acute coronary syndromes (ACSs), occur suddenly. Serial angiographic studies have shown that lesions responsible for ACSs are often non-occlusive. Consequently, the concepts of the vulnerable plaque, defined as a plaque with a high risk of causing an ACS, and vulnerable patient were introduced. However, the identification of high-risk plaque characteristics has failed to significantly increase the predictive ability of current models for the assessment of cardiovascular risk. Interestingly, few vulnerable plaques actually rupture and even fewer lead to an event, with most plaque ruptures being clinically silent.[1] As the targets of imaging, indicated by clinical imaging studies,[2,3] cannot clearly identify the unstable site, the concept of vulnerable plaque has been challenged lately.

New studies, however, have provided new elements for the identification of a vulnerable plaque and/or patient. Morphological and functional factors, including microcalcification and endothelial shear stress (ESS), seem to play an important role in plaque destabilization, both sharing local atherosclerotic plaque inflammation, as their common variable (*Figure 1*). In this review article, we aim to provide an update on new pathophysiological mechanisms implicated in vulnerable plaque formation and rupture, as derived from latest advancements in intracoronary and non-invasive imaging.

Vulnerable plaque formation and rupture—pathological insights

Two major pathological substrates of coronary thrombosis have been demonstrated by pathology: plaque rupture in the majority of the cases and plaque erosion, while a third infrequent mechanism, consisting of thrombosis on the ground of protruding calcified nodules, has also been suggested.[4,5] A rupture-prone plaque has been characterized by a thin inflamed fibrous cap (<65 μm thick), a large necrotic lipid core with abundant inflammatory cells and few smooth muscle cells, as well as spotty calcification and positive outward remodelling. Such a plaque is often described with the term thin-cap fibroatheroma (TCFA), and it is considered to be the major precursor of ACS.[1,5]

Eroded plaques are heterogeneous, scarcely calcified, have lower lipid content than ruptured plaques, and are rarely associated with

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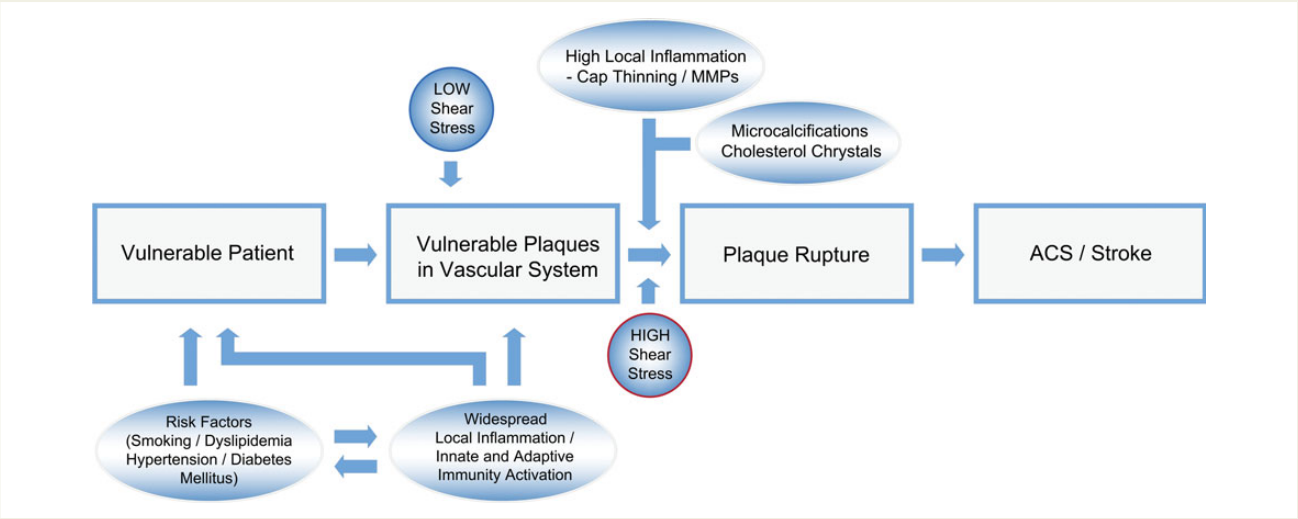

Figure 1 Schematic illustration of pathobiological mechanisms implicated in plaque vulnerability and rupture. Vulnerable patient's risk factors establish a prothrombotic microenvironment. There is a strong interplay between risk factors and widespread inflammation in the vascular tree. Haemodynamic factors (endothelial shear stress/wall stress) act locally on a vulnerable plaque, characterized by high inflammatory activation, cholesterol crystals, and microcalcifications, leading to plaque rupture and potentially to clinical events. ACS, acute coronary syndrome; MMPs, matrix metalloproteinases.

expansive remodelling, while the presence of inflammation is controversial.[5]

Inflammation

Inflammatory activation in atherosclerosis includes both innate and adaptive immunity mechanisms. Macrophages, recruited as monocytes from the circulating blood, are the predominant cell type of innate immunity in atherosclerosis. The recruitment takes place through adhesion molecules (vascular cell adhesion molecule-1) expressed by vascular endothelial cells (ECs). In the plaque, these mononuclear phagocytes express scavenger receptors necessary for uptake of modified lipoproteins and, hence, foam cell formation.[6] Macrophages of the plaque can overproduce matrix metalloproteinases (MMPs), specific enzymes with interstitial collagenase activity, causing the thinning of fibrous cap.[7]

It is now well recognized that at least three subtypes of macrophages can be found in atherosclerotic lesions: M1, the most common form, is a pro-inflammatory macrophage subtype, activated by cytokines secreted by T cells, while M2 seems to play an anti-inflammatory role, promoting tissue repair.[5] The activation of M1 macrophages is mediated by toll-like receptor-4 (TLR-4).[8] A novel third atheroprotective macrophage phenotype, haemoglobin-stimulated macrophage (M(Hb)), induced by intraplaque haemorrhage, has also been isolated in atheromas.[9]

Cumulative experimental and clinical evidence imply an important role of adaptive immunity in atherosclerosis progression and destabilization.[10] Indeed, the cells of adaptive immunity, namely T and B lymphocytes, reside in atherosclerotic plaques.[11]

Cholesterol crystals

A novel link between cholesterol metabolism and local atherosclerotic plaque inflammation has also been proposed. It has recently been shown that phagocytosis of cholesterol crystals by human macrophages leads to a dose-dependent secretion of mature human pro-inflammatory cytokine interleukin (IL)-1, through an inflammasome-mediated pathway.[12] Moreover, cholesterol crystallization alone, due to shift in local plaque temperature, pH, and hydration status, has been proposed to cause plaque fissure and thrombosis through acute volume expansion.[13] Recent advances in higher resolution modalities such as micro-optical coherence tomography (μOCT) could detect *in vivo* such structures, shedding light in vulnerable plaque evolution.[12]

Intraplaque haemorrhage

In advanced atherosclerosis, inflammation and angiogenesis often co-exist. The new microvessels usually originate from the vasa vasorum in adventitia. Intraplaque bleedings increase significantly the levels of free cholesterol and lead to rapid necrotic core expansion and plaque progression, increasing its vulnerability.[14] Thus, an association between intraplaque haemorrhage, cholesterol crystals, and inflammation has been established.

Vulnerable plaque formation and rupture—insights from imaging

A close inspection of findings of *in vivo* imaging studies reveals potential shortcomings of the TCFA paradigm, despite the association of these specific morphological characteristics with clinical presentation (*Figure 2*).

Prospective studies using intravascular ultrasound (IVUS) have shown that high plaque burden in combination with a low residual lumen area has been identified as independent predictors of adverse cardiovascular events.[15,16] In addition, this association has also been

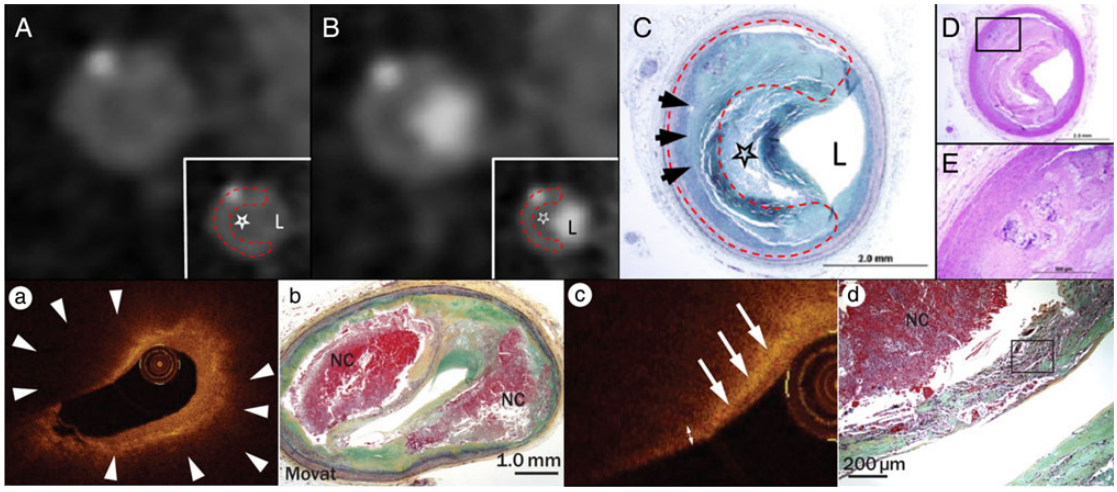

Figure 2 Intracoronary and non-invasive imaging of vulnerable plaque – Correlation to histopathology. Upper panel shows cross-sectional computed tomography images of a coronary plaque with napkin-ring-like attenuation pattern and spotty calcification. The circumferential outer rim (red dashed line) of the non-calcified plaque has a higher computed tomography attenuation in both the non-contrast (*A*) and contrast-enhanced (*B*) images compared with the attenuation within the central part of the plaque. The corresponding histological section (*C*, *D*, and *E*) revealed a late fibroatheroma, with spotty calcification (*E*). (Arrowheads indicate the vasa vasorum. Star indicates necrotic core, L, lumen). Reprinted with permission from Maurovich-Horvat *et al.*[53] Lower panel shows a thin-cap fibroatheroma with extensive haemorrhage. A low backscattering, signal-poor region with diffuse border (white arrow heads) in an *ex vivo* optical coherence tomography (OCT) image (*a*) corresponds with a large NC with extensive intraplaque haemorrhage in histological sections of human coronary plaques (*b*); section stained with MovatPentachrome. A high-power optical coherence tomography image in (*c*) shows distinct superficial high backscattering, signal-rich region (white arrows) and thin fibrous cap (double arrow = 70 μm). A corresponding high-power histological image in (*d*) shows thin fibrous cap that is heavily infiltrated by macrophages. Black square shows infiltrated macrophages. (NC, necrotic core). Reprinted with permission from Otsuka *et al.*[54]

demonstrated for tissue characteristics such as VH-TCFA plaque morphology by IVUS with virtual histology (IVUS-VH) or high-lipid content by near-infrared spectroscopy (NIRS). Both features have been prospectively associated with an adverse outcome at long-term follow-up.[15–17] However, a high prevalence of these findings and a relatively low event rate suggest a limited value of invasive imaging as a risk stratification tool. Imaging studies in ACS patients have demonstrated that not only TCFAs, but also fibroatheromas with thicker cap (>100 μm), are identified within the culprit lesion.[18] Moreover, the potential contribution of the pre-existing luminal narrowing should not be underestimated, considering its prospective association with future events and the findings of lower lumen area in STEMI vs. NSTEMI,[3] and in clinically evident vs. silent rupture.[2] Consequently, despite the prognostic value of TCFA, there is still a need to identify patients who are at high risk for cardiovascular event through identification of new imaging targets.

Widespread vs. local inflammation

Numerous clinical studies have shown that in patients who suffer an ACS, inflammation is not restricted only in culprit plaques.[19,20] Widespread vascular inflammation in atherosclerosis is also supported by non-invasive imaging methods. In patients with peripheral artery disease, inflammation, as assessed by 18F-fluoro-D-glucose positron emission tomography-computed tomography (^{18}FDG PET-CT scan), in paired arterial beds (carotid and femoral arteries) is highly correlated between left and right side.[21] Similar conclusions have also been reached by our group using another non-invasive method for evaluation of carotid atherosclerotic plaque inflammation, microwave radiometry (MWR). Microwave radiometry allows *in vivo* non-invasive assessment of internal temperature of tissues, reflecting inflammation. Indeed, patients with multi-vessel coronary artery disease have higher carotid artery temperatures, compared with patients with one vessel or no coronary artery disease (CAD).[22]

On the other hand, despite the widespread inflammatory background in atherosclerosis, culprit atherosclerotic plaques for cardiovascular events present with a higher degree of inflammation, underlining the importance of local factors in plaque destabilization. C-reactive protein has been detected in human coronary atherosclerotic plaques, suggesting its local production. Similarly, in patients with recent ischaemic stroke, culprit carotid arteries exhibit higher local temperature differences, compared with the contralateral carotids.[23]

MicroRNAs in vulnerable plaque formation and rupture

MicroRNAs (miRNAs, miRs) are small, non-coding post-transcriptional regulatory RNAs. They inversely regulate their target gene expression at the post-transcriptional level either by inhibiting translation or by causing degradation of the target messenger RNA (mRNA). Their potential role in atherosclerotic plaque formation and rupture, through regulation of inflammation, microcalcification, angiogenesis and apoptosis, and interaction with biomechanical factors, has recently been the focus of extensive research.

Table 1 MicroRNAs involved in vulnerable plaque formation/rupture[24,25,52]

Atherosclerotic plaque vulnerability	miRNAs	Proposed mechanism of action
Plaque inflammation	miRNA-126	VCAM-1 inhibition
	miRNA-155, -222- 424, -503, -9, -17, -20a, and -106a	Regulation of monocyte differentiation into macrophages within the plaque
	miRNA-147, -155, and -342-5p	Activation of plaque macrophages in M1 phenotype, TNFα, and IL-6 up-regulation
	miRNA-125a, -146a, -33, and -155	Inhibition of lipid accumulation
	miRNA-15a and -16	Modulation of macrophage apoptosis through bcl-2 receptor targeting
	miRNA-21	Production and secretion of matrix metalloproteinase-9 by human macrophages
	miRNA-34a	VSMC proliferation
	miRNA-210	Tubulogenesis and migration stimulation
	miRNA-146a	Drives peripheral blood mononuclear cells towards a Th1 response
	miRNA-29	Inhibition of elastin expression
	miRNAs-221/222	Promotion of proliferation or cellular death
	miRNA-365	Stimulation of endothelial apoptosis through Bcl2 targeting
	miRNA-100, -127, -145, -133a, and -133b	Highly expressed in symptomatic carotid plaques
Endothelial shear stress	miRNA-143/145	Promotion of VSMCs phenotype switch into the atheroprotective contractile phenotype
	microRNA-126-5p	Limitation of endothelial cell proliferation at low endothelial shear stress sites
	miRNA-92a	Associated with low endothelial shear stress, promotion of inflammation
Microcalcification	miR-125b	Differentiation of VSMCs into an osteoblast-like phenotype

TNFα, tumour necrosis factor alpha; IL-6, interleukin-6; Th1, T helper 1.

MicroRNAs seem to participate as post-transcriptional regulators in all stages of the inflamed atherosclerotic plaque formation, ending to its rupture. VCAM-1 expression by ECs, inflammatory activation of plaque macrophages in M1 phenotype, lipid accumulation in macrophages and foam cell formation, oxLDL-induced lipid uptake through regulation of scavenger receptors, and macrophage apoptosis are only few of the processes regulated (*Table 1*).[24,25]

Biomechanical factors in vulnerable plaque formation and rupture

Endothelial shear stress and plaque vulnerability

Endothelial shear stress, the tangential force derived by the friction of the flowing blood on the endothelial surface, plays a key role in the pathobiology of atherogenesis, plaque formation, and plaque progression to vulnerability. The implication of ESS in plaque vulnerability has been extensively studied in animal and human studies.[26]

Studies in swine showed that low ESS is a strong stimulus that perpetuates the atherosclerotic process in a dose–response manner leading to plaque vulnerability.[27–29] Low ESS modulates the accumulation of lipids[28] and neovascularization resulting to plaque volume expansion. In the setting of inflammation, low ESS increases the mRNA expression and activity of major elastolytic MMPs (MMP 2, 9, 12) and cathepsins (cathepsin K and S) relative to their endogenous inhibitors (TIMPs and cystatin C, respectively), thereby leading to internal elastic lamina fragmentation.[27] Through the fragmented internal elastic lamina, the inflammatory cells migrate into the media where they further promote matrix degradation and ultimately expansive remodelling, which is a key pathobiological feature of vulnerable plaque.[27,30] Moreover, low ESS increases the expression and enzymatic activity of collagenolytic MMPs (MMP 1, 8, 13, and 14), which in turn degrade the collagen in fibrous cap.[29] This effect in conjunction with the low shear-mediated smooth muscle cell apoptosis and reduction in smooth muscle cells and collagen synthesis leads to fibrous cap thinning.[28]

A large, multicentre clinical study investigated the role of ESS in the natural history of atherosclerosis. Five hundred patients with ACS underwent three-vessel IVUS examination and profiling of local ESS at baseline and at 6 months. This landmark study demonstrated that low baseline ESS is an independent predictor of plaque progression and expansive remodelling with lumen narrowing.[31] Data from OCT studies in man further elucidated the role of low ESS in plaque vulnerability. Those studies combined functional and morphological assessment of plaque using 3D OCT and showed that low ESS is associated with inflammation, thin fibrous cap, large lipid core, and expansive remodelling, all of which are key pathobiological fingerprints of vulnerable plaque (*Figure 3*).[32]

Endothelial shear stress and plaque rupture

Local ESS is a major local factor that interplays with the vascular remodelling response, leading to plaque growth and potentially plaque

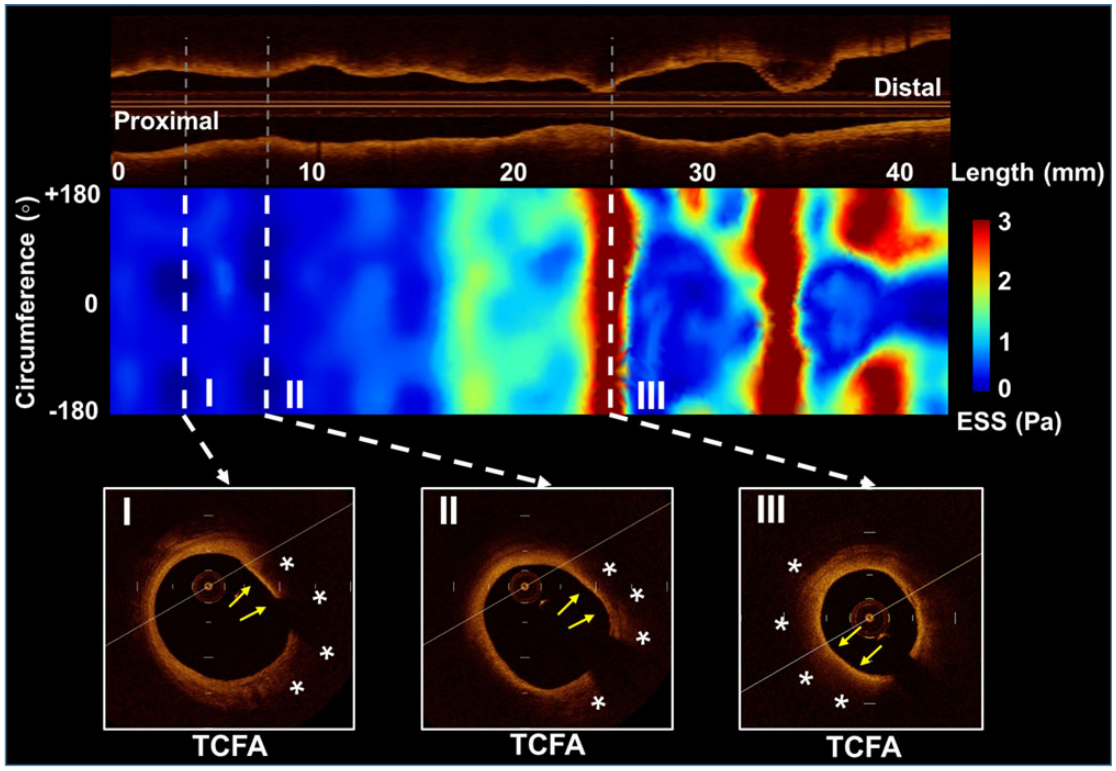

Figure 3 Co-localization of thin-cap fibroatheroma (TCFA) and local endothelial shear stress in a left anterior descending artery: Upper panel: optical coherence tomography run in longitudinal view, Middle panel: 2D map of endothelial shear stress distribution along the reconstructed segment with *y* axis corresponding to the vessel circumference and *x* axis to the vessel length. Lower panel: Sections I and II depict non-stenotic, thin-cap fibroatheroma that developed in a low endothelial shear stress environment (blue), whereas Section III shows a stenotic, thin-cap fibroatheroma that co-localizes with high endothelial shear stress (red).

rupture.[33] Non-stenotic vulnerable plaques are exposed to low ESS, which perpetuates an ongoing vicious cycle of local inflammation, matrix degradation, and expansive remodelling leading to acute plaque disruption and thrombotic occlusion of the lumen in the setting of increased thombogenicity.[1,34]

In contrast to non-stenotic vulnerable plaques, the stenotic vulnerable plaques create a heterogeneous local ESS environment along their length, which involves low ESS in the upstream shoulders, high ESS at the neck of the plaque, and low/oscillatory ESS in the downstream shoulder.[35,36] The majority of ruptures occur at the upstream shoulders of the plaque, whereas the downstream regions are more stable and therefore less prone to rupture. Plaque rupture can also occur at the most stenotic part of the plaque likely as a result of local erosion in the setting of very high flow and ESS. However, the pathobiological association of high ESS with plaque erosion is not well investigated.

Wall stress

Local wall stress is another major local haemodynamic factor that plays a key role in the pathobiology of plaque rupture.[37] Wall stress is proportional to blood pressure and inversely proportional to lumen stenosis. The highest wall stress typically occurs at the upstream shoulders of a stenotic plaque or within a non-stenotic plaque co-localizing with thin fibrous cap, increased macrophage density, and local microcalcifications.[37]

Collectively, the dynamic synergism among local haemodynamic factors (ESS and wall stress), plaque composition, and vascular remodelling is a key player in acute plaque disruption.[38] The advent of modern invasive (e.g. 3D OCT) and non-invasive imaging modalities is anticipated to advance our knowledge on these pathophysiological pathways.[32,39]

Calcification in vulnerable plaque formation and rupture

Although the degree of coronary calcification is a useful biomarker for the prediction of cardiovascular risk on a population level, the pathogenetic link between the presence of calcification in individual plaques and their potential for rupture is not well established.[4,40] Conversely, severe calcification in a plaque seems to be a factor associated with stable rather than unstable morphology.[41]

While the origin of intimal calcification is not entirely elucidated, it appears that apoptosis of smooth muscle cells and matrix vesicles released by macrophages are key mechanisms in the formation of microcalcifications.[40] Subsequent aggregation of such microcalcifications in larger masses will eventually lead to formation of calcified

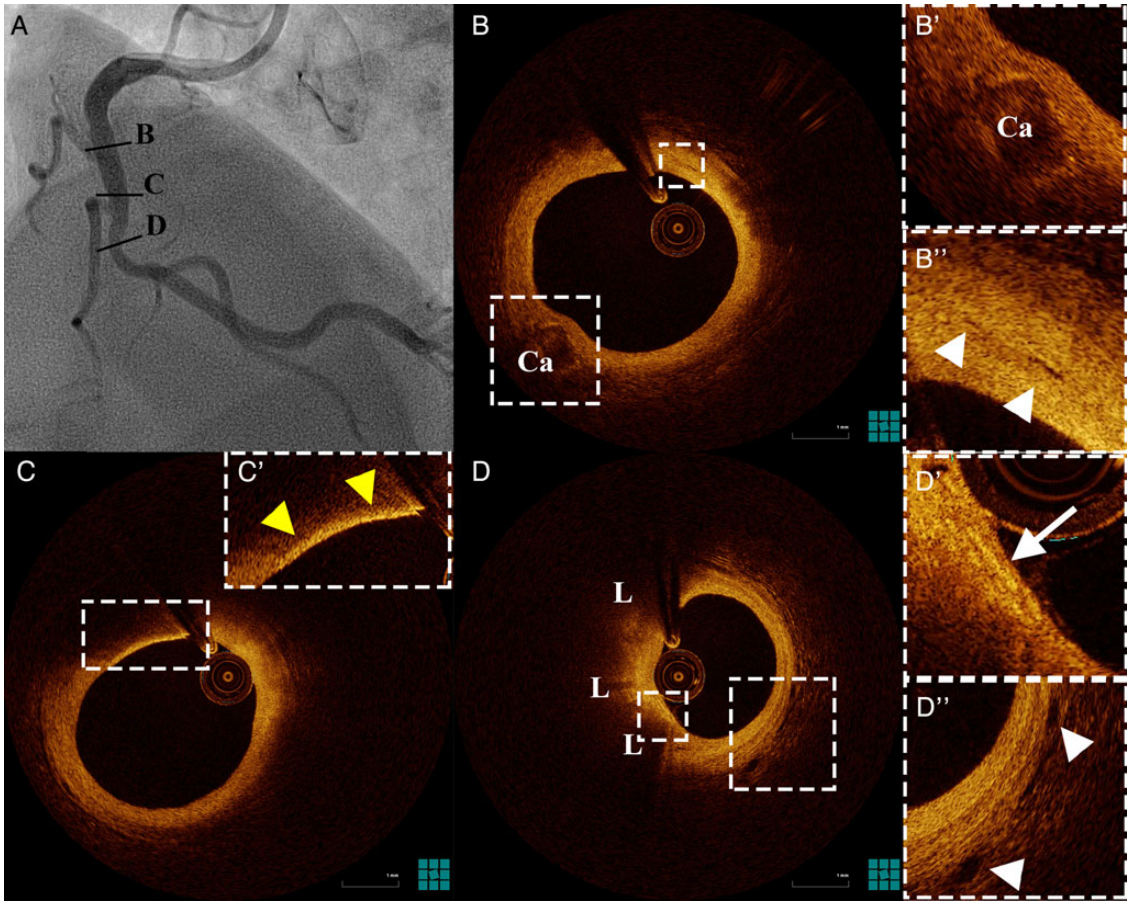

Figure 4 Features of non-culprit plaque vulnerability by optical coherence tomography. (*A*) Angiogram of a non-culprit right coronary artery lesion (fractional flow reserve: 0.95; minimal lumen area: 3.55 mm^2) in a patient with stable angina. (*B–D*) Optical coherence tomography cross-sections demonstrating several features of vulnerability in three locations across the length of the artery (noted with black lines), including: (*B′*) a protruding calcified nodule (Ca), (*B″*) intimal microvessels (white arrowheads), (*C′*) macrophage infiltration (yellow arrowheads), and (*D′*) cholesterol crystals (arrow) with (*D″*) adventitial vasa vasorum (white arrowheads) at the site of a thick-cap fibroatheroma (L).

sheets or plates,[40] a process that is more pronounced in healed plaque ruptures and fibroatheromas and rarely observed in fibrous plaques. These calcium sheets might later be broken down and form convex calcific protrusions into the lumen with cutting edges called calcified nodules. Calcified nodules, which can be identified by *in vivo* imaging modalities,[42] are a potential substrate for acute thrombosis (*Figure 4*).

Several *in vivo* imaging studies by IVUS or multislice computed tomography have indicated a potential role for calcification in plaque rupture, by demonstrating an association of 'spotty' calcification with culprit lesions of ACS.[43,44] However, pathological studies demonstrated that the amount of calcium in 'vulnerable plaques' may vary significantly and that the observed patterns of calcification often resembled other plaque types, thereby limiting the value of spotty calcification as a biomarker of 'vulnerable plaque'.[45] Recently, a PET-CT imaging study showed better discrimination between culprit and non-culprit plaques in acute coronary syndromes using ^{18}sodium floride (^{18}F-NaF), an imaging radioactive tracer targeting active calcification, rather than ^{18}F-FDG, a non-specific tracer of inflammation.[46] Interestingly, uptake of ^{18}F-NaF was also observed in coronary segments with minimal calcification, while simultaneously several heavily calcified segments had no ^{18}F-NaF uptake. Therefore, ^{18}F-NaF could specifically identify regions with active formation of calcifications, potentially reflecting a process of macrophage-initiated microcalcification deposition.[47]

Although microcalcifications, consisting of cellular-size hydroxyapatite depositions within the intima, are located in several locations within the plaque, their presence within the fibrous cap is speculated to be implicated in plaque rupture by compromising the mechanical stability of the plaque. Computational fluid dynamic studies have shown that microcalcifications within the fibrous cap exaggerate the mechanical forces applied to the cap within the cardiac cycle, thus facilitating even thicker cap disruption.[3,18,48] Subsequent studies using high-resolution micro-CT have shown that these stresses are exponentially increased in the presence of multiple microcalcifications >5 μm in high proximity with each other that can create 'explosive voids', allowing for the occurrence of plaque rupture.[49] Nevertheless, the *in vivo* detection of microcalcifications remains a

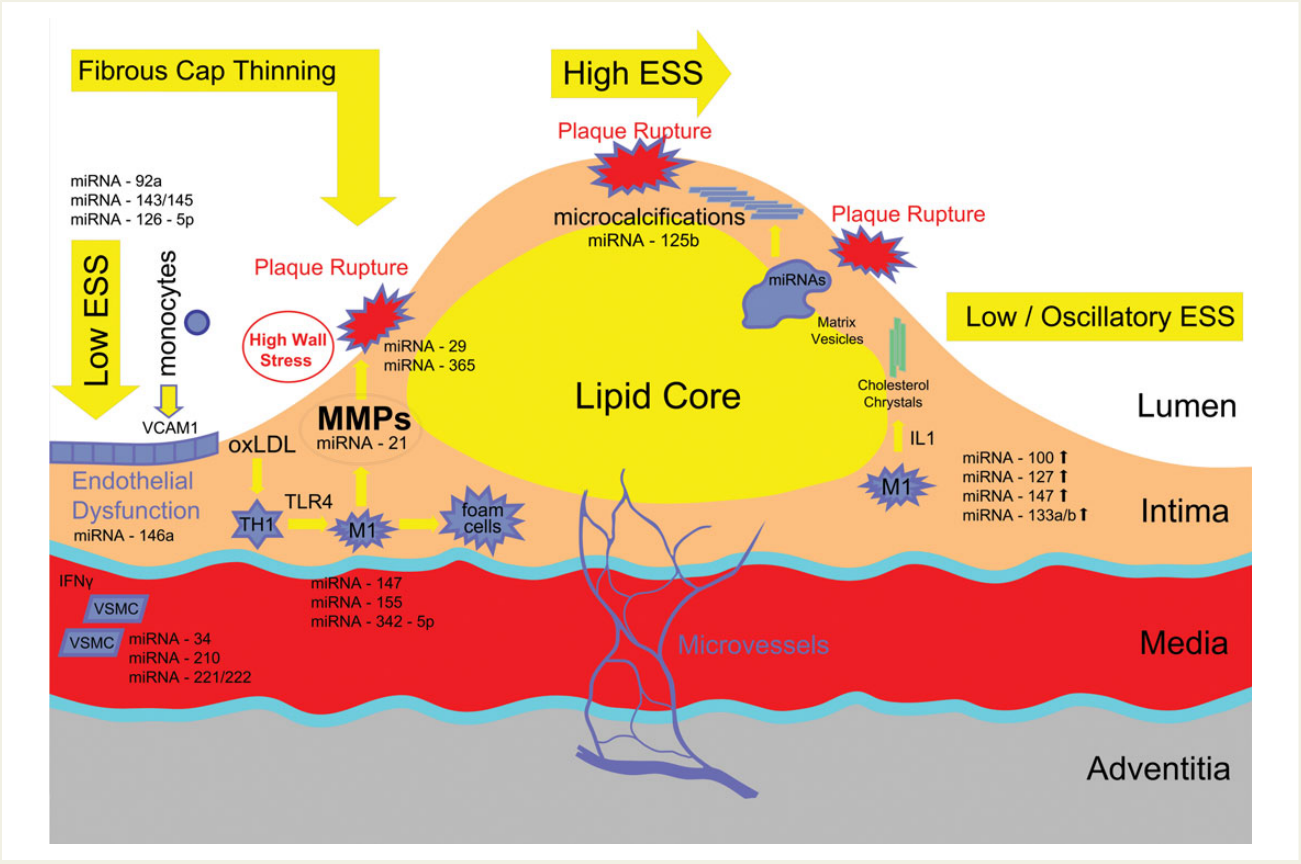

Figure 5 Review of the interactions between biomechanical forces, inflammation, microcalcifications, cholesterol crystals, and microRNAs (miRNAs) in high-risk plaque formation and rupture. Atherosclerotic risk factors including dyslipidaemia and low endothelial shear stress at the upstream shoulder of the plaque promote endothelial dysfunction and local inflammation. Recruited monocytes by endothelial adhesion molecules (VCAM-1) are activated within the plaque into M1 macrophages through toll-like receptor 4 (TLR4) and produce matrix metalloproteinases (MMPs), causing the thinning of the fibrous cap. T helper 1 (Th1) cells of innate immunity, activated by oxidized low-density lipoprotein (oxLDL) and other endogenous antigens, regulate the process. Co-localization of high wall stress with cap thinning (upstream shoulders of a stenotic plaque or within a non-stenotic plaque) can lead to plaque rupture. Microcalcifications in high proximity with each other within the plaque can create 'explosive voids', allowing for the occurrence of plaque rupture through their interaction with biomechanical forces. Cholesterol crystals through acute volume expansion could also cause mechanical instability or mediate local inflammation through interleukin 1 (IL-1) production. Specific cellular microRNAs regulate these interactions. ESS, endothelial shear stress; ACS, acute coronary syndrome; IFNγ, interferon gamma; VSMC, vascular smooth muscle cells.

challenge, as although current imaging modalities, such as OCT, could potentially discern larger microcalcifications,[50] the detection of microcalcifications $<15-20$ μm would require higher resolution modalities.[12]

Critical appraisal and conclusions

Both the extent of coronary atherosclerotic burden and the inflammatory activation in combination with risk factors that favour a prothrombotic milieu are the strongest predictors of adverse events. The factors responsible for the higher local vulnerability of the culprit plaque within an environment of widespread inflammation remain elusive. Recently, recognized local morphological characteristics of vulnerability (microcalcifications, cholesterol crystals) in combination with local haemodynamic factors (ESS, wall stress) seem to play an important role, while microRNAs are key mediators in plaque destabilization and rupture (*Figure 5*).

The identification of new targets for imaging, including microcalcification, cholesterol crystals, and ESS in addition to the local inflammatory environment might enhance the diagnostic accuracy for vulnerable, prone to rupture plaques. These new factors need to be further investigated. ^{18}F-NaF positron emission tomography scan could serve as a concise screening tool. Improvements in computational modelling and simulations can allow for more accurate ESS calculation in a time-efficient manner. Human application of μOCT could help in the identification of macrophage subtypes, microcalcification, and cholesterol crystals. Fusion of modalities, such as OCT with near-infrared autofluorescence, can offer information on both microstructural and molecular characteristics of vulnerable plaques.[51]

As the technology provides tools for the identification of these characteristics, new prospective studies are needed pertaining the aforementioned features in their predictive models. Comprehensive understanding of the mechanisms implicated in the destabilization of

atheromatic plaques can shift the current focus of research towards elements that were neglected until recently.

Limitations of the studies—open questions

As mentioned above, recent studies have challenged the vulnerable plaque paradigm. Although newly recognized targets of imaging have emerged, they also assume high-resolution invasive procedures, which preclude their application in primary prevention. Despite the concurrent evaluation of both haemodynamic and morphological characteristics, these new methods do not take into account atherosclerotic plaque burden. Most importantly, even with these new targets, high-positive predictive values are required, to justify vulnerable plaque-specific and vulnerable patient-dedicated therapies.

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