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The changing landscape of the vulnerable plaque: a call for fine-tuning of preclinical models

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ABSTRACT

For decades, the pathological definition of the vulnerable plaque led to invaluable insights into the mechanisms that underlie myocardial infarction and stroke. Beyond plaque rupture, other mechanisms, such as erosion, may elicit thrombotic events underlining the complexity and diversity of the atherosclerotic disease. Novel insights, based on single-cell transcriptomics and other "omics" methods, provide tremendous opportunities in the ongoing search for cell-specific determinants that will fine-tune the description of the thrombosis prone lesion. It coincides with an increasing awareness that knowledge on lesion characteristics, cell plasticity and clinical presentation of ischemic cardiovascular events have shifted over the past decades. This shift correlates with an observed changes of cell composition towards phenotypical stabilizing of human plaques. These stabilization features and mechanisms are directly mediated by the cells present in plaques and can be mimicked *in vitro via* primary plaque cells derived from human atherosclerotic tissues.

In addition, the rapidly evolving of sequencing technologies identify many candidate genes and molecular mechanisms that may influence the risk of developing an atherosclerotic thrombotic event - which bring the next challenge in sharp focus: how to translate these cell-specific insights into tangible functional and translational discoveries?

1. Introduction

Atherosclerosis is a chronic vascular occlusive disease that manifests clinically only at a late stage of progression. Based on risk factors, preventive interventions have the most population-wide impact before the disease manifests itself through a heart attack, peripheral vascular disease, or cerebral infarction. Lipid-lowering agents have proven their value, but there is still a residual risk that requires intervention and a deeper understanding of the natural history of the development and progression of atherosclerosis [1,2].

Meticulous post-mortem examinations of coronary arteries from myocardial infarction patients have provided invaluable insights that dominated the research field of vascular biology for decades [3–5]. Plaque rupture has long been considered the pathological substrate leading to an acute thrombotic occlusion of the artery. It has always been described as the lipid-rich, atheromatous plaque with a thin fibrous cap with local infiltration of inflammatory cells that cause proteolytic activity and degradation of the stabilizing stimulate extracellular matrix [6]. However, pathological observations have also shown significant heterogeneity of vascular lesions that can lead to an adverse event. The observation of thrombotic occlusions on non-ruptured plaque and the role of micro-calcifications destabilising the cap made it clear that there is an ambiguous description characterising all symptomatic atherosclerotic lesions [7–12]. Despite its limitations [13], the morphological characterisation of the vulnerable plaque based on pathological observational research is a gold standard for historical and current vascular

Abbreviations and acronyms: α-SMA, Alpha smooth muscle actin; ECM, Extracellular matrix; KLF4, Kruppel-like factor 4; MYH11, Myosin heavy chain 11; NSTEMI, Non-ST-segment elevation; Sca1, Stem cell antigen 1; SMC, Smooth muscle cell; STEMI, ST-segment elevation; TLR, Toll-like receptor; VSMC, Vascular smooth muscle cell.

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biological research.

The lesions included in pathological studies usually represent a snapshot at the end-stage of the disease and, by default, patients with a poor prognosis. As a result, the prevailing view of the natural course of atherosclerotic disease progression has been mostly based on the end-stage symptomatic phase. It hampers the interpretation of the casual mechanisms, which can only be modelled in non-human or *in vitro* experimental models.

Yet, recent insights into cell-specific biology and transcription in the active human plaque have provided an in-depth understanding of the role of specific cell clusters in plaque vulnerability [14–19], offering new impetus for research into destabilization mechanisms. The known cell types (macrophages, T-lymphocytes, smooth muscle cells, endothelial cells) can not be captured in a single functional framework since cells can differentiate [20–25], form sub-clusters [14–19] and undergo (clonal) expansions [26]. The insights, mostly based on sequencing efforts of human plaques and cell lineage tracing in mouse models, provide tremendous opportunities to search for new therapies that will further reduce the residual risk of developing an atherosclerotic thrombotic event. However, generating lists of many genes and pathways will be followed by another challenge: how to translate these cell-specific insights from transcription into functionality and prioritise the functional targets? This review will map the rapidly evolving landscape of the unstable human atherosclerotic plaque description and then discuss the challenges that await us to translate this wealth of data towards dedicated therapeutic studies targeting residual risk.

2. The thin capped atheromatous inflammatory plaque: a dogmatic gold standard in experimental research

The traditional model for the vulnerable plaque, conserved for decades, has served as the starting point for countless studies attempting to unravel the mechanisms of atherosclerosis. Plaque characteristics observed in coronary lesions are applied as the surrogate endpoint in experimental research. However, the fixed histological measures of lesion characteristics may have narrowed our viewpoint (Fig. 1). Notably, this standardised approach was and still is considered a basis for developing animal models and technological innovations.

The emergence of genetically modified animal models resulted in the development of the ApoE and LDL receptor knockout mice that, when combined with a high-fat diet, develop lesions harbouring many components of the lipid-rich inflammatory plaque described in *post mortem* human studies [27,28]. These murine models were used for research into the initiation and progression of arteriosclerosis and did not spontaneously develop myocardial infarction, or plaque rupture in the timeframe studied. Even though these studies have provided a great deal of information regarding the possible causal role of hundreds of genes, they are more valuable to model specific mechanisms (*e.g.* atherogenesis, response to injury, cell recruitment, *etc.*) than simulate the full features of human cardiovascular disease pathogenesis.

Genetic variability, exposure to risk factors, lifestyle and drugs add an extra dimension to the heterogeneity that characterises humans' atherosclerotic process, and modelling these elements has proven elusive. This complexity is not easily captured in a genetically homogeneous mouse population where mostly only one variable, the gene of interest, varies between experimental groups. Partly because of this, the extrapolation of mice studies into human atherosclerosis has been a matter of debate [29,30].

Moreover, technological developments in imaging techniques have often focused on detecting determinants, as described in the classical description of the human vulnerable and ruptured plaque [31,32]. The description of the vulnerable plaque characteristics that form the substrate for a thrombotic event has led to an enormous impulse in technological developments in imaging to detect the plaque at risk for rupture [31,32]. Spectral analysis of intravascular ultrasound [33], palpography [34], temperature measurement [35], near-infrared spectroscopy [36] are just a few of the many imaging modalities that have found their way to catheterisation labs. All those techniques aim to detect one or more components of the vulnerable plaque that could significantly contribute to the generation of surrogate endpoints in clinical studies to test new plaque stabilizing drugs at an early development stage. The gold standard of the vulnerable plaque has been

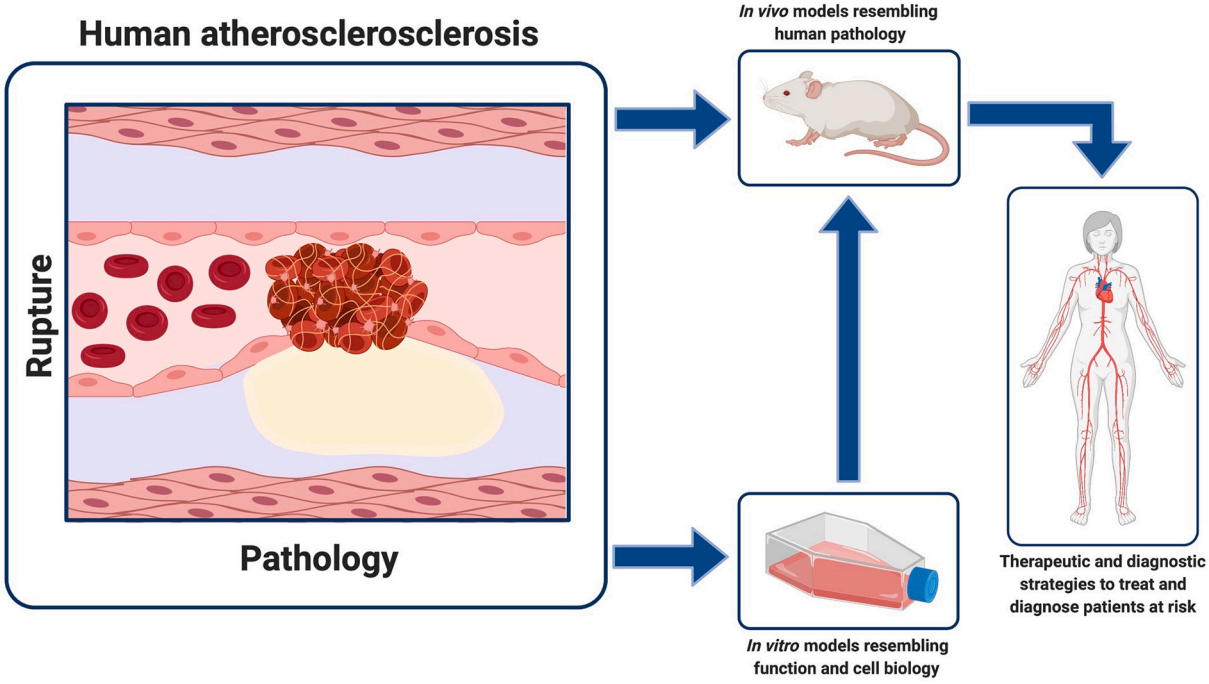

Fig. 1. The vulnerable plaque: the gold standard in atherosclerosis research. The vulnerable ruptured plaque as a standard model to develop animal models and technological innovations in the atherosclerosis field. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

described by imaging plaques in dead patients, showing high sensitivity for myocardial infarction but still low specificity. The PROSPECT studies reveal that the absolute risk for a thrombotic event on top of a lesion that fulfils the traditional description of the vulnerable plaque is limited. We have to acknowledge however, that the resolution of intravascular ultrasounds may be too limited to accurately detect all characteristics that represent vulnerability in atherosclerotic lesions. The percentage of plaques that fulfil the intravascular ultrasound-based geometrical parameters that should reflect a measure of plaque vulnerability and results in target lesion failure does not exceed 5% [37,38]. Therefore, it can be questioned if the currently applied vascular imaging modalities have been chasing a dogma [39] and that vision was limited by gaze constriction.

3. The heterogeneity of atherosclerotic lesions

Since the original observation that plaque rupture is a primary underlying substrate of acute coronary thrombotic occlusion, insights in pathophysiology have changed significantly. Clinical studies revealed a shift in presentation from STEMI to NSTEMI coinciding with the increased prevalence of stable symptomatic lesions. The following observations in the era of next-generation sequencing mandate a recalibration of "plaque vulnerability".

First, nowadays most patients survive their first myocardial infarction, and the incidence of non-ST-segment elevation (NSTEMI) increased compared to patients presenting ST-segment elevation (STEMI) [40,41]. Interestingly, NSTEMI has been more often observed in patients with partially occluded vessels, commonly occurring with stable atherosclerotic plaques. In contrast, STEMI associates with full vessel blockage, often seen in patients with an unstable plaque phenotype. The shift in the clinical presentation of atherosclerotic disease corresponds to the observed temporal changes in the characteristics of human plaques [42]. These clinical and pathological time-dependent changes are likely the consequence of prevention measures, lifestyle changes and improved therapeutic interventions adopted over the past decades. Indeed, serial imaging and pathological studies revealed that statin treatment reduces lipid content and increases the relative collagen content of human plaques, which is thought to stabilize the lesions [43–45]. These morphological alterations further underline the necessity to fine-tune and revisit the mechanisms that lead to atherosclerotic thrombotic events in the current era [46].

Second, pathology studies showed that also fibrous plaques without rupture can underly a thrombotic event. Previous autopsy data show that eroded lesions are prevalent in younger individuals, particularly in women who died of acute myocardial infarction [8,10]. Eroded plaques are characterised by the absence of the fibrous cap and the formation of a platelet-rich white thrombus that does not interact with the plaque's core. Despite the identification of plaque erosion as an important alternative mechanism for thrombosis and a significant cause of sudden death, its underlying pathophysiological mechanisms are not well understood [10].

The leading hypothesis for erosion is that inflammation may have a different role and involve other mechanisms than plaque rupture [11]. Of note, the combination of multiple mechanisms, including endothelial dysfunction, TLR signalling, leukocyte activation, and modification of sub-endothelial matrix by endothelial cells or smooth muscle cells (SMCs), may trigger loss of adhesion to the extracellular matrix (ECM) or endothelial apoptosis leading to erosion [47]. For example, in atherosclerotic plaques, endothelial cells commonly express Toll-like receptor 2 (TLR2) that can sense both bacterial products and extracellular matrix (ECM) glycosaminoglycans - such as hyaluronan. The activation of TLR2 triggers endothelial dysfunction, which is further enhanced by activating neutrophils that attack the endothelium. The activated neutrophils release proteases - forming the neutrophil extracellular traps (NETs), damaging endothelial cells and trap leukocytes [48]. As a result, endothelial cells may detach and expose the subendothelial matrix and thrombogenic components, ultimately leading to the formation of a white-thrombus and vessel occlusion [49].

Third, current insights into atherosclerosis mechanisms are still primarily based on the assumption that the vast majority of cells in the atherosclerotic plaque are in the final stage of differentiation. However, lineage tracing animal experiments show that substantial numbers of the cell populations in the atherosclerotic vessel wall result from transdifferentiation of cells from different origins [20,21]. For instance, the majority of the cap stabilizing SMCs are thought to be originated from medial SMCs [50]. However, endothelial cells may also be under some circumstances a significant source of cap SMCs by undergoing endothelial to mesenchymal transition (EndoMT) [51–53].

In cross-heart transplant patients, *circa* 20% of $CD68^+$ cells in advanced coronary plaques have smooth muscle rather than myeloid origins [54]. This study has highlighted that VSMC-derived foam cells comprise a substantial component of the plaque core and that the conventional view of the macrophage-rich necrotic core may need to be revisited. Indeed, VSMCs within the vessel wall may have a progenitor subpopulation capable of proliferating and accumulating in the plaque [54]. In mice, the cells derived from medial VSMCs, that lack detectable expression of some SMC reporter genes, express markers of mesenchymal stem cells (*e.g.*, Stem cell antigen 1 ($Sca1^+$), $CD105^+$), as well as myofibroblasts (α-$SMA^{+/-}$, platelet-derived growth factor β $receptor^+$ ($PDGFRB^+$) [55]. Moreover, a specific SMC H3K4me2 myosin heavy chain 11 (MYH11) epigenetic signature in 38% of the $CD68^+$ α-SMA^+ cells isolated from murine plaques suggests their non-myeloid derivation [55]. Besides, several lineage tracing studies have shown that mature $MYH11^+$ VSMCs are not terminally differentiated and are capable of transdifferentiation in culture [54,55], in atherosclerosis [56], and after vascular injury [22]. In addition, Bennet et al. have shown that myeloid-derived cells do not acquire MYH11 epigenetic signature and SMC-specific markers in advanced human plaques, suggesting that they do not behave like vessel wall VSMC-derived cells [57].

The presumed role of cell plasticity in the progression of atherosclerosis results in several challenges for vascular biology research, underlining the complexity of atherosclerotic disease: "one size does not fit all". Therefore, it will be fundamental to unravel the primary determinants and cascades that are key players in these differentiation processes.

Much insight into vascular cells' differentiation capabilities has been generated in genetically modified mice on an atherosclerotic background. Lineage tracing is technically challenging in human disease, but the upcoming insights from single-cell sequencing of atherosclerotic plaques are creating a valuable opportunity for in-depth cross-validation between mice and men. Although cross-sectional and observational, the comparison of gene architecture of cell populations in diseased tissues between human plaques and experimental models will support (or debunk) extrapolation towards human disease.

Altogether, the temporal changes in clinical presentation and the diversity of lesions observed in pathological examination demonstrate the complexity and heterogeneity of molecular and cellular substrates that result in plaque vulnerability and subsequent clinical events. The role of cell plasticity and clonal expansion [26] in arteriosclerosis may still be strongly underestimated and requires a further deepening of knowledge of molecular and cellular vessel wall biology. To understand and study the relevance of these observations, this pathological heterogeneity and cellular biology should be optimally mirrored in *in vitro*, animal and human lesions.

4. Can an understanding of the cellular composition of atherosclerotic lesions contribute to a fine-tuning of the vulnerable plaque definition?

For a considerable amount of time, our understanding of the cellular composition of the atherosclerotic plaque was based on histological and flow cytometry studies coupled with the expression of several cell-

specific markers. In 2000, Bonnano et al. characterised cell populations in human carotid plaques using flow cytometry, showing that approximately half of the cells are inflammatory mononuclear cells (26.16 ± 14.2% lymphocytes; 17.34 ± 12.6% monocytes/macrophages), and the other half consists of vascular smooth muscle cells (VSMCs) (56.50 ± 20.3%) [58]. With the advances in flow cytometry technologies, namely the introduction of cytometry by time of flight (CyTOF), the number of labelling parameters vastly increases. Indeed, it allows for more advanced characterisation of different cell populations, especially in immune cells, and identifies an even higher T cells content in human lesions than previously anticipated [18].

Even though highly multiparametric approaches will increasingly be instrumental in revealing the cellular composition of the lesions, they do not directly reflect the cell functions and state. Thus, they might miss unexpected cell (sub-)types. Advancements in molecular techniques, primarily the single-cell transcriptomics (scRNA-seq), have further aided our understanding of plaque composition on a cellular level beyond the defined set of markers. In 2016, the first scRNA-seq effort characterised a subset of plastic IFNγ^+ Th1/Tregs in murine aortic tissue [59]. Later, three independent groups characterised the CD45+ population cells in plaques from murine models using scRNA-sEq. [14–16]. They described numerous leukocyte populations that included T cells, macrophages, B cells, dendritic cells, and NK cells. These studies have increased our understanding of cholesterol uptake and T cell recruitment but provide no insight into CD45- populations. Later, Wirka et al. identified 12 cell populations in murine plaques, including SMCs, fibroblasts, and endothelial cells. They further describe that a growing pool of modulated SMCs appeared during disease progression, of which 11% did not have SMC origin [17].

Most recently, the scRNA-seq studies on human atherosclerotic plaques from coronary arteries of cardiac transplant recipients identified populations of vascular tissue cells, leukocytes, endothelial cells, and SMCs. In concordance with murine data, a portion of SMCs were undergoing phenotypic modulation to a fibromyocyte state [17]. The CD45+ landscape of carotid artery plaques has been described using a combination of CyTOF, CITE-seq, and scRNA-sEq. [18]. Finally, our group has recently published an in-depth annotation of the cellular landscape of carotid artery plaques. We described several macrophage and T cell populations confirmed in a different cohort of patients using scATAC-seq. Also, we highlighted distinct populations of endothelial and SMCs. Notably, a subcluster of the SMC population was partially depleted of SMC markers ACTA2 and MYH11. Sub clustering showed that the total population blanketed two sub-populations of synthetic and contractile SMCs [19]. Simultaneously, a subcluster of endothelial cells showed upregulation of SMC markers - likely representing the cells undergoing either EndoMT or MET. This, together with mouse studies that revealed the differentiation potential of VSMCs [20,26], strengthens the evidence of an extensive level of phenotypic cell plasticity in atherosclerotic lesions. Therefore, it is conceivable that cell plasticity and transdifferentiation events will be keys in future studies to define the cellular landscape of stable and unstable plaques.

5. Animal models unravelling mechanisms of plaque vulnerability: verification in human-like models, an unmet need

The complexity of the human atherosclerotic disease, with lesions that have been exposed to numerous medical interventions, risk factors and inherited genetic variability, is challenging to mimic in animal models.

Together with the pathological characterisation, the in-depth transcriptomic analysis of the human lesion associated with clinical presentation, *e.g.* myocardial infarction and stroke, will lead to a broad range of novel phenotypic descriptions of the vulnerable atherosclerotic lesion. These patho-transcriptome based phenotypes come with a challenge and opportunity for *in vitro* and animal models: *how can the complexity and heterogeneity of atherosclerotic disease be captured in a standardised in vitro or animal model?*

The search for model systems with an optimal match in cell-specific transcriptomes between human plaques, cell cultures and animal models will have strong added value that strengthens the translational perspective of experimental observations. A recent study associating the natural genetic architecture of over 100 inbred mice strains with phenotypical traits or atherosclerotic disease [60] provided a resource for studies that examine the complex molecular interactions underlying atherosclerotic disease in a controlled environment.

Similarly, phenotyping of cell cultures and animal models that go beyond morphologic and pathological descriptions and includes a transcriptomic characterisation can now be compared with gene expression profiles of cell-based clusters in human plaques. A large scale -omics effort unravelling the proteome and (cell-specific) transcriptome in atherosclerotic mice strains can facilitate the search for the best match with the different human atherosclerotic lesions characteristics that lead to a clinical event (Fig. 2) [61].

6. Primary plaque cells: advantages in experimental research

The use of human plaque derived cells in functional experiments will likely aid the search for experimental models that best resemble the gene expression profiles and underlying molecular processes in human atherosclerotic plaque. The concept of using primary human cells from diseased tissue for functional studies is not new [62,63] – as it is a widely accepted approach mainly in cancer research, immunology, and numerous mendelian disorders. At the same time, this concept is less applied in atherosclerotic research. In principle, primary atherosclerotic plaque cells from patients hold the key to unearth human-specific pathways to disease and screen therapeutics. They, indeed, offer an opportunity to mimic plaque's cellular architecture and functionalities *in vitro*.

In recent years, the new insights from -omics related studies clearly unfold the strong complexity and variability in the mechanisms that underlie atherosclerotic disease development. The diversity of mechanisms likely also results in diverse pathological substrates of myocardial infarction. However, in experimental research, we still follow the simplified concept of one gold standard for lesion destabilization: the lipid-rich, smooth muscle cell poor and inflammatory plaque. The strong transcriptomic variation observed in human atherosclerotic lesions can be explained by genetic diversity, risk factors and environmental triggers that all influence lesion phenotype. In experimental research, it can be questioned what mouse strain reflects the human disease at best? The genetic background of the strain of wild-type mice being used should be taken into account, as this can significantly affect the development of atherosclerosis. The diverse processes promoting atherosclerosis in humans demand for different mouse models. Primary cells isolated from plaques can be used to establish the *in vitro* assays to study candidate genes' functions and prioritise the most promising ones for further animal and human studies. This way, the *in vitro* models can be used to perform genetic and drug screening studies and represent an ideal bridge between *in silico* and *in vivo* studies. For example, the analysis of atherosclerotic coronary arteries from the STARNET cohort found different molecular networks that underlie these plaques and hint towards a pivotal role of SMCs [64]. Similarly, the integration of single-cell transcriptomics and genetic data directed towards the involvement of endothelial cell, SMCs and plaque macrophages in the genetic component of atherosclerotic disease [19], identified several novel candidate genes. Due to the large number of candidate genes and processes, a medium or high throughput system is required to preselect the most promising candidates before a follow up *in vivo* study. Although some phenotypical characteristics may be lost in prolonged cultures, especially if cell passaging is required, several donor characteristics will maintain by epigenetic and transcriptomic features. Those will reflect age, medical history, race, and sex, making primary plaque cells a potential *in vitro* platform to mimic atherosclerotic disease [65]. Thus, they

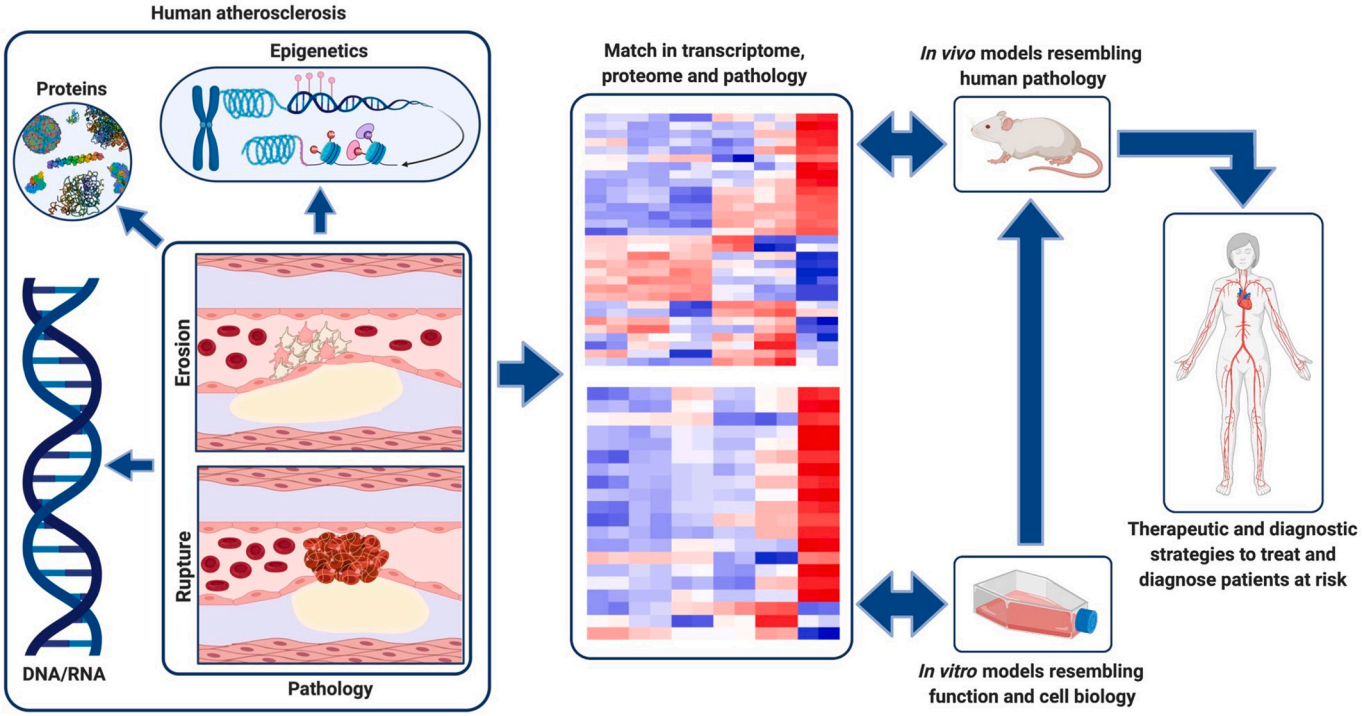

Fig. 2. Translating the complexity of human atherosclerotic lesions to *in vitro* and *in vivo* models. Phenotyping of human atherosclerotic plaques based on RNA, epigenetic modifications and protein in addition to the pathological assessment will lead to a recategorisation of plaque types. This further differentiation of vulnerable lesions leading to a clinical event will require broader phenotyping of existing and future *in vitro* and *in vivo* models to be developed, seeking the best match that best mirrors the human vulnerable plaque.

Table 1
Identification of plaque cells. The table summarises the studies done on the isolation, culture methods, and characterisation techniques used to obtain and identify the cells dissociated from human atherosclerotic plaque bodies.

Year First author	Plaque extraction	Patients N. ♂ ♀	Isolation method	Culture medium	Characterisation technique	Dissociated cells	Ref.
1988 Libby P.	CEA FEA AAA	13 12	Culture Outgrowth	DMEM + 25 mM Hepes + 10% FCS	IHC	α-SMA$^+$ vWF$^-$	[67]
1990 Dartsch P. C.	FEA	Primary Stenosis Lesions 11 8 Restenosis Lesions 3 2	Culture Outgrowth or 3 h digestion mix: Collagenase 3 + Elastase 3 + Trypsin Inhibitor	W-MB/H-F12 (*v*/v) + 10–15% FCS	IHC	α-SMA$^+$ Vim$^+$ Des$^-$	[69]
1995 Bennet M. R.	DCA	3 2	16 h digestion mix: Collagenase 1 + Elastase 3 + Trypsin Inhibitor	W-MB 752/1 + 20% FCS + 10 mM Hepes + 2% ECGS	IHC	α-SMA$^+$ Vim$^+$ MYH11$^+$ Des$^-$ vWF$^-$	[68]
2000 Bonanno E.	CEA	20	O/N digestion in: Collagenase 1	None	IHC FC	α-SMA$^+$ CD3$^+$ CD68$^+$	[58]
2004 Monaco C.	CEA FEA	Nd	Digestion mix: Collagenase 1 + Elastase 3 + DNase + Trypsin Inihibitor	RPMI 1640 +10% FCS +10% Hum. Ser.	IF FC	α-SMA$^+$ CD3$^+$ CD68$^+$	[70]
2011 Pankajakshan D.	CEA	12	3 h digestion in Collagenase	ScienceCell SMCM: 10% FBS 5 ml SMCGS 5 ml P/S	IHC	α-SMA$^+$ CNN1$^+$	[71]
2019 Novikova O. A.	CEA	Stable Plaque 20 15 Vulnerable Plaque 46 10	O/N digestion in: Collagenase 2	IMDM + 10% FBS + 10 μg/ml P/S	RNA-seq IF qPCR	α-SMA$^+$ CD14$^+$ VEGF-A$^+$ CD105$^+$ CD31$^+$ CD34$^+$	[72]

AAA = Abdominal Aortic Aneurysm; CEA = Carotid Endarectomies; DCA = Directional Coronary Atherectomies; FC = Flow Cytometry; FEA = Femoral Endarectomies; H-F12 = Ham F12 Nut Basal Medium; IF = Immunofluorescence; IHC = immunohistochemestry; W-MB = Waymouth's MB 752/1 Basal Medium.

can serve as a candidate to link outcomes of human "-omics" studies with an *in vivo* experimental design. A similar approach was taken by Aherrahrou et al. [66] who quantified 12 atherosclerosis-relevant phenotypes related to calcification, proliferation, and migration in VSMCs isolated from 151 multiethnic heart transplant donors. They have demonstrated that genetic variants from individual donors have significant influences on SMC function *in vitro*, relevant to the development of atherosclerosis.

7. Isolation of primary cells from human atherosclerotic plaque

The employment of primary cells from the plaque directly isolated from human atherosclerotic tissue may be an emerging approach to better understand lesions' progression mechanisms by performing disease-specific functional tests.

Several groups have shown how to isolate and characterise plaque cells over the past few decades. Interestingly, the studies reported in Table 1 have shown that despite the differences in sample derivation, processing, culture conditions and characterisation techniques, plaque cells commonly express alpha-smooth muscle actin (α-SMA; specific for VSMCs) (Fig. 3) and lack the expression of von Willebrand factor (vWF; specific for endothelial cells) [67,68].

Two main approaches can be used to isolate plaque cells – 1.) enzymatic digestion of atherosclerotic plaque and subsequent culturing of isolated cells or 2.) outgrowth method where plaque cells spontaneously migrate out of the small pieces of plaque immobilised on a surface of a cell culture dish. The digestion of plaque tissue allows for a higher yield of cells. However, it may lead to more heterogeneous cultures - since a pool of cells includes, besides the plaque cells, also other cell types. Those cells include medial VSMCs, endothelial cells, fibroblasts, macrophages, myeloid and immune cells. This can explain why the plaque cells isolated *via* tissue digestions seem to morphologically differ and express non-related markers such as CD31, VEGF-A, CD14, CD34, and CD3 and become more homogenous during sub-culturing [58,68,70–72].

In contrast, even though the culture outgrowth method initially provides a lower number of cells, it eventually leads towards a more homogeneous cell population with a lower inter donor variation [67,69].

8. Diversity in the origin of plaque cells

To understand the origins of cells in atherosclerotic plaque, several studies have been performed in humans and mice to explore whether VSMCs can *in vivo* transdifferentiate into other cell types involved in atherogenesis [54–56]. Interestingly, as mentioned previously, this process occurs at a relatively high frequency in animal experiments and human lesions. [54]. Thus, although cultured cells reflect the transcriptomic signature of mesenchymal cells, their origin may differ. For example, adventitial cells, including pericytes and fibroblasts, may contribute to the formation of neointimal lesions within atherosclerotic plaques, for instance, after vascular injury. Interestingly, the existence of an adventitial Sca1+ cell population has been found to increase the expression of numerous SMC-marker genes *in vitro*. Indeed, the fact that adventitial pericytes and activated fibroblasts (myofibroblasts) express multiple SMC-markers reinforces the hypothesis that they may give rise to a VSMC-like cell type involved in atherogenesis [57]. On the other hand, Wang et al. have shown that Sca1+ vascular cells do not do not give rise to any SMC-like cells in atherosclerosis *in vivo* [73].

In conclusion, human plaque cells' origins are still controversial, despite their characteristics and similarities with VSMCs. However, although it may be precarious in the search for standardised atherosclerotic platforms, it could be significantly advantageous since the plaque cells may be capable of transdifferentiating into specific cell types involved in plaque progression.

9. Possible mechanisms underlying the differentiation of plaque cells

The phenotypic switching of VSMCs is a transdifferentiation process regulated by the myocardin expression (Fig. 4). Myocardin is a serum response factor (SRF) transcriptional coactivator specific for SMCs and cardiac cells, playing a key role in activating SMC reporter genes and, hence, in the differentiation to a contractile SMC phenotype during embryogenesis [23,74].

Contractile VSMCs, resident in the blood vessel wall (tunica media), usually express myocardin and other SMC specific markers, including MYH11, 22-kDa SMC lineage-restricted protein (SM22α), α-SMA, smoothelin, and others [23]. The loss of myocardin significantly reduces the expression of SMC markers. It upregulates various inflammatory pathways, triggering the VSMC transdifferentiation from a contractile towards a more synthetic phenotype, with enhanced migration, proliferation and secretion (ECM and cytokines) capacities [24,25]. This switching process is regulated by the Kruppel-like factor 4 (KLF4) transcriptional program, which leads to the inhibition of myocardin gene activation in response to platelet-derived growth factor-BB (PDGF-BB), oxidised phospholipids, or interleukin (IL)-1β *via* down-regulation of SMC-marker genes [20,76]. Of interest, the transdifferentiation of VSMCs to macrophage-like cells may be driven by lipid accumulation in the plaque. It was shown that the cholesterol loading of cultured VSMCs

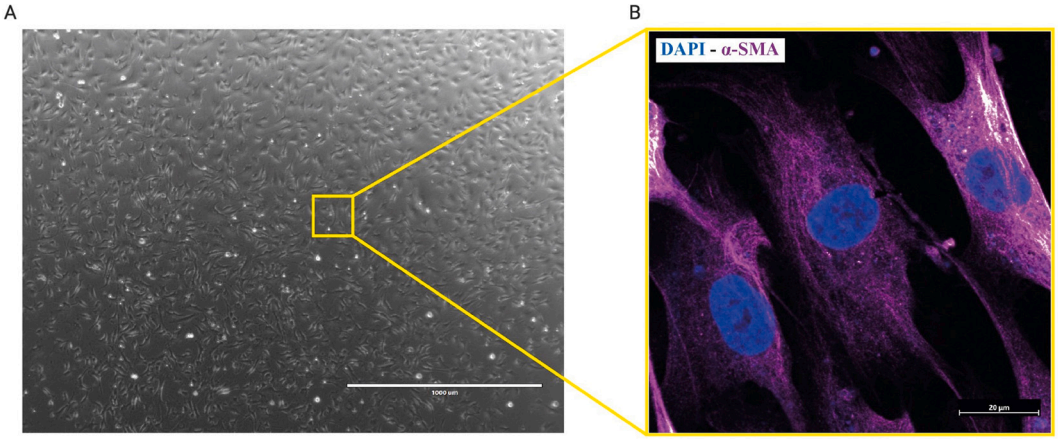

Fig. 3. Representative images of isolated plaque cells. (A) Transmitted light microscopy image of plaque cells in culture. (B) Immunofluorescence image of plaque cells labelled with alpha-smooth muscle actin (α-SMA; violet) and DAPI (blue) for nuclear staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

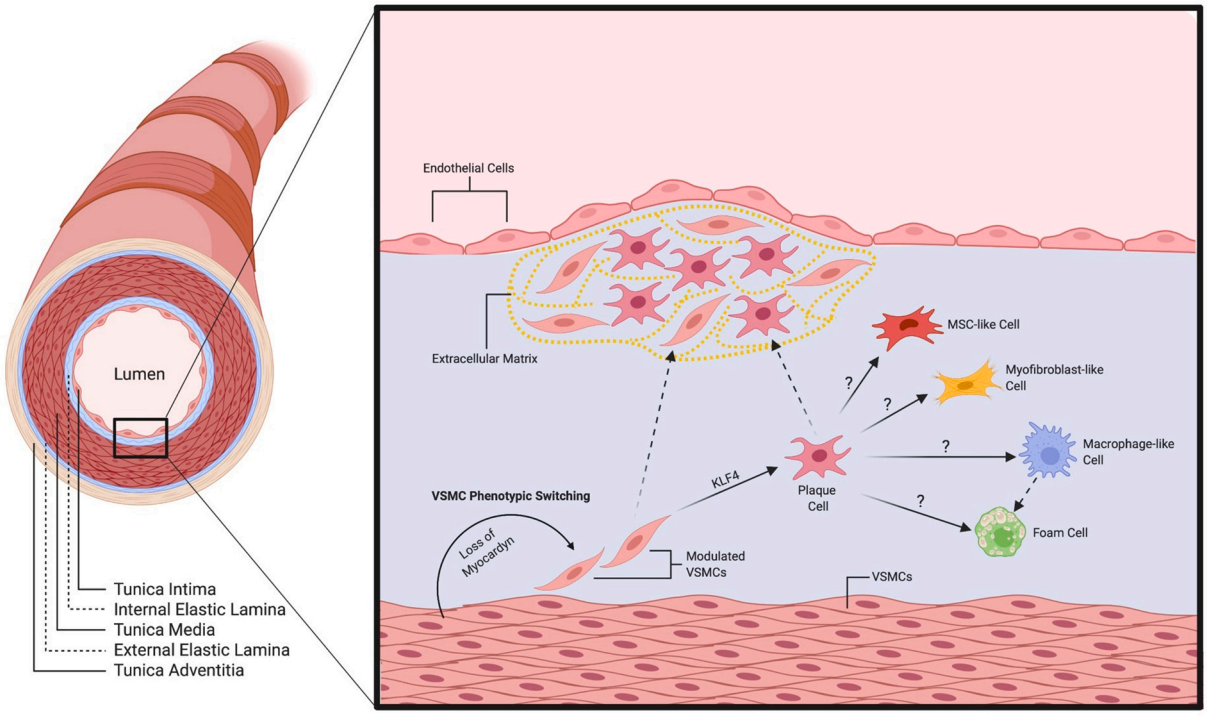

Fig. 4. How VSMCs may switch to plaque cells. The transdifferentiation of VSMCs to a pro-atherogenic cell type is a KLF4-dependent process that may give rise to plaque cells. Those body cells can express a broad range of specific markers and produce connective tissue present in the plaque. Adapted from Bennet et al. [75].

activates multiple pro-inflammatory genes, suppresses the expression of SMC-marker genes, activates macrophage markers, and induces phagocytic activity, all of which are KLF4-dependent processes [21]. However, these VSMC-derived macrophage-like cells' gene expression is distinctly different from classical monocytes, macrophages, and dendritic cells. In addition, these cells have reduced phagocytic capacity compared with activated peritoneal macrophages [75,77]. Although VSMCs within lesions may play a beneficial role, for example, by protecting the fibrous cap from rupture and promoting plaque stability and repair, it has been shown that the original VSMC features can vary dramatically depending on the mechanism of the phenotypic switching [75]. Accordingly, cultured plaque cells should be very plastic cells that actively affect atherosclerosis progression and plaque stability. However, further studies are needed to demonstrate their origin and how the plasticity capacities are modulated *in vitro* [67,78].

The characterisation and functional properties of cultured plaque cells could be an opportunity to fill this knowledge gap regarding the emerging role of cell differentiation and complement the atherosclerosis research pipeline. Their gene expression profile has shown the expression of mRNAs encoding for markers of VSMCs, macrophages, fibroblasts, endothelial cells, chondrocytes, and osteoblasts [72]. This complex profile of plaque cells could be related to their plasticity since, during atherogenesis, cells may lose or acquire specific markers [54]. The RNA-seq data on the expression of both SMC- and macrophage-markers in plaque cells are supported by positive immunofluorescence staining of the cultured plaque cells for α-SMA and CD14. Generally, it is assumed that the intimal proliferative VSMCs and the macrophages contained into plaque bodies originated from medial contractile VSMCs, and from monocytes that transmigrate through the endothelial layer upon vascular injury, respectively [56]. However, it has been recently shown that classifying plaque cells into macrophage-like and SMC-like cells based on the traditional markers' expression is inaccurate [57]. Indeed, the formation of cultured plaque cells could represent a viable group of mesenchymal cells with transdifferentiation capacity that can acquire a pro-inflammatory phenotype, losing the expression of some SMC markers while gaining other markers such as macrophage ones.

The study of somatic mutations can be used as lineage barcodes to track the origin of plaque cells (*e.g.*, VSMCs). Thus, assuming that plaque cells originated from medial VSMCs, exposure to coactivators such as myocardin would be helpful to study their plasticity. In addition, it would be an essential step to identify the factors and mechanisms to promote VSMCs to transdifferentiate to plaque cells.

In light of this, identifying potential cues that might regulate VSMCs to switch to plaque cells would be the key to determine how to therapeutically manipulate them to reduce plaque burden and inhibit plaque erosion.

10. Limitations and future challenges

Generally, the major limitation of using primary cells is the significant variability induced by the genetic architecture of each donor [79]. This is applicable to plaque cells as well and it may hamper generalizability towards the general population.

Besides, the finite division number and shorter lifespan can complicate the genetic manipulation efforts and requires a continuous source of patient's material. The same phenomenon is related to phenotypic changes during prolonged culturing, eventually leading to senescence and limited self-renewal and differentiation potential. Over time, primary cells may show morphological and functional changes, and as such, only early passages can be used to have reliable and reproducible results [80].

Moreover, the use of *in vitro* VSMC differentiation models may be hindered and can differ depending on the VSMC origins and their ability to undergo phenotypic transition during cultures [52]. It remains to be questioned to what extent these *in vitro* models recapitulate SMC differentiation and maturation *in vivo*. Therefore, although *in vitro* models are great tools to provide insights into the molecular mechanisms of atherosclerotic cell types that differentiate in a controlled environment, *in vivo* experiments are always required to confirm the observations done *in vitro* [66].

In addition, although VSMC models may elucidate fundamental mechanisms in response to different external stimuli and give rise to

other cell types, such as macrophage-, chondrocyte-, foam-like cells that can be game-changers when considering disease progression and clinical implications, they do not provide any insights on hematopoietic stem cell derived inflammatory cells, which also have important roles in atherogenesis.

Despite these limitations, primary cells are a widely used crucial tool in experimental studies in numerous medical fields. This way, although the diversity of lesion characteristics and cellular biological responses of primary plaque cells, their potential in atherosclerotic research may not have entirely been met.

11. Future outlook: how plaque cells may complement the atherosclerosis research pipeline

The biofabrication of tissues and diseases mimicking human pathophysiology *in vitro* has become a reality. It has gained the scientific community's attention because the establishment of these human-like models might offer significant advances to refine animal studies. To date, the topic has been extensively investigated. It is known that replacing the traditional 2D cultures with the recapitulation of 3D micro-environments can move a step forward in understanding the progressions and the regeneration of several human genetic-based diseases [81].

Plaque cells dissociated from patients who underwent endarterectomies carry a donor-specific genetic and epigenetic signature, that is partially shaped by environmental factors [82], offering the basis to better screen disease markers, design precision treatments and develop novel screening tools. This way, plaque cells could be fundamental to address current clinical needs for novel approaches to comprehend the mechanisms behind the temporal shift towards more stable phenotype features and close an open modelling gap in the field of atherosclerosis research. A future outlook may foresee the establishment of functional models and study their capability to differentiate into the other cell lineages. This way, we might recapitulate atherosclerosis features that have recently emerged and provide a mechanistic understanding of its pathophysiology. The increasing knowledge in cell-specific genetic architecture generated by human plaque cell sequencing will provide a phenotype of plaque vulnerability that reflects its diversity as observed in clinical presentation and heterogeneous vascular pathology. This will also provide a means to validate the translational value of *in vitro* and *in vivo* models by matching the transcriptional profile observed in human lesions. However, although they resemble the human lesion best, plaque-derived cells in mechanistic research will come with inherited limitations that will necessitate validation *in vivo*.

Author contributions

M.F.B., M.M., G.P., and H.M.R. conceptualised and designed the manuscript. M.F.B. generated the figures and drafted the manuscript. M. M., G.P., H.M.R. and L.S.: write-review and editing; M.M., G.P., H.M.R., C.M., M.W. and R.J.G.H. revised it critically for important intellectual content; M.M., G.P. and H.M.R. final approval of the submitted manuscript.

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Authors disclosures

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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