Two-faced Janus: the dual role of macrophages in atherosclerotic calcification

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Abstract
Calcification is an independent predictor of atherosclerosis-related cardiovascular events. Microcalcification is linked to inflamed, unstable lesions, in comparison to the fibrotic stable plaque phenotype generally associated with advanced calcification. This paradox relates to recognition that calcification presents in a wide spectrum of manifestations that differentially impact plaque’s fate. Macrophages, the main inflammatory cells in atherosclerotic plaque, have a multifaceted role in disease progression. They crucially control the mineralization process, from microcalcification to the osteoid metaplasia of bone-like tissue. It is a bilateral interaction that weighs heavily on the overall plaque fate but remains rather unexplored. This review highlights current knowledge about macrophage phenotypic changes in relation to and interaction with the calcifying environment. On the one hand, macrophage-led inflammation kickstarts microcalcification through a multitude of interlinked mechanisms, which in turn stimulates phenotypic changes in vascular cell types to drive microcalcification. Macrophages may also modulate the expression/activity of calcification inhibitors and inducers, or eliminate hydroxyapatite nucleation points. Contrarily, direct exposure of macrophages to an early calcifying milieu impacts macrophage phenotype, with repercussions for plaque progression and/or stability. Macrophages surrounding macrocalcification deposits show a more reparative phenotype, modulating extracellular matrix, and expressing osteoclast genes. This phenotypic shift favours gradual displacement of the pro-inflammatory hubs; the lipid necrotic core, by macrolcification. Parallels to bone metabolism may explain many of these changes to macrophage phenotype, with advanced calcification able to show homeostatic osteoid metaplasia. As the targeted treatment of vascular calcification developing in atherosclerosis is thus far severely lacking, it is crucial to better understand its mechanisms of development.

Keywords: Macrophages • Inflammation • Calcification • Vascular remodelling • Atherosclerosis

1. Introduction
Atherosclerosis is a slowly progressing, chronic inflammatory disease that affects large- and middle-size arteries, 1 featuring the accumulation of fatty and fibrous elements together with immune cells, and structural vascular smooth muscle cells (VSMCs) in the intimal layer of the arterial wall. During disease progression, atherosclerotic plaques develop regions of mineralization, a process which has been traditionally linked to an increased risk for heart disease, atherosclerotic plaque rupture, and stroke. 2–3 Rather than a mere by-product of the development and changing inflammatory environment of the plaque, calcification impacts grievously on disease progression and pathogenesis, particularly through mediating biomechanical destabilization and directly impacting plaque inflammation. Calcification, be it bone related or ectopic, is an active process involving interplay between multiple cell types, 4 with an important role for osteoclast-like macrophages in bone. Osteoclasts, the specialized resorptive cells found in bone, derived from a common myeloid progenitor with macrophages. In plaque, both multinucleated giant cells

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and macrophages are observed to emulate osteoclast traits \cite{5,9} induced through the RANK/RANKL/OPG signalling axis, \cite{10,11} as present in late-stage calcification.

Although micro- and macrocalcification often occur side-by-side during plaque progression, microcalcification is largely observed in earlier-stage lesions, \cite{12} while the latter predominates in late-stage plaque. \cite{13} Microcalcification particles, defined as \(<50 \mu m\) in size, \cite{14} are developed in a four-stage process, involving calcifying extracellular vesicle (cEV) accumulation, aggregation, membrane fusion, and finally, mineralization. \cite{15}

During mineralization, amorphous calcium phosphate transforms into mature crystal-like form hydroxyapatite 'microcalcification' particles, present in spherical and needle-like morphology types, 0.5–15 \(\mu m\) in size. \cite{16} They become larger as lesions progress, as 'speckled calcification' (\(>15 \mu m\) to \(<2 \text{ mm in diameter}\)). \cite{17} Microcalcification particles in the fibrous cap increase the risk of plaque rupture. \cite{18,19} These particles coalesce into larger sheet-like or nodular structures, up to several millimetres in diameter. Such macrocalcification has been linked with healing response and plaque stability. \cite{20,21} However, increased coronary artery calcification (CAC) score is related to atherosclerotic plaque burden, which has also been linked with all-cause mortality and is a broadly adopted predictor of cardiovascular events. \cite{21,22}

Like Janus, the Roman God of duality, macrophages in the atherosclerotic plaque are seen to have both accelerative and decelerative, bilateral relationship with calcification. On the one hand, they may trigger and exacerbate vascular calcification onset, as calcification first develops in inflammatory hotspots throughout the plaque; \cite{23} whereas on the other hand, macrophages may limit calcification by encapsulation, internalization, and resorption of macro and microcalcites. \cite{24,25}

Macrophages co-localize with calcium phosphate crystals in developing atherosclerotic lesions. \cite{26-28} The presence of inflammatory macrophages has even been used as a surrogate marker for early microcalcification. \cite{29} Vice versa, a high score of intimal macrocalcification can help to pinpoint the most inflamed \cite{28} and likely to rupture plaque areas. \cite{30} Multimodal \(^{18}\text{F}-\text{NaF}\) and \(^{18}\text{F}-\text{Fluorodeoxyglucose}\) PET imaging of both measures allows detection of highly metabolically active inflamed areas and microcalcified areas in plaque in one shot. \cite{31} In contrast, areas of macrocalcification have been largely observed to feature fewer inflammatory cells, more reparative macrophages, including osteoclast-like cells, \cite{32} more fibrosis, \cite{33} and presentation of osteoid metaplasia. \cite{34} This was confirmed by transcriptional analysis of human high- vs. low-calcified carotid atherosclerotic plaques, showing repressed inflammation, lipid transport, and chemokine signalling pathways. \cite{35} Hence, a better comprehension of exactly how macrophages engage with calcification throughout disease progression will offer more opportunities for highly necessary, novel therapeutics. In this review, we will outline current literature on macrophage crosstalk with intimal calcification in atherosclerosis, including both direct and indirect interactions, and its impact on disease progression.

2. Macrophage phenotypic plasticity in response to a calcified microenvironment

Macrophages’ remarkable plasticity and functional heterogeneity render them adaptive, according to specific microenvironment stimuli, to different subsets or phenotypes. \cite{34} To understand how macrophages behave in calcified plaques, \textit{in vitro} assessment of macrophage response to individual calcifying stimuli has been performed. In this, it is important to note that the outcome appears to greatly depend on the initial phenotype of the cells being studied, but this is not regularly factored into account. The M1/M2 macrophage model, \cite{35} whilst being now considered a too-broad descriptor of macrophage’s full phenotypic spectrum, has been shown to have opposing effects on extracellular endogenous mechanisms of calcification as further elaborated on below and may respond differently to a calcified/calcifying environment.

 Attempting to mimic macrophage responses to a microcalcified environment, some \textit{in vitro} studies have shown M2-like phenotypic shift in hyperphosphataemia. Macrophages had increased phosphate-handling ability and enhanced arginine hydrolysis, which both may dampen crystal nucleation within the plaque. \cite{36} These phosphate-polarized cells produced higher levels of secreted adenosine triphosphate (ATP) and increased pyrophosphate (PPi) synthesis, inhibiting calcium phosphate deposition. \cite{37} PPi is produced by the enzyme ectonucleotide pyrophosphatase/phosphodiesterase 1 (eNPP), which hydrolyses extracellular ATP to generate Ppi and adenosine monophosphate. Therefore, PPi inhibits the precipitation of calcium phosphate, preventing the formation of hydroxyapatite and favouring its dissolution. Phosphate-polarized cells also showed enrichment in oxidative stress handling genes. \cite{36}

Inversely to the response seen to hyperphosphataemia, incubation of macrophages with calcium phosphate-supplemented medium could induce the release of calcifying matrix extracellular vesicles, and increased interleukin (IL)-6 expression in M1 polarized cells, while M2 polarized cells had reduced induction of Arginase-1 expression upon the same stimulation, \cite{38} both pointing to net M1 skewing. Alone, increased extraacellular Ca\(^{2+}\) could trigger NLR family pyrin domain containing 3 (NLRP3) activation in monocytes and increase IL-1β secretion after lipopolysaccharide stimulation. \cite{39} However, the combined exposure to the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α), plus CaPO\(_4\) stimulated the transformation of macrophages into osteoclast-like cells \textit{in vitro}, in an RANK-L independent manner. \cite{40} Importantly, \textit{in vitro} cell culture models reliant on supplementing additional calcium phosphate to alter normal equilibrium may better reflect a medial calcification environment, as it is observed during kidney dysfunction. \cite{41}

Calcium phosphate crystals can be internalized actively by human monocyte-derived macrophages through phagocytosis. Induce a pro-inflammatory M1 phenotype, and activate the NLRP3 inflammasome complex to release IL-1β, amongst others. \cite{25,42,43} IL-1 molecule release in response to cholesterol crystal phagocytosis and NLRP3 activation drives the recruitment of neutrophils, and early lesion formation. \cite{44} This pro-inflammatory response to calcium phosphate particles could be reversed/dampened by co-incubation with Fetuin A or Gla-Rich Protein (GRP), both natural calcification inhibitors. \cite{45,46} Stimulation of THP-1 derived macrophages with hydroxyapatite nanoparticles, the naturally occurring mineral form of calcium phosphate, alone could also induce the expression of GRP and Matrix Gla Protein (MGP), \cite{46} a potent vitamin K-dependent protein inhibitor of vascular calcification produced by VSMCs and chondrocytes. The mechanism behind the hydroxyapatite induced pro-inflammatory response is not fully understood, nor is it known if macrophages can directly sense and respond to hydroxyapatite particles, or if pro-inflammatory responses are instead a by-product of frustrated phagocytosis due to the inability to effectively breakdown ingested hydroxyapatite particles. However, the physicochemical properties of hydroxyapatite particles are highly variable, with sizes ranging from 0.1 to 100 \(\mu m\) and needle-shaped/spherical morphology and smooth/rough surface topography, all factors that can modulate the degree of inflammatory response. \cite{47}
As the atherosclerotic plaque milieu is so complex and difficult to model in culture, it is still largely unclear how direct macrophage-calcification stimulation seen in vitro is taking place in the inflammatory state of the plaque itself. Particularly, as plaque macrophages are likely to be highly inflammatory, this may skew responses to calcification stimuli in vivo. Deep phenotyping studies of plaque macrophages in proximity to early and advanced plaque calcification would help mapping this causality dilemma. Several ground-breaking single-cell sequencing studies in atherosclerotic plaque have helped to highlight the macrophage spectrum in this disease, but as of yet, studies comparing cellular heterogeneity of calcified vs. non-calcified plaques are lacking. A second outstanding question is to what extent the calcification-related macrophage phenotype in vivo is dependent on the physicochemical features of the calcium phosphate particle (e.g. charge, size, composition). The distinct response of macrophages to hydroxyapatite particles and inorganic minerals suggests that pathological atherosclerotic calcification is not merely a passive consequence of chronic inflammatory disease but may lead to a positive feedback loop as a result of the active interplay between calcification and inflammation during the disease progression.

### 3. Macrophage contribution to intimal calcification

The critical step in the formation of an atherosclerotic plaque is the infiltration of macrophages in the subendothelial space. In this sense, macrophage infiltration is a sine qua non for vascular calcification. Direct causal involvement of macrophages in vascular calcification is conceivable. This section will review the diverse mechanisms in microcalcification initiation (i) cEV release, (ii) apoptotic body nucleation, (iii) endogenous inhibitor dysregulation, and (iv) osteogenic transdifferentiation, involving both direct and indirect macrophage engagement. All these mechanisms, occurring simultaneously in actively calcifying plaques, have been shown to be initiated and driven by macrophage interaction with the microenvironment and contained cells.

### 3.1 Macrophage extracellular vesicles

Macrophages can directly contribute to atherosclerotic plaque calcification through the release of cEVs. These macrophage extracellular vesicles are characterized by markers CD9, CD63, CD81, TSG101, and CD68, and are loaded with S100A9 and Annexin-5 proteins. As well as possessing high calcification potential, accumulation and aggregation of cEVs initiate nucleation of hydroxyapatite particles, promoting the mineralization process within plaques.

Parallels between extracellular vesicles released in the pro-inflammatory atherosclerotic plaque milieu, and matrix vesicles in bone formation can be drawn, since they share many commonalities: high mineralization potential, annexin expression, and acidic lipids such as phosphatidylinerine. However, extracellular vesicles are highly variable: recent high-throughput technologies highlighted phenotypic differences, consistent with their originating cell type; including immunopositivity for cell markers, protein, and RNA content. Furthermore, differences between vesicles from the same origin cells can be seen in an altered microenvironment. Comparative proteomic profiling analysis of extracellular vesicles released from primary mouse aortic smooth muscle cells upon different pro-osteoergic conditions demonstrated significant differences in protein composition, such as endocytosis-associated proteins reduced vesicles released from phosphate-stimulated cells. In agreement, proteomic analysis of cEVs from human VSMCs and valvar interstitial cells cultured in osteogenic media revealed an enrichment of annexins including ANXA1 and its calcium-dependent binding partner, S100 calcium-binding protein A11 (S100A11) that could tether extracellular vesicles. Interestingly, ANXA1 knockdown attenuated extracellular vesicle microcalcification and therefore human SMCs and VICs calcification. More research to that direction is needed in the developing multi-omics era, for further characterization of these vesicles, their loading molecules as well as their emerging role in and beyond the vascular calcification pathology. Extracellular vesicles’ ability to contain proteins, lipids, nucleic acids, and other signalling molecules, as well as their capability to circulate and transmit specific molecular information to other cell types influencing their function, is of great interest for potential and promising diagnostic and prognostic biomarker evaluation.

### 3.2 Macrophage lipid handling and cell death driving microcalcification

Lipid infiltration and modification in early atherosclerosis trigger an inflammatory response, monocyte recruitment, and macrophage differentiation, as well as foam cell generation. The lipid-rich necrotic core is a key site of early calcification, with high hydroxyapatite nucleation potential. Lipids may enhance the deposition of calcium crystals serving as an extra scaffold for calcification, by triggering osteogenic differentiation of VSMCs. Similarly, by affecting foam cell effrocytosis and stimulating inflammation, the lipid core increases the calcification propensity of surrounding cells and extracellular matrix. Apoptosis of VSMCs in culture was shown to be a key regulator of the initiation of vascular calcification with apoptotic bodies acting as nucleation sites for calcification. Parallels have also been drawn between apoptotic bodies and matrix microvesicles that induce calcification in bone. Failure of macrophages to clear apoptotic bodies, as observed in advanced atherosclerosis, allows calcium crystal growth to progress, and may also be a significant inflammatory spur leading to the release of cytokines such as TNF-α, also a potent inducer of osteogenic gene expression in VSMCs.

Uptake of ox-LDL triggers apoptosis in macrophages and VSMCs. In early lesions, macrophage apoptosis can reduce overall plaque size and lesion inflammation; however, in more advanced lesions, with compromised effrocytosis, apoptosis will transition to secondary necrosis, which is detrimental to plaque development and increases calcification. Macrophages are highly effective in effrocytosis and have a high capacity for continued clearance of apoptotic cells in the plaque; upon uptake of apoptotic cells, they release anti-inflammatory cytokines IL-10 and transforming growth factor-beta (TGF-β). Thus, targeting effrocytosis in macrophages may have promise in reducing vascular calcification, as well as overall plaque progression. Effrocytosis-targeting strategies such as blockage of CD47 ‘don’t eat me’ signalling dramatically reduced atherosclerosis in ApoE−/− through the improvement of debris clearance by macrophages. CD47 inhibition is already considered for cancer therapy, making clinical translation to atherosclerosis potentially easier, and recently CD47-interference nanotherapy was shown to have a favourable outcome in atherosclerotic ApoE−/− mice.

Beyond influencing cell death, macrophage lipid handling also impacts the inflammatory nature of the plaque. OxLDL has a chemotactic effect on monocytes, and is a TLR4 agonist; stimulation of both macrophages and VSMCs with oxLDL increases their expression of TLR4. It activates NF-kB signalling, producing a pro-inflammatory phenotype in macrophages, and increasing osteoblastic differentiation and calcification in VSMCs, as well as increasing foam cell formation in both. Moreover, oxLDL uptake and subsequent lysosomal cholesterol crystal generation
are inflammasome activating factors in macrophages, allowing maturation and secretion of IL-1β and IL-18. IL-1β production is a key factor in perpetuated atherosclerotic calcification, as mentioned, as it is also induced in response to hydroxyapatite stimulation. Suggestive of positive feedback during microcalcification establishment. Indeed therapeutic inhibition of IL-1β in Ldr⁻/⁻ mice using a monoclonal antibody showed greatly diminished calcification burden within plaques. Inflammasome associated IL-1β production is kept in check by Rho GTPases RAC1 and 2, the expression of which was seen to be down-regulated with plaque progression, potentially accelerating atherosclerotic calcification. A neutralizing IL-1β antibody increased macrophage presence within the fibrous cap and promoted M2 macrophage polarization; whereas IL-1 signalling in VSMCs is essential for their migration and collagen secretion into the fibrous cap in advanced atherosclerotic plaques. Of interest, no difference in the lesion calcification was observed, compared to IL-1 signalling and inflammasome modulation in early plaques.

3.3 Macrophage impact on endogenous calcification inhibitors

Several endogenous mechanisms exist throughout the body to prevent ectopic calcification. Macrophage-driven inflammation causes several vascular cell types—including smooth muscle cells, endothelial cells and pericytes—to undergo phenotypic changes resulting in altered expression of calcification modulating factors. Macrophage-produced inflammatory drivers initiate simultaneous loss of VSMCs-expressed calcification inhibitors, such as MGP, osteopontin (OPN) and PPI, and gain of inducers such as osteoprotegerin (OPG). Stimulation of VSMCs with macrophage conditioned medium simultaneously increased bone morphogenic protein-2 (BMP-2) and inhibited MGP expression. As in macrophage response to a calcified microenvironment, polarized macrophages can exert opposing pro- and anti-calcifying activity via endogenous inhibitors. M1 macrophages have higher expression and activity of the enzyme ectonucleoside triphosphate diphosphohydrolase 1 (eNTPD1, a.k.a. CD39), which hydrolyses ATP to AMP and Pi. Thus, macrophages may promote calcification by not only producing Pi, a calcification substrate, but also lowering ATP availability for eNPP1 to produce Pi, a potent calcification inhibitor. In the aortic wall, more than 90% of extracellular ATP is degraded to Pi, more than the rate of Pi synthesis and insufficient for the inhibition of hydroxyapatite formation. Co-culture of M1 macrophages, or M1-derived TNF-α enhances the TNAP activity of VSMCs; augmenting calcification in vitro. Contrastingly, co-culture of VSMCs with M2 macrophages stimulated the synthesis of extracellular ATP and PPI and enhanced the activity of eNPP1 in VSMCs.

Macrophages secrete large amounts of the inhibitors OPN and Fetuin-A in calcified plaques, which have been suggested to enhance microcalcification opsonization for the purposes of phagocytosis. Although OPN can have pro-atherogenic effects, it has been shown to be anti-calcifying in atherosclerosis, and specifically in macrophages can induce carbonic anhydrase II expression, attenuate inflammatory activation, and regulate osteoclast formation. Exogenous OPN exerts a significant role as an inflammatory mediator of vascular injury; it is induced in the differentiation of peripheral monocytes into an M2-like phenotype.

M2 macrophages release anti-inflammatory mediators and phagocytize necrotic fragments or apoptotic cells to prevent the formation of calcified nucleation sites. Of interest, macrophage-derived OPN binding to calcium phosphate or hydroxyapatite particles functions as an opsonin and facilitates their ingestion through the phagocytosis process. In accordance, fetuin/x2-HS glycoprotein, another vascular calcification inhibitor, enhances phagocytosis of apoptotic cells and macrophocytes by macrophages, reducing the accumulation of pro-calcifying apoptotic vesicles.

3.4 Macrophage impact on smooth muscle cell osteogenic transdifferentiation

Macrophage interaction with VSMCs heavily contributes to plaque calcification and is perhaps their most impactful indirectly calcifying activity. Macrophages release a vast variety of pro-osteogenic cytokines, that stimulate smooth muscle cells to transdifferentiate into an osteogenic phenotype. VSMC-osteogenic/chondrogenic phenotype is accompanied by genetic lineage reprogramming involving up-regulation of chondrogenic markers (RUNX2, SOX9, ALP, osteocalcin, osterix, type II, and X collagen), down-regulation of VSMC markers (SM22a, SMa actin, etc.), and secretion of calcifying microvesicles. It was shown that co-culture of macrophages with VSMCs profoundly affected the ability of the latter to calcify. Inflammatory macrophages especially induced VSMC chondrogenic switch, as well as active calcification.

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4. Observed macrophage phenotype in macrocalcified plaque areas

In advanced calcified atherosclerotic plaques, macrophages surrounding areas of macrocalcification generally have acquired markedly less inflamed, more reparative phenotypes. The transition from inflamed micro- to stable macrocalcification is highly understudied. Macrocalcified plaque environments have not yet been successfully modelled in culture. Also, little research has been documented on how microcalcification in the arteries can transition into nodular or sheet-like structures, and whether this is influenced by the inflammatory state of the plaque, or if the structures themselves are influential factors. Certainly, differing
Macrophage-osteoclast phenotypic switch

As mentioned above, lipids are important factors in the mineralization process, histologically, early calcification can be detected in atherosclerotic lesions. The lipid-rich necrotic core is the highest risk area to precursor micro- and macrocalcification deposits, and undergo long-term transformation into dense calcium phosphate. This process is enhanced from studies showing that lesions with a higher load of calcification contain less lipid core. High serum LDL-cholesterol is highly correlated to vascular calcification, and both serum LDL and total cholesterol have been independently associated with CAC incidence. However, it remains elusive whether lipids are causative in atherosclerotic calcification or just represent an epiphenomenon, although they have been linked to calcification-associated phenotypes in macrophages.

Lipid handling equivalent processes can be drawn between macrophages and observed osteoclast-like traits. Although it is vastly under-investigated, this parallel may help to better understand the origin and role of osteoclast-like cells in the plaque. More specifically, foam cells, expressing the lysosomal protease CATK, have been shown to contribute to plaque remodeling. Much like activated macrophages and osteoclast-like cells, plaque multinucleated giant cells express markers such as TRAP and CATK along with their distinctive osteoclast morphological overlap. In culture, lipids and modified lipids have been shown to promote osteoclastogenesis through VSMC RANK-L up-regulation, direct macrophage osteoclast gene up-regulation, and promoting osteoclast survival. Similarly, lipid exposure in murine bone marrow-derived macrophages could trigger multinucleated giant cell formation in culture, a phenotype that could be greatly exacerbated by megalin Mcl-1 depletion in Ldlr<sup>−/−</sup> mice where a lipid accumulating, giant cell forming and apoptosis prone phenotype in macrophages was demonstrated. Furthermore, hyperlipidemia in Ldlr<sup>−/−</sup> mice, which is associated with increased plasma oxLDL levels, was shown to increase osteocalcification potential in osteoclasts co-expr. In culture, lipids and modified lipids have been shown to promote osteoclastogenesis through VSMC RANK-L up-regulation, direct macrophage osteoclast gene up-regulation, and promoting osteoclast survival. Similarly, lipid exposure in murine bone marrow-derived macrophages could trigger multinucleated giant cell formation in culture, a phenotype that could be greatly exacerbated by megalin Mcl-1 depletion in Ldlr<sup>−/−</sup> mice where a lipid accumulating, giant cell forming and apoptosis prone phenotype in macrophages was demonstrated. Furthermore, hyperlipidemia in Ldlr<sup>−/−</sup> mice, which is associated with increased plasma oxLDL levels, was shown to increase osteocalcification potential in osteoclasts co-expr. In culture, lipids and modified lipids have been shown to promote osteoclastogenesis through VSMC RANK-L up-regulation, direct macrophage osteoclast gene up-regulation, and promoting osteoclast survival. Similarly, lipid exposure in murine bone marrow-derived macrophages could trigger multinucleated giant cell formation in culture, a phenotype that could be greatly exacerbated by megalin Mcl-1 depletion in Ldlr<sup>−/−</sup> mice where a lipid accumulating, giant cell forming and apoptosis prone phenotype in macrophages was demonstrated. Furthermore, hyperlipidemia in Ldlr<sup>−/−</sup> mice, which is associated with increased plasma oxLDL levels, was shown to increase osteocalcification potential in osteoclasts co-expr. In culture, lipids and modified lipids have been shown to promote osteoclastogenesis through VSMC RANK-L up-regulation, direct macrophage osteoclast gene up-regulation, and promoting osteoclast survival. Similarly, lipid exposure in murine bone marrow-derived macrophages could trigger multinucleated giant cell formation in culture, a phenotype that could be greatly exacerbated by megalin Mcl-1 depletion in Ldlr<sup>−/−</sup> mice where a lipid accumulating, giant cell forming and apoptosis prone phenotype in macrophages was demonstrated. Furthermore, hyperlipidemia in Ldlr<sup>−/−</sup> mice, which is associated with increased plasma oxLDL levels, was shown to increase osteocalcification potential in osteoclasts co-expr. In culture, lipids and modified lipids have been shown to promote osteoclastogenesis through VSMC RANK-L up-regulation, direct macrophage osteoclast gene up-regulation, and promoting osteoclast survival. Similarly, lipid exposure in murine bone marrow-derived macrophages could trigger multinucleated giant cell formation in culture, a phenotype that could be greatly exacerbated by megalin Mcl-1 depletion in Ldlr<sup>−/−</sup> mice where a lipid accumulating, giant cell forming and apoptosis prone phenotype in macrophages was demonstrated. Furthermore, hyperlipidemia in Ldlr<sup>−/−</sup> mice, which is associated with increased plasma oxLDL levels, was shown to increase osteocalcification potential in osteoclasts co-expr. In culture, lipids and modified lipids have been shown to promote osteoclastogenesis through VSMC RANK-L up-regulation, direct macrophage osteoclast gene up-regulation, and promoting osteoclast survival. Similarly, lipid exposure in murine bone marrow-derived macrophages could trigger multinucleated giant cell formation in culture, a phenotype that could be greatly exacerbated by megalin Mcl-1 depletion in Ldlr<sup>−/−</sup> mice where a lipid accumulating, giant cell forming and apoptosis prone phenotype in macrophages was demonstrated. Further study with a VSMC-specific runx2 deletion model showed reduced calcification, but no change in lipid metabolism, lesion size, or macrophage...
This highlights a mechanism of vascular calcification possibly separable from the inflammatory and lipid-driven mechanisms. It is possible that the osteoid metaplasia, driven by the pathways here highlighted, acts as a compensatory mechanism to control calcification progression, and moreover, as a mechanism of inflammation control and wound healing. The observations linking lipid handling to osteoclastogenesis may help to bridge the gap between highly inflammatory microcalcification-associated macrophages, and the osteoclastogenic switch capable of taking place. Understanding this association, and how it affects calcification development and progression, may be critical in future efforts to clinically modulate plaque inflammation and calcification.

6. Conclusions and future perspectives

Whilst calcification is an independent predictor of clinical cardiovascular events, the overall risk to plaque rupture or stability critically depends on the actual calcification phenotype. Bone-like vascular calcification has been shown to be a typical feature of more stable plaques and asymptomatic disease. However, even fibrocalcific plaques have an associated risk of adverse events, such as rupture, occlusion, or thrombosis through calcified nodules. Plaque regression studies and meta-analysis showed that a common feature of a regressing plaque is an increase in dense calcium volume and CAC score, which is inversely correlated to event risk. Treatment and rupture prevention, largely relies on aggressive lipid-lowering statin therapy, shown to stabilize plaques but also increase calcification. However, combination therapy with protein convertase subtilisin/kexin type 9 (PCSK9) could inhibit statin-induced calcification progression in 16 subjects, compared to statin monotherapy (n = 15) in a paired longitudinal study.

Drugs to specifically treat or reverse atherosclerotic calcification are still currently missing. However, treating atherosclerotic calcification effectively at later stages will likely also not rely on purely targeting calcification, but through a better understanding of the greatest risks at each stage of the disease, so that treatment can be more targeted. Reducing...
inflammation and improving beneficial macrophage functions could represent a more powerful tailored strategy to prevent and reduce macrophage calcification, particularly in atherosclerosis patients with a high degree of vascular calcification, unresponsive to regular lipid-lowering therapy.

A research explosion has occurred based on the role of macrophages in the process of vascular calcification and is summarized in Figure 1. The phenotypic plasticity and functional heterogeneity of macrophages according to the microenvironment variables led to the understanding of their pleiotropic effects in the atherosclerotic plaque calcification. Inflammatory macrophage activity accelerates plaque calcification profoundly, through many mechanisms that also couple to plaque growth and risk of rupture. Meanwhile in-kind, a calcified microenvironment reinforces these processes and produces calcification-associated macrophage phenotypes linked to macrocalcification. As such, net M2 skewing in atherosclerosis through clinical intervention may reduce not only plaque progression but also calcification growth, both in early and late stages. Reducing microcalcification generation, and enhancing fibrotic activity associated with established stable calcification; specifically through inflammation reduction, direct calcification inhibition activity and regulation of plaque cell death. The precise modulation mechanisms that allow for in vivo differentiation of macrophages into a phenotype in a manner that is more protective for the patient is still an unmet need and an urgent problem to be solved. Therefore, deep phenotyping of macrophages subsets with high-resolution omic methodologies like single-cell technology in calcified plaques is still an open research area and represents a clear benefit for better disease understanding and assessment of clinical risk. However, the relative contribution of macrophages to late-stage calcification and disease state is also yet to be comprehensively elucidated. Comparative assessment of cellular phenotypes presenting at all stages of vascular calcification can help to fill in many gaps in understanding of how this contributes to disease progression, risk of plaque rupture (i.e. clinical events) and how current therapeutic strategies may be improved. Conclusively, additional investigation of the potential molecular mechanism and function of how macrophages modulate the progression and regression of vascular calcification is expected not only to bridge the gap between in vitro and in vivo observations but also to uncover a new notion for the prevention and treatment of vascular calcification.

Authors’ contributions
O.J.W. and N.T.S. wrote and revised the manuscript, M.M.P.C.D. and E.A.L.B. revised the manuscript.

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