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SYMPOSIUM REVIEW

Zooming in on the genesis of atherosclerotic plaque microcalcifications

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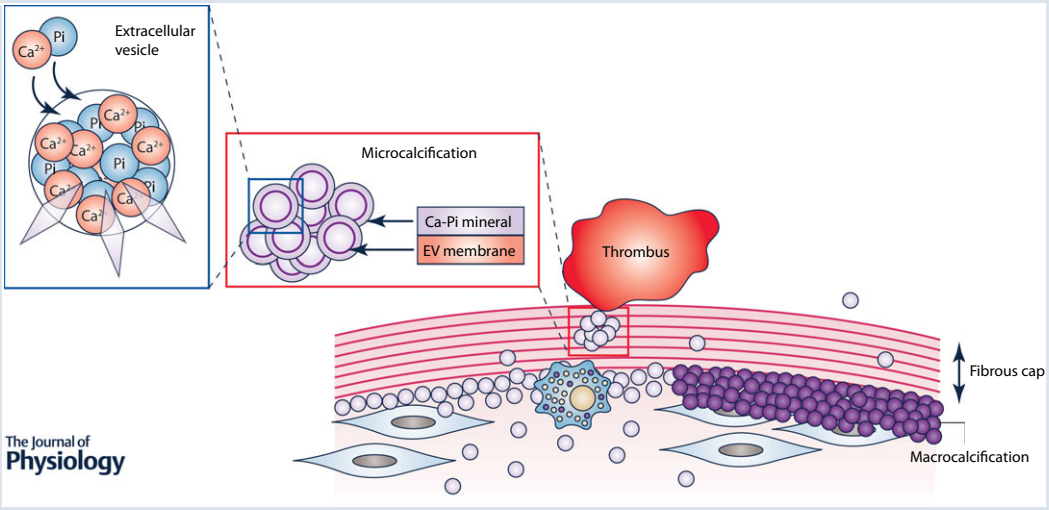

Abstract Epidemiological evidence conclusively demonstrates that calcium burden is a significant predictor of cardiovascular morbidity and mortality; however, the underlying mechanisms remain largely unknown. These observations have challenged the previously held notion that calcification serves to stabilize the atherosclerotic plaque. Recent studies have shown that microcalcifications that form within the fibrous cap of the plaques lead to the accrual of plaque-destabilizing

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mechanical stress. Given the association between calcification morphology and cardiovascular outcomes, it is important to understand the mechanisms leading to calcific mineral deposition and growth from the earliest stages. We highlight the open questions in the field of cardiovascular calcification and include a review of the proposed mechanisms involved in extracellular vesicle-mediated mineral deposition.

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Abstract figure legend Microcalcifications in atherosclerotic fibrous caps lead to plaque rupture and thrombus formation. Extracellular vesicles aggregate to build microcalcifications with each vesicle serving as a nidus for calcium phosphate mineralization.

Abbreviations ACP, amorphous calcium phosphate; ANK, ankylosis protein; CT, computed tomography; ECM, extracellular matrix; EV, extracellular vesicle; FetA, fetuin A; HU, Hounsfield unit; MGP, matrix Gla protein; MV, matrix vesicle; NC, nucleational core; NPP, nucleoside pyrophosphohydrolase; PChol, phosphocholine; PEA, phosphoethanolamine; PET, positron emission tomography; PHOSPHO1, phosphatase orphan 1; P_i, phosphate; PP_i, pyrophosphate; TNAP, tissue non-specific alkaline phosphatase; VSMC, vascular smooth muscle cell.

Vascular calcification predicts cardiovascular morbidity and mortality

Rupture of vulnerable atherosclerotic plaques is the leading cause of heart attacks and strokes (Lloyd-Jones *et al.* 2010). The root cause of this acute rupture event is a failure of the collagen-rich fibrous cap that lies at the boundary of the plaque and the vessel lumen. Rupture of the fibrous cap exposes the underlying plaque contents to blood flow, leading to platelet-mediated thrombin generation and fibrin deposition. The resulting thrombus occludes the vessel, reducing blood flow and oxygen supply to downstream tissues. Despite the once prevailing clinical view that calcification of the vessel wall is inconsequential to plaque rupture, and possibly plaque stabilizing, recent data indicate that calcium score is a better predictor of acute cardiovascular events than lipid score (Vliegenthart *et al.* 2005; Martin *et al.* 2014). Inclusion of calcium score in risk assessments significantly improves the prediction of coronary heart disease and cardiovascular events in at-risk patients (Elkeles *et al.* 2008; Gepner *et al.* 2015; Matsushita *et al.* 2015). Traditionally, computed tomography (CT) identifies arterial calcium as high attenuating regions ($>$ 130 Hounsfield units (HU)) within the relatively low attenuating vascular tissue (Fig. 1) (Rumberger *et al.* 1999; Kelly-Arnold *et al.* 2013; Han *et al.* 2015).

In a study of 1795 asymptomatic participants, compared to participants with an Agatston score lower than 100, the relative risk of coronary heart disease increased to 3.1 for scores of 101–400 HU, to 4.6 for scores of 401–1000 HU, and to 8.3 for scores higher than 1000 HU (Vliegenthart *et al.* 2005). Further, a retrospective study of 23,057 patients found a significant relationship between the number of coronary arteries (right, left main, left anterior and left circumflex) with calcification in each patient and all-cause mortality within 7 years of the initial measurement of calcium burden (Tota-Maharaj *et al.* 2015). The compiled data from these studies suggest a strong link between cardiovascular calcification and morbidity/mortality that has challenged previously held notions that calcification stabilizes atherosclerotic plaques. Indeed, biomechanical models predict that large calcifications that form within the lipid pool/necrotic core of atherosclerotic plaques increase plaque stability by reducing the deformation of the fibrous cap during systolic pressure (Imoto *et al.* 2005; Wong *et al.* 2012; Holzapfel *et al.* 2014; Ruiz *et al.* 2015) (Fig. 2). Studies into this apparent contradiction have revealed that calcification

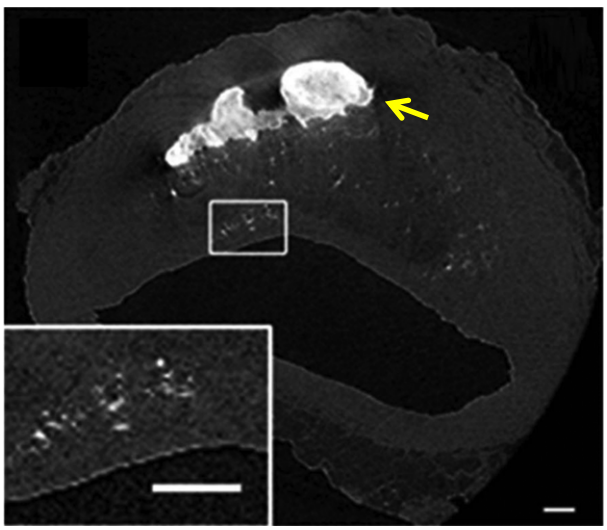

Figure 1. High resolution micro-computed tomography (CT) of explanted human coronary artery
The yellow arrow indicates a large calcification, and the box highlights spotty microcalcifications. (Adapted from Kelly-Arnold *et al.* 2013.)

morphology and location within the plaque determine the impact of the calcification on plaque stability.

Microcalcification and rupture of the atherosclerotic fibrous cap

The first indication that calcification morphology influences plaque stability came from the observation that 'spotty' calcification identified by CT (Ferencik *et al.* 2012) and ultrasound (Ehara *et al.* 2004) correlates with cardiovascular events. Further, prospective clinical data from the Multi-Ethnic Study of Atherosclerosis trial found calcium density to be inversely related to the incidence of cardiovascular events (Criqui *et al.* 2014). The calcium density score was determined by dividing the Agatston score by the total area score in each patient. Including the density score in the prediction models significantly improved risk prediction for both coronary heart disease and cardiovascular disease events. These data support the predictions that larger, denser calcifications may indeed stabilize atherosclerotic plaques (Imoto *et al.* 2005; Lin *et al.* 2006; Wong *et al.* 2012; Holzapfel *et al.* 2014), whereas smaller, disperse calcifications seem to contribute to plaque destabilization (Ehara *et al.* 2004). Thus the morphology of the calcification directly determines the associated cardiovascular risk.

Ultrasound and CT are currently able to image only calcifications larger than 30 μm, whereas clinically relevant microcalcifications that lead to plaque rupture are largely 5–15 μm and visible either in histology or high resolution microCT (Kelly-Arnold *et al.* 2013; Maldonado *et al.* 2013). However, it is possible that a cloud of numerous closely spaced, smaller microcalcifications could appear as a single larger microcalcification in CT. This same observation was made when what was thought to be a single microcalcification at 6.7 μm resolution microCT was shown to be a collection of numerous smaller microcalcifications at 2.1 resolution microCT (Kelly-Arnold *et al.* 2013). Future work is needed to connect the observation of 'spotty' calcification to the presence of clinically unidentifiable microcalcifications that may provide the link between calcification and biomechanical failure of the atherosclerotic plaque.

Vengrenyuk *et al.* (2006) provided the first explanation of the biomechanical mechanisms through which sub-cellular 'microcalcifications' within the fibrous cap contribute to the rupture of atherosclerotic plaques. *In silico* modelling of the mechanical stress distribution within the fibrous cap by finite element analysis shows increased stress surrounding microcalcifications (Fig. 3). The stiff microcalcifications present impurities within the relatively soft vascular tissues (Vengrenyuk *et al.* 2006). Modelling the stresses under different scenarios indicates that calcification size, morphology, spacing and collagen content combine to determine the likelihood of plaque rupture (Kelly-Arnold *et al.* 2013; Maldonado *et al.* 2013). The largest increase in tissue stress occurs when two closely spaced microcalcifications aligned along the tensile axis of the fibrous cap are pulled apart. The tissue in the intervening gap between the particles can undergo an amplification of stress that is more than fivefold depending on their spacing (Kelly-Arnold *et al.* 2013; Maldonado *et al.* 2013). Large calcifications beneath the cap are stabilizing because they attach to the boundaries of the lipid pool/necrotic core and support the cap from below (Maldonado *et al.* 2015). Therefore, more work is needed to determine how different calcification morphologies arise and the factors that determine calcification location – especially regarding the development of fibrous cap microcalcifiations.

Recently, nano-analytical electron microscopy techniques revealed structural detail of calcific mineral within cardiovascular tissues that included large regions of calcification and small microcalcifications (Bertazzo

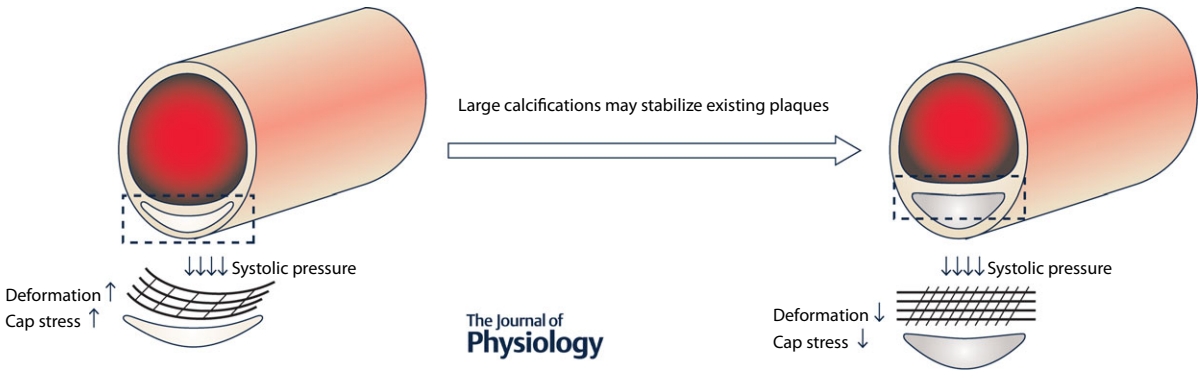

Figure 2. Schematic illustration of the mechanism of atherosclerotic plaque stabilization by a large calcification
The lipid pool/necrotic core of a non-calcified plaque is deformable, which allows for high tissue strain in the fibrous cap during systole. Large, dense calcifications help counteract the resulting stress by limiting the degree of fibrous cap deformation that occurs under systolic pressure. (From Ruiz *et al.* 2015.)

et al. 2013). The formation of large calcifications and microcalcifications may involve cell-derived extracellular vesicles (EVs) that serve as nucleating foci for mineralization (Anderson, 2003; Kapustin *et al.* 2011; New *et al.* 2013). Work from our laboratory showed that collagen within the atherosclerotic plaques acts as a scaffold, directing the aggregation of calcifying EVs and thus influencing calcification morphology (Hutcheson *et al.* 2016). Our laboratory used structured illumination microscopy to directly visualize the aggregation of individual EVs, which appeared to nucleate calcium phosphate mineral in a spherical morphology (Hutcheson *et al.* 2016), and both nanoparticle tracking analyses and spectroscopic analyses revealed mineral formation and maturation concomitant with EV aggregation. Further, optical and electron micrographs of calcific human arterial plaques showed calcifications composed of aggregated EVs. It remains unclear whether calcification builds solely by aggregation and fusion of EVs or if it is possible for mineral to grow directly from a single EV under certain conditions. MicroCT and histopathological analyses of excised human plaques revealed large macrocalcifications in areas corresponding to the lipid pool/necrotic core of the plaque (Kelly-Arnold *et al.* 2013; Maldonado *et al.* 2015). Detailed histopathological analyses of atherosclerotic plaques showed that lipid pools often contain microcalcifications (Otsuka *et al.* 2015). Lipid pools of atherosclerotic plaques often transition into necrotic cores over time, though many factors regulating this transition remain poorly understood (Sakakura *et al.* 2013). The necrotic core does contain both microcalcifications and larger calcifications. Further, small microcalcifications in regions of calcification development preferentially form and align at the borders of the lipid pool/necrotic core (Maldonado *et al.* 2015). Oxidized forms of cholesterol have shown the propensity to accelerate calcification by osteogenic reprogramming of vascular smooth muscle cells (VSMCs) and interactions with calcifying EVs (Hsu, 2003). Our recent work also showed the formation of microcalcifications in spaces between collagen fibres and in regions of collagen fibre degradation within the fibrous cap of atherosclerotic plaques (Hutcheson *et al.* 2016). The microcalcifications that form within the fibrous cap preferentially form near the lipid pool/necrotic core (Maldonado *et al.* 2015). The observation of microcalcifications in the fibrous cap and lipid pool/necrotic core may suggest a role for both VSMCs (Kapustin *et al.* 2011; Hutcheson *et al.* 2014) and macrophages (New *et al.* 2013) in mineral deposition.

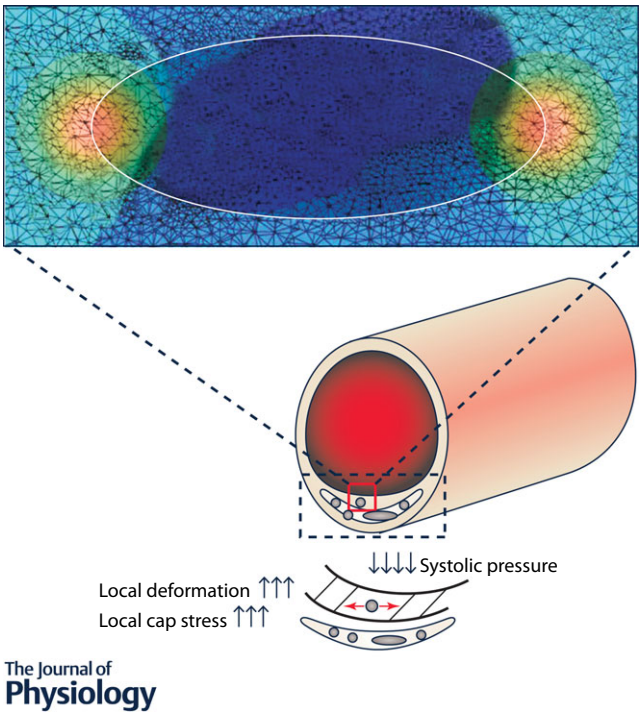

Figure 3. Microcalcifications within the fibrous cap
Microcalcifications serve as foci for high levels of local stress within the fibrous cap. Finite element analysis of the local stress levels surrounding microcalcifications within a fibrous cap. The gradient from blue to red indicates a transition from low to high mechanical stress. (Adapted from Vengrenyuk *et al*. 2006; Ruiz *et al*. 2015.)

Calcification progresses over time in newly formed lesions as microcalcifications appear first in the lipid pool. As the plaque continues to develop the late necrotic core can contain large macrocalcifications with microcalcifications at the periphery. Therefore, within a single patient, as a plaque develops along the lumen, multiple calcification morphologies can be observed as calcifying vesicles diffuse slowly through the lesion to form microcalcifications that build into macrocalcifications (Maldonado *et al.* 2015). As predicted, lipid pool/necrotic core calcification that occurs beneath a collagen-rich fibrous cap may serve to stabilize the plaque; however, collagen breakdown at localized regions in vulnerable fibrous caps due to the presence of inflammation and cell migration may lead to the formation of stress-inducing microcalcifications that cause plaque rupture (Hutcheson *et al.* 2016). More work is needed to better understand the factors driving EV aggregation and association with extracellular matrix and other plaque components to build calcification. Though these recent studies have provided some explanation of the mechanisms by which EVs may contribute to calcification genesis and growth, the specific processes leading to calcifying EV generation and mineral nucleation remain largely unclear, and traditional clinical imaging modalities cannot readily detect early calcific remodelling to identify at-risk patients.

Recent advances using positron emission tomography/CT (PET/CT) with ^{18}F-labelled sodium fluoride (Na^{18}F), an established PET tracer for bone formation and remodelling, may provide new strategies for identifying

regions of microcalcification and plaque vulnerability (Chen & Dilsizian, 2013). Coronary uptake of $Na^{18}F$ was found overlaying, adjacent to and distal from regions of CT identified calcifications (Dweck *et al.* 2012). Additionally, large areas of calcification with no $Na^{18}F$ uptake were observed. This suggests that, as with bone, $Na^{18}F$ uptake in the vasculature is a marker of ongoing calcific remodelling (Dweck *et al.* 2012). Large, stable calcifications do not exhibit $Na^{18}F$ uptake, whereas active regions of mineralization accumulate $Na^{18}F$. Future studies are needed to identify cellular and extracellular mechanisms associated with $Na^{18}F$ uptake. The $Na^{18}F$ regions far away from the CT identified calcific regions may represent processes that lead to the development of clinically relevant microcalcifications that cannot be detected by traditional imaging modalities (Dweck *et al.* 2012). In support of this hypothesis, a prospective clinical trial showed high $Na^{18}F$ accumulation in the culprit coronary plaques in cases of myocardial infarction and in ruptured carotid artery plaques (Joshi *et al.* 2014). Histological evaluation of these plaques revealed active calcification processes and regions of microcalcifications, indicating that these processes associate with $Na^{18}F$ uptake.

Cell-mediated processes actively drive mineral nucleation

It is generally believed that many aspects of vascular calcification mirror that of bone formation. Namely, both processes appear to utilize cell-derived, calcifying vesicles to nucleate and transport mineral to a collagen-rich matrix. While a competing theory posits that bone mineralization is an acellular process directed by specific zones of the collagen fibril (Glimcher, 1984; Dey *et al.* 2010; Nudelman *et al.* 2010), there exists a body of evidence demonstrating the requisite role of cell activity. This hypothesis is exemplified by studies in which the inhibition of cell metabolism or the disruption of cytoskeletal regulation led to a decrease in mineral deposition measured *in vitro* (Stanford *et al.* 1995; Drabek *et al.* 2011). These cellular processes culminate in the generation of vesicles, loaded with enzymatic machinery that concentrates calcium and phosphate ions within the lumen for mineral nucleation.

Similarly, calcifying vesicles have been visualized in and isolated from atherosclerotic vessels (Kim, 1976; Tanimura *et al.* 1983; Hsu & Camacho, 1999). Inflammation within the atherosclerotic environment may trigger an osteochondrogenic transformation of VSMCs (Tintut *et al.* 2000; Aikawa *et al.* 2007). Both osteogenic VSMCs (Tanimura *et al.* 1986; Kapustin *et al.* 2011) and macrophages (New *et al.* 2013) have been implicated in producing calcifying vesicles. The degree of mechanistic overlap between bone and vascular mineralization is unclear. However, literature from the bone mineralization field contains the most extensive exploration of the nucleational properties of bone-derived vesicles. These findings provide a concrete starting point for unravelling the mechanism of vascular calcification, and as such, we will explore key findings from both fields below. For clarification, moving forward we will refer to cardiovascular extracellular vesicles as EVs and osteochondral matrix vesicles as MVs.

Calcific conditions within an atherosclerotic lesion result in the production of vesicles that are mineralization competent (Fig. 4). Specifically, the pro-calcific vesicles are proposed to contain lipid, protein and ionic components necessary to form membrane-anchored structures that are the starting point for calcium phosphate mineral formation, known as the nucleational core (NC) (Wu *et al.* 1993, 1997*a*). The NC is fed with Ca^{2+}/P_i ions from multiple sources. Within the vesicle, phosphatase orphan 1 (PHOSPHO1) cleaves P_i ions primarily from two phospholipids enriched in the EV membrane: phosphoethanolamine (PEA) and phosphocholine (PChol) (Mebarek *et al.* 2013). Enzymes bound to the external EV membrane, particularly tissue non-specific alkaline phosphatase (TNAP), nucleoside pyrophosphohydrolase (NPP) 1 and NPP3, liberate P_i from ATP, ADP and PP_i in the extravesicular space (Ciancaglini *et al.* 2010). Pit1 and Pit2 transporters pump these P_i ions into the EV (Anderson, 2003, 2005). Calcium is supplied to the NC by annexins A2, A5, and A6, as well as other calcium transporting enzymes (Kirsch *et al.* 2000*b*). As extensive intraluminal Ca^{2+}/P_i mineral forms, the EV membrane undergoes a loss of structural integrity, and mineral crystals are propagated out onto the collagen-enriched matrix, guided by proteins that attach the EV membrane to collagen (Wu *et al.* 2002; Mebarek *et al.* 2013). The continued extra-vesicular propagation of mineral is regulated by the ratio of pyrophosphate (PP_i), an inhibitor of crystal growth (Francis, 1969), and its derivative, P_i.

Membrane-bound nucleational core is the primary unit of vesicle mineralization

Pivotal studies conducted two decades ago subjected chondrocyte-derived MVs to a series of chemical and detergent extraction steps to reduce MVs to their most basic unit that retained the ability to nucleate mineral formation (Wu *et al.* 1993, 1997*a*). This and subsequent studies by Wuthier and colleagues demonstrated that this NC is composed of three key components (Genge *et al.* 2007): amorphous calcium phosphate (ACP); CPLX, a membrane-associated complex of PS–Ca^{2+}–P_i; and annexin A5 (Wu *et al.* 1993, 1997*a*). While ACP comprises 91.8% of the NC (Wu *et al.* 1997*a*) experiments with *in vitro* synthesized NCs demonstrated that ACP mediates only 20% of mineral formation (Wu *et al.*

2008), concluding that CPLX predominately exerts the nucleating power of the NC. Furthermore, annexin A5, and to some degree annexin A6, catalysed the onset and increased the extent of CPLX mineral nucleation by 10- to 20-fold (Genge *et al.* 2007). Ultimately, Wutheir and colleagues hypothesize that the atomic arrangement of the CPLX–annexin A5 complex provides a molecular template that guides the arrangement of Ca^{2+}/P_i ions into a crystalline pattern (Genge *et al.* 2007). These hypotheses have been supported by electron microscopy images that demonstrate calcium phosphate deposits localized to the inner MV membrane (Anderson *et al.* 2005).

Furthermore, it has been proposed that MV budding and NC formation occur simultaneously within the mineralizing chondrocyte. These cells demonstrate an increase in intracellular phosphate (Wuthier, 1977) and a marked increase in intracellular Ca^{2+} levels near the plasma membrane right before MV budding (Wu *et al.* 1997*b*). The elevated peripheral calcium may drive simultaneous clustering of PS within the cell membrane and binding of annexin A5 to these PS-rich regions, leading to NC formation that is timed with MV budding (Wu *et al.* 1993). Indeed, when comparing MVs from mineralizing *vs.* non-mineralizing chondrocytes, only those from mineralizing cells contained annexins, demonstrating the specialized loading of mineral-nucleating MVs with pro-calcific components (Kirsch *et al.* 2000*a*). EVs elaborated by VSMCs are similarly enriched in annexins A2, A5 and A6, hinting at a shared mechanism of EV loading and mineral nucleation by these two cell types (Chen *et al.* 2008; Kapustin *et al.* 2011). However, the presence of an NC has not been identified within vascular EVs, requiring more extensive investigation.

Concerted effort of vesicle machinery concentrates ions for mineral nucleation

Annexins mediate Ca^{2+} influx via direct and indirect activities. Annexins are hypothesized to contribute to two other bone-derived MV functions: collagen binding and Ca^{2+} influx. Annexin A2, A5 and A6 are all known components of MVs, and have all individually been demonstrated to mediate Ca^{2+} influx into PS-rich liposomes and non-calcifying MVs that were supplemented with exogenous annexin A2, A5 and A6 (Kirsch *et al.* 2000*b*). This hypothesis is supported by structural analyses, which revealed that the hydrophilic pore located within the structure of the annexin core is biochemically similar to other Ca^{2+} channels

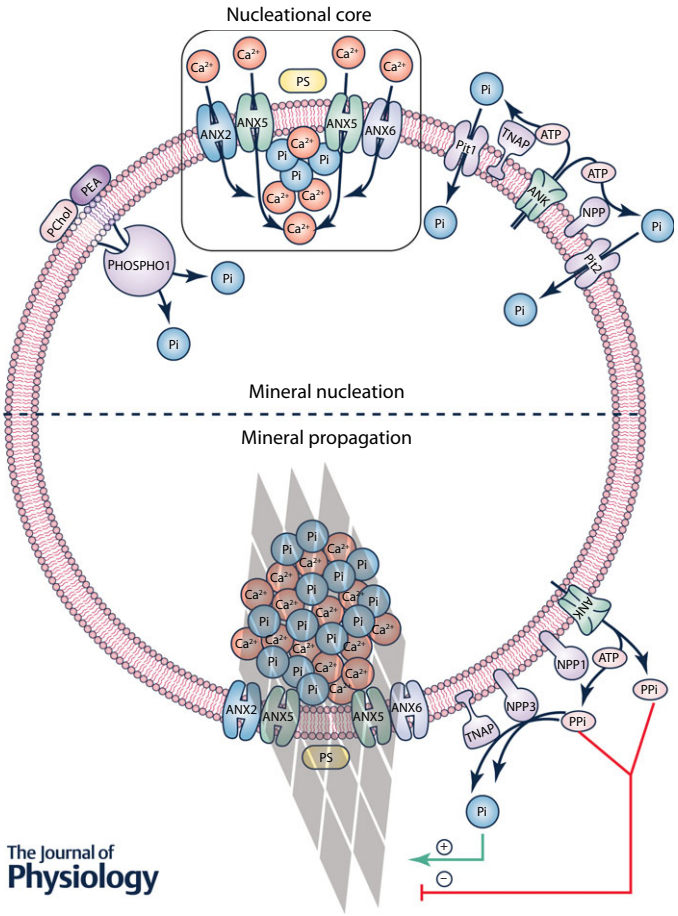

Figure 4. Proposed mechanism of vesicle-mediated mineral formation
Top, cell-derived vesicles are preloaded with a nucleational core (NC), composed of annexins A2, A5 and A6, as well as a complex of PS, Ca^{2+} and P_i. Calcium and phosphate ions are accumulated within the vesicle lumen via multiple routes: intravesicular P_i cleavage from PChol and PEA via PHOSPHO1; P_i cleavage from ATP in the extravesicular space via TNAP and NPP1/3, and import via Pit1/2; and calcium import via annexins A2, A5 and A6. These ions are patterned as calcium phosphate mineral at the NC. Bottom, over time, mineral crystals propagate through the vesicle membrane, where their continued growth is regulated by the ratio of PP_i, an inhibitor of crystal growth, to its derivative P_i. Note that the hypothesis that annexins form transmembrane ion channels, as depicted in this figure, is controversial (given that annexins are believed to primarily function as peripheral membrane proteins), thus meriting further investigation.

(Gerke, 2002). However, the postulated role of annexins as Ca^{2+} channels has yet to be demonstrated in living cells (Gerke, 2002). Furthermore, the annexin-specific Ca^{2+} channel blocker K-201 did not suppress EV mineralization (Kapustin *et al.* 2011), calling to question the function of annexins as mediators of EV Ca^{2+} influx. Indeed, annexins are primarily thought of as peripheral membrane-binding proteins. A dynamic interplay of several factors has been proposed to mediate potential annexin membrane insertion, including lipid bilayer composition, annexin concentration, Ca^{2+} concentration, a hyperpolarization of transmembrane voltage, membrane destabilization and intracellular/intravesicular acidification (Gerke, 2002). Further studies are necessary to definitively determine if annexins have the capacity to function as cellular and/or vesicular Ca^{2+} channels.

Only annexin A5 binds types II and X collagen, anchoring MVs to the extracellular matrix (ECM). This collagen binding enhances Ca^{2+} influx via annexin A5 (Kirsch *et al.* 2000*a*), demonstrating an active interplay between the ECM and MV calcification. Interestingly, EVs isolated from bovine vascular smooth muscle cells (BVSMCs) were only capable of inducing mineral formation along type I collagen fibrils, not type II collagen (Chen *et al.* 2008). Given that type I collagen is up-regulated in the setting of atherosclerosis and medial calcification (Rekhter *et al.* 1993), these findings indicate that EVs are produced with an affinity to promote calcification specific to the ECM environment into which they are released (Chen *et al.* 2008).

Additionally, there is evidence that annexin A2 may act to induce TNAP activity in MVs. Annexin A2 and TNAP have been shown to colocalize at the periphery of calcifying SaOSLM2 osteoblastic cells. Membrane fractionation has demonstrated that both are more specifically localized to cholesterol-stabilized lipid rafts. An overexpression of annexin A2 led to an increase in TNAP activity in the lipid-raft membrane fraction (Gillette & Nielsen-Preiss, 2004). Similarly, VSMC-EVs treated with the annexin Ca^{2+} channel inhibitor K201 have demonstrated a decrease in TNAP activity (Chen *et al.* 2008).

Intraluminal PHOSPHO1 generates phosphate ions that initiate mineral nucleation. The phosphate ions (PO_4^{3-} or P_i) that combine with Ca^{2+} to build mineral at the NC are sequestered in larger molecular structures to prevent spontaneous, indiscriminate calcium phosphate precipitation. Enzymes positioned on the inner and outer MV membrane liberate P_i for mineral nucleation. Mice deficient in these enzymes do not undergo skeletal mineralization, even in the presence of abundant serum P_i (Millan, 2013), highlighting the requirement for active, 'peri-vesicular' generation of P_i.

PHOSPHO1, a Mg^{2+}-dependent hydrolase (Stewart *et al.* 2003), is the only enzyme thus far identified to generate P_i within MVs and EVs. This enzyme primarily cleaves P_i from PEA and PChol (Mebarek *et al.* 2013), poorly hydrolysing more classical P_i-providing substrates associated with bone mineralization, including ATP, ADP and PP_i (Ciancaglini *et al.* 2010). Indeed, recent studies have used MVs from autopsy samples from TNAP deficient patients and from TNAP knockout mice to demonstrate that TNAP is not essential to achieve intraluminal mineralization (Anderson *et al.* 1997, 2004). Furthermore, calcifying VSMCs have demonstrated an up-regulation of *Phospho1* expression and activity (Kiffer-Moreira *et al.* 2013). Both PHOSPHO1 knockout (*Phospho1*$^{-/-}$) VSMCs and VSMCs under pharmacological PHOSPHO1 inhibition undergo a significant reduction in their ability to calcify (Kiffer-Moreira *et al.* 2013). Because PHOSPHO1 drives the initial, intravesicular stage of mineralization, it has been proposed that targeting PHOSPHO1 may be more effective than inhibiting TNAP at preventing vascular calcification (Kiffer-Moreira *et al.* 2013).

Reduced skeletal mineralization seen in *Phospho1*$^{-/-}$ mice is not corrected by a superimposed overexpression of TNAP, indicating that these two enzymes fulfil distinct roles in the process of vesicle-mediated mineralization (Millan, 2013). However, *Phospho1*$^{-/-}$ EVs exhibit a 70% reduction in TNAP activity (Ciancaglini *et al.* 2010), and PHOSPHO1 inhibition leads to a reduction of TNAP mRNA (Yadav *et al.* 2011). Thus, just as with the interplay between annexin A2 and TNAP, the complex relationship between PHOSPHO1 and TNAP activity merits further investigation.

Extravesicular P_i is regulated by a host of membrane-bound enzymes and pumps. PHOSPHO1-driven P_i generation takes place in conjunction with the coordinated activity of several enzymes and pumps interacting with the extravesicular space. Within osteochondrogenic mineralization, ankylosis protein (ANK) has been proposed to pump ATP (Villa-Bellosta *et al.* 2011) and PP_i (Zhao *et al.* 2012) out of MV-releasing cells, and perhaps MVs themselves. ATP is the principal source of P_i channelled back into MVs for mineral nucleation, with additional contributions from ADP and PP_i (Ciancaglini *et al.* 2010; Millan, 2013). P_i liberation from these molecules is predominantly driven by TNAP, which is expressed in osteoblasts and chondrocytes (Ciancaglini *et al.* 2010), as well as calcifying VSMCs (Villa-Bellosta *et al.* 2011; Haarhaus *et al.* 2013; Kiffer-Moreira *et al.* 2013). However, MV-mediated ATP hydrolysis persists in the absence of TNAP and PHOSPHO1 (Ciancaglini *et al.* 2010), and mice with a combined deficiency in PHOSPHO1 protein and TNAP function (*Phospho1*$^{-/-}$ *Akp2*$^{-/-}$) still underwent a small degree of skeletal mineralization (Yadav *et al.* 2011). Thus

a third P_i-generating mechanism likely contributes to mineralization.

Recent studies have demonstrated that the NPP family may also play a role in osteochondrogenic and vascular mineralization. NPP has demonstrated ATPase, ADPase and PP_iase activity in MVs, but only when TNAP is absent or inhibited (Ciancaglini *et al.* 2010). TNAP outcompetes NPP1 to bind ATP, ADP and PP_i (Ciancaglini *et al.* 2010), limiting the degree to which NPP1 can contribute to P_i generation. Applying inhibitors of TNAP, PHOSPHO1 and NPP to wild-type MVs led to a complete inhibition of ATPase activity (Ciancaglini *et al.* 2010). Further, not only do rat and mouse aorta express NPP1–3 (Villa-Bellosta *et al.* 2011), but NPP1 knockout (*Enpp1*$^{-/-}$) VSMCs cultured with PHOSPHO1 and TNAP inhibitors demonstrate a 93% decrease in mineralization, *versus* only 65% in wild-type cells cultured with both inhibitors (Kiffer-Moreira *et al.* 2013).

While Ciancaglini *et al.* argued that the observed compensatory phosphatase activity in TNAP-deficient bone-derived MVs was mediated by NPP1 (Ciancaglini *et al.* 2010), the possible contribution of NPP3 in addition to NPP1 was not addressed. Indeed, while Ciancaglini *et al.* found that purified NPP1 mediated PP_i hydrolysis, Villa-Bellosta and colleagues demonstrated that PP_i hydrolysis was carried out only by NPP3-transfected HEK cells, observing no phosphatase activity in NPP1 cells (Villa-Bellosta *et al.* 2011). Furthermore, explanted aortas from *Enpp1*$^{-/-}$ mice did not demonstrate altered PP_i hydrolysis (Villa-Bellosta *et al.* 2011). Further studies are needed to tease apart the exact roles of NPP1 and NPP3 in vascular calcification.

Nevertheless, these studies have demonstrated that the vast majority of extravesicular P_i generation is driven by TNAP and NPPs (Millan, 2013). Additional MV enzymes that act to liberate P_i in the extravesicular space include NTPDases, which convert ATP to AMP and 2 P_i (Villa-Bellosta *et al.* 2011), and ecto-5′-nucleotidase, which cleaves AMP to adenosine and P_i (Fish *et al.* 2013). Liberated P_i may subsequently be pumped into the vesicle via type III sodium phosphate cotransporters Pit1 and Pit2 (Anderson, 2003; Anderson *et al.* 2005). Several reviews describe the bone forming activity of these phosphate-handling enzymes in greater detail (Anderson, 2003; Golub, 2011; Wuthier & Lipscomb, 2011; Fish *et al.* 2013; Millan, 2013).

To become nucleation competent, forming EVs may also require a reduction in the concentration of mineralization inhibitors that they contain, most notably including fetuin-A (FetA) and matrix Gla protein (MGP). FetA is a circulating glycoprotein produced predominantly by the liver (Dziegielewska *et al.* 1987). It inhibits MV and EV mineralization by binding to calcium phosphate crystals and preventing further crystal growth (Reynolds *et al.* 2005). Importantly, several studies have demonstrated that there exists a negative correlation between serum concentration of FetA and severity of cardiovascular calcification (Kapustin *et al.* 2015). Kapustin *et al.* recently observed the internalization of FetA by VSMCs via a receptor-independent mechanism (Kapustin *et al.* 2015). The FetA was observed in both the late endosomal and lysosomal compartments, and some of it was ultimately packed into EVs, which the authors identified as exosomes. Further, an earlier study from the same group demonstrated that EVs containing FetA were less likely to contain calcium phosphate mineral (Reynolds *et al.* 2005).

Similarly, MGP is a (vitamin K-dependent) mineralization inhibitor that regulates MV and EV calcification. Produced directly by VSMCs, MGP is loaded into forming EVs (Reynolds *et al.* 2004), where it is proposed to bind growing calcium phosphate crystals and prevent further crystal growth (Wuthier & Lipscomb, 2011). The stresses that promote an osteochondrogenic conversion of VSMCs have been hypothesized to lead to a decrease in the amount of FetA and MGP loaded into EVs. Further, calcifying VSMCs tend to produce undercarboxylated MGP, which is non-functional (Kapustin & Shanahan, 2012). The loss of inhibitors packed into EVs is permissive to EV mineralization and microcalcification.

Restructuring of MV lipid membrane permits crystal escape beyond the intraluminal space

As bone-derived MVs undergo intraluminal mineral nucleation, the membrane lipid composition undergoes significant turnover to become more permissive for the outgrowth of calcium phosphate crystals (Wu *et al.* 2002; Mebarek *et al.* 2013). MVs are loaded with phospholipases, including phospholipase A and C (PLA and PLC), which degrade membrane phospholipids to lysophospholipids (LPLs) that alter membrane curvature and compromise membrane integrity (Wu *et al.* 2002; Mebarek *et al.* 2013). The exact means by which calcium phosphate crystals emerge from the MV membrane is poorly understood. Kapustin *et al.* demonstrated that annexin A6 and PS can be isolated from the inner and outer membranes of VSMC-derived EVs. Furthermore, several studies used TEM to visualize mineral nucleation that occurred on both the inner (Fig. 5*A*) and outer membranes (Fig. 5*B*) of EVs (Aikawa *et al.* 2007; Kapustin *et al.* 2011; New *et al.* 2013). Thus, active NCs may be present on both sides of the EV membrane. Phospholipase activity may externalize membrane-associated NCs, which would allow NC-guided crystal growth to progress in the extravesicular space (Wuthier & Lipscomb, 2011). Subsequently, annexin would mediate binding of the NC to collagen, which would then further guide crystal outgrowth (Wuthier & Lipscomb, 2011). Similarly, TNAP binds collagen via its crown domain, an interaction that is critical for the activity of the enzyme (Mornet *et al.* 2001). Calcium phosphate

crystals may similarly emerge onto the ECM via this TNAP connection. Ultimately, these calcium phosphate crystals form clusters centred upon the nucleating MV, progressing outward within and along the surrounding collagen fibrils (Wu *et al.* 2002; Millan, 2013). The collagen meshwork contains hole zones that are aligned to form channels sized appropriately for the entry of calcium phosphate nanocrystals (Golub, 2011). These crystals then propagate within the collagen to fill all available intrafibrillar spaces (Golub, 2011). Therefore, our limited insight into how mineral crystals emerge from nucleating vesicles comes exclusively from the field of bone mineralization. EV-specific studies are needed to determine if these proposed osteochondrogenic mechanisms are also at play in vascular calcification.

Extravesicular mineral propagation regulated by the PP_i/P_i ratio

The propagation of any calcium phosphate mineral crystal once it has emerged from the vesicle lumen is dictated by the ratio of PP_i to P_i in the extravesicular environment. PP_i inhibits mineral propagation by directly binding to the crystal surface and preventing the further addition of Ca^{2+} and P_i. PP_i is supplied to this environment via two mechanisms: ANK, which releases intracellular PP_i; and NPP1 and -3, which generate PP_i chiefly from ATP (Prosdocimo *et al.* 2009). Studies using explanted aortas in an organ culture model have demonstrated the role of NPP in vascular calcification. Aortic tissue exposed to an NPP inhibitor or isolated from an *Enpp1*$^{-/-}$ mouse failed to synthesize PP_i from ATP (Villa-Bellosta *et al.* 2011). Accordingly, children born with the inability to produce NPP1 suffer from pervasive, fatal arterial calcification (Rutsch *et al.* 2001). Mouse models that carry a homotypic ANK mutation (ank/ank) that restrict the PP_i channelling function of the protein also demonstrate extensive soft tissue calcification (Harmey *et al.* 2004).

Certain tissue environments are permissive to calcification due to the expression and activity of TNAP, which has been found to conduct 50% of PP_i hydrolysis (Villa-Bellosta *et al.* 2011). The remaining 50% of PP_i hydrolysis is accounted for in party by NPP3. HEK cells transfected with NPP3, but not those with NPP1, demonstrated an increase in PP_i hydrolysis. Further, aortic explants from *Enpp1*$^{-/-}$ mice hydrolysed PP_i to the same degree as wild-type tissues. Thus, NPP3 appears to be unique in its ability to both generate and hydrolyse PP_i. Deficiencies in PP_i hydrolysis result in the failure of mineralization to progress past the EV membrane, due to the suppression of extravesicular crystal expansion via PP_i binding (Yadav *et al.* 2011).

New therapeutic targets may be hidden in the process of EV formation

Our increasing knowledge of the mechanisms driving ectopic calcification reveals multiple potential points of intervention. One approach is to target the array of EV membrane-associated pumps and enzymes that fuel mineral nucleation. Direct targeting of mineral propagation via physicochemical means, which has previously been attempted with bisphosphonates (Lomashvili *et al.* 2009), is another possible approach. These attempted therapies have failed so far, however, because they compromised bone mineralization. Therefore, more specific therapeutic targets will come to light only after we develop a refined understanding of the differences that delineate calcification driven by bone-derived MVs *versus* that by soft tissue-derived EV.

A third potential target is at the level of vesicle formation, but this is arguably the least understood step in the process of tissue mineralization. Within

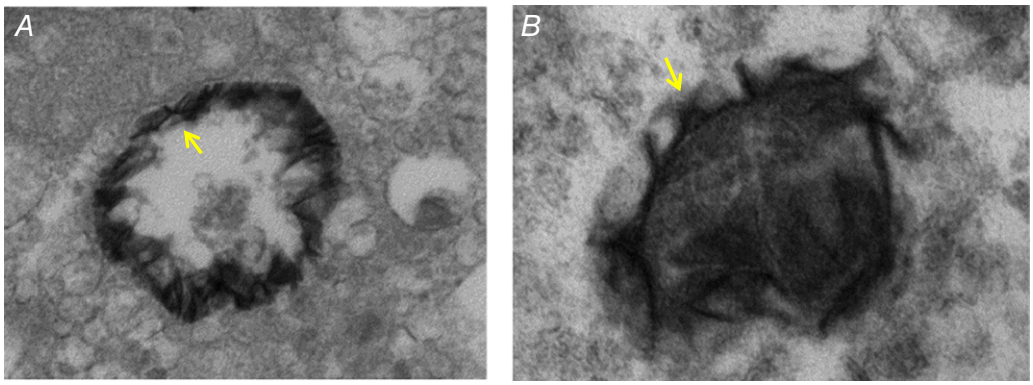

Figure 5. Electron micrographs of mineral formation on EV inner membrane (*A*) and outer membrane (*B*)
(Adapted from Aikawa *et al.* 2007.)

the context of endochondral ossification, it has been demonstrated that vesicles can bud directly from microvilli of chondrocytes (Wuthier & Lipscomb, 2011). However, electron microscopic studies of osteoblasts have demonstrated the presence of intracellular vesicles loaded with calcium phosphate mineral (Rohde & Mayer, 2007; Boonrungsiman *et al.* 2012; Nollet *et al.* 2014). In some cases, intracellular mineral aggregates appeared to form freely within the cytoplasm before being engulfed by a vesicle membrane. Others have observed calcium phosphate mitochondrial granules that may subsequently be loaded into intracellular vesicles. Still others have demonstrated a potential involvement of autophagy (Nollet *et al.* 2014), cytoskeletal rearrangement (Drabek *et al.* 2011) and intracellular vesicle trafficking mediated by the ER (Stenbeck & Coxon, 2014) and golgi (Stanford *et al.* 1995; Rohde & Mayer, 2007). Many of these investigators have observed the exocytosis or secretion of what appear to be non-membrane-bound apatite crystals into the ECM (Stanford *et al.* 1995; Rohde & Mayer, 2007; Nollet *et al.* 2014). Finally, other studies conducted with osteoblasts and VSMCs provide evidence that calcifying vesicles are exosomes derived from the generation and release of multivesicular bodies (Xiao *et al.* 2009; Kapustin *et al.* 2015). Given the complex interplay between many of these systems of intracellular trafficking, more targeted studies are required to determine the origin of calcifying vesicles specifically within vascular tissues before we can design solutions to inhibit their production or disease-causing activity.

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Additional information

Competing interests

We have no conflicts of interest to declare.

Author contributions

J.L.R. and J.D.H. wrote and compiled the manuscript text. S.W. provided critical review of the text. E.A. provided critical review of the text and recommendations on manuscript figures. All authors edited the final text. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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