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Multiple Pathways for Pathological Calcification in the Human Body

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Abstract

Biomineralization of skeletal components (e.g., bone and teeth) is generally accepted to occur under strict cellular regulation, leading to mineral-organic composites with hierarchical structures and properties optimized for their designated function. Such cellular regulation includes promoting mineralization at desired sites as well as inhibiting mineralization in soft tissues and other undesirable locations. In contrast, pathological mineralization, with potentially harmful health effects, can occur as a result of tissue or metabolic abnormalities, disease, or implantation of certain biomaterials. This Progress Report defines mineralization pathway components and identifies the commonalities (and differences) between physiological (e.g, bone remodeling) and pathological calcification formation pathways, based, in part, upon the extent of cellular control within the system. These concepts are discussed in representative examples of calcium phosphate-based pathological mineralization in cancer (breast, thyroid, ovarian, and meningioma) and in cardiovascular disease. In-depth mechanistic understanding of pathological mineralization requires utilizing state-of-the-art materials science imaging and characterization techniques, focusing not only on the final deposits, but also on the earlier stages of crystal nucleation, growth, and aggregation. Such mechanistic understanding will further enable the use of pathological calcifications in diagnosis and prognosis, as well as possibly provide insights into preventative treatments for detrimental mineralization in disease.

Graphical Abstract

Physiological biomineralization is a tightly regulated process characterized by multiple levels of synchronized cellular controls. In contrast, pathological calcification, occurring in non-skeletal tissues including vasculature and cancers, occurs via multiple pathways, with varying degrees of cellular regulation. Identifying “pathway components”, e.g., cell death, mineralization-proteins,

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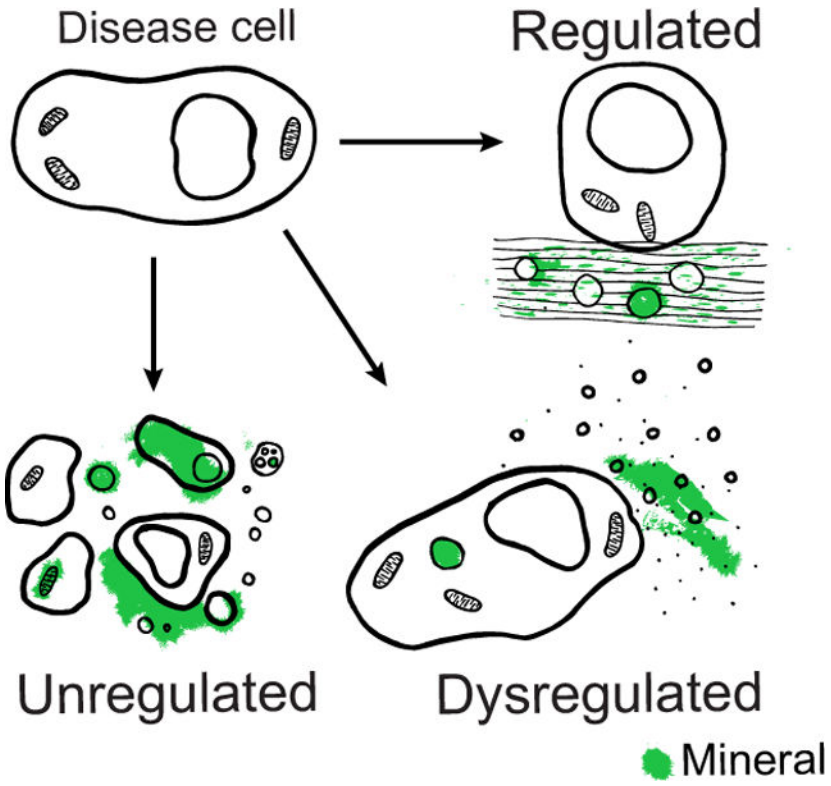
vesicle secretion, and mineralized-collagen, provides a framework with which to describe pathological mineralization pathways.



Keywords

breast cancer microcalcifications; biomineralization; psammoma bodies; apatite; cardiovascular calcifications

1. Introduction

Our understanding of the mechanisms underlying biomineralization processes, which yield functional biominerals such as bones, teeth, and shells, has greatly advanced over the past decade[1–6]. Across organisms, common biomineralization processes are observed including regulation by soluble and insoluble biomacromolecules (proteins[7], polysaccharides[7], lipids[8]), oriented mineral nucleation on substrates (both intra- and extracellular)[9,10], exploitation of precursor phases[2,11,12], and concentration of mineral building blocks from solutions within vesicles[13–16], in which crystal nucleation and growth may proceed under confinement[17–19]. In healthy conditions, biology promotes mineralization at desired sites (physiological mineralization), while simultaneously inhibiting unwanted mineral precipitation at sites such as cardiovascular and other soft tissues[4,20]. In pathological mineralization, however, minerals form in these tissues that otherwise do not calcify. This type of mineralization can occur in association with disease conditions or in benign medical conditions such as injury, inflammation and aging[21–27]. Pathological mineralization is much less well-studied than physiological mineralization, yet mechanistic studies of some

types of pathological mineral formation suggest commonalities among pathological and physiological mineral formation[28,29]. Mechanistic studies of pathological calcifications are an active area of research, and there are still ongoing debates regarding many aspects of pathological calcification, including the chemical composition and crystal properties of the deposited mineral, the types of cells involved, the extent of cellular control, the characteristics of gene expression, the mineralization timeline, the interactions between the mineral and surrounding tissue, and the relationships among all of these factors and disease progression.

In this Progress Report, we explore the similarities and differences between physiological and pathological mineralization pathways, using tools from biomineralization and materials science. As a representative example of physiological mineralization, we use the bone-remodeling pathway, the process by which mature bone is turned over and replaced with new bone tissue. Using the bone-remodeling pathway, we identify a series of "pathway components", e.g., ion sources, cells, matrix production, and vesicle secretion, which are then pieced together in a precise, coordinated order to yield the final bone structure consisting of hierarchically arranged, mineralized collagen fibers. With this framework in hand, we turn our focus to cardiovascular and cancer-related pathological calcifications, primarily composed of calcium phosphates. For these pathological calcifications, we describe a continuum of mineralization pathways ranging from those under tight cellular control, more analogous to physiological pathways, to dysregulated pathways in which only a subset of pathway components appear, to mostly unregulated processes in which, for example, mineral is deposited on cellular debris in necrotic tissue. We argue that a better understanding of the pathway components contributing to the formation of pathological calcifications will bridge common medical knowledge and mechanistic views of biomineralization and may contribute to new approaches towards diagnosing and treating these diseases.

2. Bone Remodeling as an Example of a Physiological Mineralization Pathway.

Biomineralization in humans follows highly regulated, yet differing pathways, depending on the system (*i.e.,* bones or teeth), stage of life, and bone type[30–36]. Bone remodeling and osteonal growth are examples of well-studied, tightly regulated physiological processes under strict cellular and microenvironmental control that share mechanistic similarities[4,30,31]. For this review, we focus on bone remodeling as a physiological touchstone with which to compare pathological processes and to introduce several key concepts that underpin many studies of biomineralizing systems.

2.1. Bone structure and composition.

Most studies of biomineralizing systems begin with a structural and compositional analysis of the mineralized tissue. The basic components of bone are apatite nanocrystals (~60 wt%), collagen (~22 wt%), water (~15 wt%), and non-collagenous organics (~3 wt%) including proteins, lipids, and small molecules[37–39]. These components are organized into more than ten levels of hierarchy[40]. The exact formula of the apatite nanocrystals is, perhaps

surprisingly, still a matter of debate, though it is agreed that it is a carbonated apatite with numerous ionic substitutions reported including acid phosphate, water, sodium, fluorine, chlorine, zinc, and others[41,42]. Molecular water is also present either as a stoichiometric constituent, a surface bound nano-interphasic component, a matrix component, or some combination. These ongoing debates are reflected in the conflicting formulas put forth for bone apatite (e.g. $Ca_{10-x}[(PO_4)_{6-x}(CO_3)_x](OH)_{2-x} \cdot nH_2O$, where n ~ 1.5 or alternatively $Ca_{10-x}(PO_4)_{6-x}(HPO_4,CO_3)_x (OH,½CO_3)_{2-x}$ with $0 \leq x \leq 2$)[38,43]. Regardless, the bone apatite crystallites are anisotropic particles (e.g., platelets, needles) sized in the nanometer range and are structurally supported by type I collagen to form mineralized collagen fibrils[44–46]. The collagen provides a structural and chemical template in which mineralization proceeds, both intrafibrillar and interfibrillar, and the crystallographic *c* axes of the apatite nanocrystals are aligned with the long axes of the collagen fibrils[47]. These fibrils are then further assembled into a diversity of architectural motifs and patterns depending on specific microscopic location, the general location within the bone (e.g., cortical versus trabecular), and the type of bone (e.g., long bone versus vertebrae)[40]. Finally, all of these compositional and structural characteristics of bone crystals are known to vary with tissue age, as well as disease state. For example, the apatite crystals in older bone tissue are known to be larger and contain fewer substitutions and defects as compared to crystals in newly deposited bone tissue[48–50].

2.2. Biomineralization Pathways and Pathway Components.

The term “pathway” comprises multiple meanings depending on context and field. In the field of biomineralization, a mineralization pathway is comprised of a coordinated series of events that begins with bound or free ions and ends with the final biomineralized tissue[2]. In general, for this process to occur, the following three conditions must be met: local increase in supersaturation, decrease in mineralization inhibitors, and exposure to mineralization promoters. There are multiple “pathway components” that are pieced together in the proper order during physiological mineralization to achieve the final product. More specifically, physiological formation of biominerals involves some combination of the following “pathway components”[2,4,51]: ion sources (e.g., from extracellular fluids[2] or sea water[52]), mineralizing cells[2,53–55], structural (insoluble) extracellular matrix[56,57], mineralization biomacromolecules[58] and in some cases, intracellular mineralization[16,59], vesicle secretion[60] and amorphous mineral precursor phases[11,61].

2.2.1. Bone Remodeling Biomineralization Pathway.—

The bone remodeling biomineralization pathway follows a series of coordinated events, with one or more “pathway components” involved in each step (Figure 1): 1) Calcium and orthophosphate containing-species are sequestered from the blood stream by the osteoblasts. 2) Osteoblasts deposit the structural extracellular matrix (ECM), called the osteoid, which is primarily composed of oriented type I collagen fibers. Mineralization regulating non-collagenous proteins (NCPs) and other small molecules are also secreted into the osteoid. NCP expression is a dynamic process and so the timing of these events depends on the specific protein and the stage of mineralization[62]. 3) Mineral building blocks and/or amorphous precursors are most likely transported through the cell in membrane-enclosed structures, e.g., intracellular vesicular structures, for release into the site of mineralization, i.e., the

osteoid. 4) In a poorly understood step, the mineral precursors are released from the osteoblasts, perhaps enclosed within vesicles, into the osteoid where mineralization of the collagen fibers occurs. 5) The mineral precursors finally transform from a poorly ordered phase into the final, crystalline apatite within the collagen fibrils, resulting in mineralized collagen fibrils.

2.2.2. "Pathway Components".—In the following paragraphs, we describe in more detail the "pathway components" that participate in the bone-remodeling pathway. These pathway components are described here in the context of physiological mineralization, but many will also appear in pathological mineralization (see Section 3.2).

Ion Sources: Blood serum is the main source of calcium and orthophosphate ions for bone formation and remodeling. In serum, ions are free as well as bound to ion-complexes, chelators, and mineralization-inhibitory proteins[63–65]. Although serum is supersaturated with respect to apatite, mineral does not precipitate in normal conditions due to the presence of crystal nucleation and growth inhibitors and the solution being kinetically metastable[20]. For example, Fetuin-A is a well-known serum protein, which is produced in the liver[66] and actively inhibits mineralization in the serum[67–69]. Fetuin-A is thought to bind preformed nanoparticles of calcium phosphate, preventing further growth, and possibly stabilizing these "calciprotein" particles, the functions of which are still being uncovered[70]. Recent microscopy studies have also suggested that there are dense calcium-phosphate nanoparticles circulating within the blood, as was demonstrated during chicken embryonic bone and murine epiphyseal growth plate mineralization[32,71,72].

Cells Involved in Mineralization: The main cell types orchestrating bone remodeling are osteoclasts[73], osteoblasts[55,74], and osteocytes[75–80]. Their actions are locally and systemically regulated by a slew of hormones and growth factors (e.g., parathyroid hormone, calcitonin, sclerostin, and osteocalcin) as well as being inexorably linked to calcium and phosphate homeostasis in the body[81–86]. Osteoclasts are multinucleated cells of the macrophage lineage responsible for bone resorption of damaged or old bone. They perform this function by "sealing" to the bone site in question, acidifying the sealed environment (effectively dissolving mineral) followed by internal degradation of demineralized matrix and trafficking of degraded components out of the cell[73,87]. Osteoblasts, differentiated from mesenchymal stem cells, are the gate keepers and workhorses of bone formation[55]. They are responsible for depositing the insoluble, collagen-rich matrix (osteoid), maintaining the osteoid environment through a concerted multicellular seal via tight junctions, regulating the pH and the influx (and export) of ions/precursors to form the apatite mineral[74], and releasing promotors and inhibitors of mineralization (Figure 1). Osteoblasts also contain polyphosphate reserves[65,88] that can be tapped enzymatically to generate orthophosphates, and may serve other functions as well[89]. Many of the osteoblasts further differentiate into osteocytes, housed within the mineral with processes extending outward to neighboring osteocytes and osteoblasts, and comprising a vast interconnected sensory and communications network that permeates and monitors bones and helps mediate bone homeostasis globally[75,76,79,80].

Matrix Components: As already described, type 1 collagen fibrils are the main component of the bone ECM[90]. The regulatory non-collagenous proteins (NCPs)[7,91] warrant additional discussion, particularly as many are also found associated with pathological mineralization. Many NCPs, by sequence or post-translational modifications (*e.g.*, phosphorylation), are acidic, or contain negatively charged domains, which are thought to bind calcium, while other domains bind cells and collagen[39,92,93]. Well-known bone matrix proteins include glycoproteins [e.g., alkaline phosphatase (ALP), osteonectin, fetuin-A, osteopontin (OPN)[68,94–98], and bone sialoprotein (BSP)[93,99,100] where the last two are members of the SIBLING protein family (Small Integrin-Binding Ligand and N-linked Glycoproteins)[93], Gla proteins (proteins enriched with gamma-carboxyglutamic acid; predominantly osteocalcin), and proteoglycans (e.g., decorin). These NCPs, identified in mineralized bone, provide physiochemical regulation of mineralization, structuring the organic matrix, defining the sites of nucleation in the matrix, and controlling the growth of mineral[46,58,101,102]. Most of these macromolecules are thought to be directly expressed by osteoblasts[103], while some, such as fetuin-A are trafficked from the serum[66]. Many also serve as signaling molecules[104,105]. The specific mineralization functions of these regulators are spatially and temporally interconnected and dependent on many factors. In addition to these NCPs, there are small molecules, such as pyrophosphate and citrate, that are important participants in bone-remodeling. Pyrophosphate is a known mineralization inhibitor[106–109] and is also a substrate for ALP[110,111]. Citrate is either trafficked or synthesized by osteoblasts; it is reservoired in bone[112], potentially serving as an apatite stabilizer[113,114].

Vesicle Transport of Proteins and Mineralization Precursors: Vesicular structures have been implicated in both the transport of mineral precursors intracellularly as well as in the release of these materials into the mineralizing osteoid. These structures may contain soluble mineral precursors or a dense amorphous mineral phase, which may serve as an easy-to-transport ion source[14,115]. Recent work suggests that amorphous calcium phosphate serves as a precursor phase to crystalline apatite in both bone and tooth formation[12,14,115]. There is a large body of evidence that matrix vesicles shed from the osteoid-facing (apical) side of the osteoblast, containing mineralization proteins (including ALP) and amorphous calcium phosphate or mineral precursors, can also take part in bone mineralization[13,51,115,116], though the extent of involvement remains unclear and also depends on the type of bone[74,117].

To summarize, physiological mineralization is an actively regulated, multi-step process, in which specialized cells create a microenvironment in which mineral formation is favored, as described here for bone remodeling. The microenvironment at the mineralization site is designed to direct the formation of minerals with specific composition and structure. As described, physiological biomineralization pathways involve some combination of the following components: 1) ion sources, 2) mineralizing cells, 3) matrix and mineralization proteins, 4) vesicles and mineralization precursors. In bone, the final product is a functional tissue with hierarchically organized, mineralized collagen fibers. In pathological mineralization, we will see that multiple different pathways are possible, with many of these

same pathway components, as well as several additional components that are identified more frequently in pathological conditions.

3. Pathological Calcification.

In contrast to physiologically formed biominerals, like bone, pathological calcifications are presumed to have no clear function and are related to abnormalities in the tissue (termed “dystrophic calcification”) or abnormalities in the metabolic levels of calcium and phosphate ions in the blood serum (termed “metastatic calcification”)[118–121]. Dystrophic calcification is used to describe processes ranging from ectopic bone formation to calcifications found in necrotic regions of skin tissue, tuberculous lymph nodes, and regions of injury and inflammation. Metastatic calcification usually occurs in kidneys, blood vessels, corneas, or lungs[23,121,122]. Pathological calcification can also result from genetic disorders such as X-linked hypophosphatemia, the softening of bones, or from external factors such as vitamin D deficient rickets[4,83]. Biomaterials and prosthetic devices such as breast implants and heart valves are also subjected to pathological calcification, which may lead to damage and device failure[123–125]. Unlike the highly regulated formation of physiological minerals, pathological calcifications are heterogeneous in nature and form via multiple pathways, often involving only a subset of the pathway components identified in Section 2.2.2.

3.1. Structure, Composition, and Anatomical Locations of Human Pathological Calcifications.

As we did with bone, we will begin with a discussion of the structure and composition of pathological minerals. The composition, crystal phase, structure, and morphology of minerals can provide useful information that can help us to trace back the mineralization pathway and original tissue microenvironment and/or disease conditions. For example, it was demonstrated for kidney stones that the mineral morphology has an important connection to the disease state[126–129]. The environmental, chemical, and biological conditions affect the mechanism by which pathological calcifications form, i.e. crystal nucleation, growth, and aggregation pathways[25]. In turn, these pathways determine mineral characteristics such as structure, crystal phase, morphology, and size distribution. Intra-tissue interactions, which may be related to disease progression such as metastatic cancers, are affected by the mineral characteristics *in vitro*[130,131]. To gain sufficient understanding of these mineral characteristics (composition, phase, structure, morphology) multiple characterization techniques have to be combined. One has to be extremely careful when drawing conclusions regarding the nature of an observed mineral without combining at least one reliable technique for each of the four mineral characteristics.

The detection of pathological mineral deposits and the characterization methods used to analyze them range from imaging (high and low resolution) to analytical and structural techniques. As one reads the literature regarding pathological mineralization, it is important to be familiar with the strengths and weaknesses of the different characterization techniques and distinguish between structural/morphological imaging and chemical characterization methods[132]. Calcifications in soft tissues are routinely imaged and used for medical diagnosis and screening by utilizing techniques such as ultrasound, computed tomography

(CT), magnetic resonance imaging (MRI), and mammography. Radiologists use calcification characteristics to assess the presence of kidney stones and to detect breast and thyroid cancers. Higher resolution imaging techniques, requiring tissue biopsies, include histological staining methods such as H&E and von Kossa and electron microscopy (Scanning Electron Microscopy - SEM and Transmission Electron Microscopy - TEM). These imaging techniques can inform us (over very different length scales) regarding the calcification morphology, size, location, distribution, and local pathology in the tissue (or cells), but not regarding the chemical composition and crystal properties. X-Ray diffraction (XRD), X-Ray absorption spectroscopy, and electron diffraction can inform us regarding the crystal properties and phase of the mineral, including the presence of ionic substitutions within the crystal and their effect on the crystal structure. X-ray fluorescence and elemental analysis such as Energy Dispersive Spectroscopy (EDS) can provide the mineral elemental composition including trace element substituents, but inference of phase by these methods alone is unreliable. Vibrational spectroscopies, e.g., infrared and Raman, can be used to characterize the mineral phase, crystal properties, and organic components.

Unlike bone, which is characterized by a fairly narrow range of mineral and matrix compositions, pathological minerals vary in composition and structure as a function of the type and anatomical location of the deposit[25,26]. In this review, we are focusing on calcium-containing minerals (calcifications), however, there are also pathological crystal deposits formed by the crystallization of organic small molecules[25], and proteins[133,134]. Examples of molecular crystals include cystine[135,136], cholesterol[137], and uric acid crystals[138–141], which are found in kidney stones[142] (renal disorders), blood vessels (atherosclerosis), and in joints and periarticular tissues (Gout disease) (see reviews[21,25,27,143]). There are other class of pathological crystallization in which certain drug molecules themselves crystallize, usually within the urine and/or kidneys, or in which drugs induce the formation of pathological mineral deposits, both of these classes are outside of the scope of the current review[144,145]. Pathological calcifications are often poorly crystalline calcium phosphates in the form of apatite (for example in cardiovascular tissues, some kidney stones, and breast cancer) and calcium oxalates (for example in some kidney stones and in the breast) or even a mix of the two (some kidney stones), but there are also other examples such as calcium pyrophosphate dihydrate found in the joints[146–149]. Table 1 gives representative examples of pathological calcifications, their anatomical location and related disease, as well as the characterization techniques used to identify them. While some calcifications were characterized using multiple techniques (breast and ovarian cancer, cardiovascular, kidney stones, pineal gland, macular degeneration, and testicular microliths), others have almost no chemical characterization (thyroid cancer). Note that Table 1 is not an exhaustive tabulation of all types of pathological calcifications found in the human body, and focuses only on pathological calcifications identified in human tissue, i.e., it excludes pathological calcifications formed in animal models or *in vitro* studies (some examples can be found elsewhere[20,150–156]). Additionally, several representative phases found in kidney stone are listed; comprehensive tabulation of kidney stone phases can be found elsewhere[144,157].

3.2 Pathological Mineralization Pathways and Pathway Components.

The heterogeneity of pathological calcifications is demonstrated in Table 1 – several different mineral types are often associated with one disease state, and similar structures, e.g., psammoma bodies, can be found in vastly different tissue types. To begin to unravel the connections between calcification characteristics and mechanistic aspects of their formation, the approach we will take is to identify “pathway components” present in different types of pathological calcifications (Figure 2). In addition to revisiting the components identified in physiological mineralization pathways (Section 2.2), we also introduce apoptosis, necrosis and intracellular mineralization, three components identified in multiple pathological cases. The pathway components illustrated in Figure 2 can be put together in different combinations to define the overall mineralization pathway for a range of pathological calcifications.

3.2.1. Ion Sources in Pathological Mineralization.—Analogous to bone, we begin with a discussion of ion sources for pathological calcifications (Figure 2a). Understanding where the ions originate from, and how the disease state is altering the normal balance is critical for deducing a mineralization pathway. The chemical conditions at the mineralization site can direct the crystallization process towards different products. Not only the ion concentrations, but also local pH will affect supersaturation levels and crystallization – in bone, the pH is very tightly controlled by osteoblasts[74], but some of the diversity of mineral phases in pathology could be due to pH variations[214]. Possible sources for calcium and phosphorus are the blood, the skeleton, and the intracellular content. Imbalances in ion concentrations in the blood serum can occur through local concentration fluctuations and/or degradation of mineralization inhibitors that regulate the free ion concentrations in blood, such as fetuin-A, and/or in the tissue, such as matrix Gla protein[20,67]. Calcium and phosphate serum levels may also increase through the dissolution of the skeleton. In some pathological conditions such as hypophosphatemia, systemic imbalance of calcium/phosphate serum levels occurs[83]. Drug molecules and drug metabolites are also a possible source for phosphate ions leading to local accumulation of phosphate in, e.g., the kidneys[215]. Certain types of kidney stones are thought to be associated with drug-induced metabolic imbalances, brought on by use of calcium and vitamin D supplements or carbonic anhydrase inhibitors among others[144,216]; induced systemic imbalances such as these may impact other tissue sites as well. Additional possible sources for phosphorus include the DNA backbone, polyphosphates, pyrophosphates, adenosine triphosphate (ATP), guanosine triphosphate (GTP), and phospholipids[64]. The cellular contents, whether vesicle secretions or intracellular calcium-rich organelles, such as the mitochondria[65], can also locally provide calcium and phosphate. Mitochondria are known to balance cytoplasm calcium levels and can accumulate calcium phosphate[217,218], especially prior to cell death[219]. Finally, other ions such as magnesium can be supplied from the blood, and plasma levels of magnesium can affect its incorporation in pathological calcifications[220,221].

3.2.2. Cells Involved in Mineralization.—Just as the composition and structure of pathological calcifications are more heterogenous than in physiological minerals like bone, the cell types involved in their deposition are more diverse as well and in many cases under ongoing debate. Unlike in bone mineralization, in which there is a dedicated

cell-type, e.g., osteoblasts, for depositing the mineral and the matrix, in pathological mineralization, there is a range of cell types that can be involved, depending on the type of pathological calcification, namely, the disease, anatomical location, and the tissue type. For example, in cardiovascular calcification, the cells suggested to be involved in mineralization are endothelial cells, vascular smooth muscle cells, myofibroblasts, and immune cells (macrophages)[28,222–225]. In breast, thyroid, and ovarian cancers, stromal cells[172] as well as epithelial cells that become cancerous, are thought to be involved in malignant-associated mineralization[150,156,226–228]. The role of cells in close proximity to calcifications, particularly in cancer where cell populations among patients have differing mutational underpinnings (even within the same clinical classification)[229], is often unclear. Endothelial and/or vascular smooth muscle cells may differentiate into osteogenic-like cells[28], and it has also been suggested that epithelial breast cancer cells may behave in a similar fashion[162,230]. It should be noted that many tissues contain calcifications under benign conditions and the cell types associated with these are non-cancerous.

3.2.3. Mineralization proteins.—The majority of the proteins found in bone (and described in Section 2.2.) are also expressed elsewhere in the body, and are thought to be chemically different than their non-skeletal counterparts[231]. In the context of pathological mineralization, many of these proteins have been found to be directly associated with calcifications observed within vascular calcifications (Section 3.3.1) and human cancers (See Sections 3.3.2 and 3.3.3). For example, numerous bone matrix proteins have been found associated with or have hypothesized roles in vascular calcification, including Fetuin-A (Figure 2c), bone morphogenetic proteins (BMPs), and OPN[232]. Fetuin-A and Matrix Gla protein (MGP) are both thought to inhibit vascular calcification[20,233], however pathologically, the association of MGP with calcified vasculature may depend on whether or not it is carboxylated[234]. In cancer, Fetuin-A may have a context-dependent role in tumor progression, in which certain tumors may even synthesize their own Fetuin-A[235], though the association with cancer-specific calcifications has not been studied to date.

The SIBLING proteins have been implicated in cancer progression and are also known to be associated with pathological calcifications. OPN was found to be both highly expressed and correlated with disease severity in at least 15 types of human cancer[236], including breast, prostate, thyroid, and ovarian cancer, all of which can have conspicuous associated pathological mineralization[237]. SIBLING proteins such as OPN are considered multifunctional in both their skeletal and non-skeletal diversity of roles. In bone, OPN and BSP, can bind cells, collagen, and calcium, and thus help to coordinate adhesion to the ECM, among other things[39,92,93]. This multifunctionality may partially explain why OPN isoforms in cancers have been implicated in adhesion, migration, metastasis, and angiogenesis[236,238].

The specific roles of these bone proteins in pathological contexts remain difficult to classify. Even mineral-protein colocalization, while indicative of an association, may not be specific to promotion of mineralization. Fetuin-A, for example, is generally considered inhibitory and yet is a major component of bone[231]. Furthermore, it is known in bone, for example, that although seemingly structurally comparable, osteopontin (OPN)[94–96] and bone sialoprotein (BSP)[68,93,99,100,239] inhibit and initiate crystal growth, respectively. Also

in bone, multiple levels of physiological control delineate function and it is unclear how or if these controls are employed in the context of disease: the inhibitory function of osteopontin for example, may depend on extent of both phosphorylation[98] and polymerization[97]. The heterogeneous nature of disease combined with the known complex temporal and chemical controls of physiological mineralization suggest that the pathological roles these proteins play in mineralization are likely to be context dependent.

3.2.4 Apoptosis and Necrosis.—Physiologically, apoptosis is an important pathway component in endochondral ossification, a physiological mineralization process that is responsible for bone formation during skeleton development and bone elongation in growth plate structures. This pathway involves the formation of bone via a template of cartilaginous tissue[32], where chondrocyte apoptosis plays an important role in mineralization[240,241]. Pathologically, the release of high concentrations of calcium and phosphate concurrent with apoptosis or necrosis has traditionally been considered to be a cause of some types of calcification[119]. These processes are often described as dystrophic or degenerative mineralization, though the terminology is not consistent in the literature. Calcium accumulation is known to occur within apoptotic cells and necrotic tissues[51,120,219,242], in some cases leading to extensive intracellular mineralization or total cellular disintegration[219]. Charged phospholipid-containing membranes present within cells or within cellular debris after cell death[118], as well as stromal components such as collagen[243–245] and elastin, can serve as organic substrates on which apatite crystals can nucleate (Figure 2b).

Calcium phosphate precipitation within mitochondria, organelles important for trafficking and “buffering” cellular calcium ions, is known to occur in both physiological and pathological processes[51,54,65,246–249]. For the latter, the presence of intramitochondrial calcification is often associated with injured and dying cells[250], and, due to the active cellular mechanisms required to sequester calcium within mitochondria, potentially dependent on the mechanism of cell death[242]. Some have suggested intramitochondrial calcification to be casual[120,251] while, in other contexts, authors have proposed it to be secondary to cell death processes[242].

Within the context of cell death, the distinction between what is regulated and what is not can become blurred. Orphaned pathway components, in which direct cellular regulation is unclear or temporally offset, may be present. For example, since not all necrotic tissues calcify, additional pathway components may be required for calcification to occur, such as an imbalance of mineralization proteins. These proteins could have come directly from neighboring cells (implying a more regulated process that could be therapeutically interrupted) or could have been present in the tissue long before cell death occurred (of cellular origin, but temporally offset). Regardless, for our purposes, calcifications associated with cell injury and death are considered less regulated than those in which the timing and combinations of cellularly regulated pathway components present within the pathway are closer to those seen in physiological calcification.

3.2.5. Intracellular Mineralization.—Physiologically, intracellular mineralization is common in some types of organisms[19,51,252]. Within humans, intracellular mineralization

is often thought to be associated with cell injury and death (see Section 3.2.4). Outside of apoptosis and necrosis, there have been few reports of intracellular mineralization. Figure 2d shows an example of an e*x vivo* ultrastructural study of breast cancer in which intracytoplasmic membrane bound and free apatite or calcium phosphate crystals were identified[164]. These crystallization events, occurring within breast cancer-specific intracytoplasmic lumina, are thought to be distinct from intramitochondrial and necrosis-based precipitation[163], though leukocytic debris (that could conceivably calcify) has been reported within similar structures[253]. It has also been proposed that excretion of calcium and phosphorus-rich intracytoplasmic vesicles may be involved in breast microcalcification formation[162]. Intralysosomal calcium deposits have been found in malakoplakia, a rare disease in which the deposits themselves are diagnostic[120,219]. Another rare form of soft tissue mineralization, tumoral calcinosis, was found to have intracellular apatite deposits within mononuclear histiocytes associated with the disease. These deposits were unoriented and located near granular endoplasmic reticulum[254].

3.2.6. Vesicle Secretion.—As described in Section 2.2, vesicle secretion by osteoblasts is thought to be a critical pathway component for physiological bone formation. In pathological calcification, secreted vesicles have been observed in the extracellular matrix in proximity to multiple types of calcifications, for example vascular calcifications, shown in Figure 2e. In vascular medial calcifications, from patients with additional renal disorders, electron-dense vesicular bodies mixed with collagen were present in the extracellular matrix[255]. Mineralized vesicles were only abundant in the extracellular matrix at sites of vascular calcification, and not in healthy arteries[198,256]. In cancer-related calcifications, TEM studies of psammoma bodies showed mineral-containing extracellular vesicles in meningioma[257] and TEM images of serous cystadenocarcinoma of the ovary showed mineralization in association with lipid-rich vesicles[172]. Several recent studies using high resolution TEM to examine the early stages of kidney stone formation have also reported vesicular structures, possibly containing mineral, associated with incipient Randall's plaques[258–260].

3.2.7. Collagen and Mineral.—Figure 2 illustrates examples of key pathway components in pathological mineralization. In physiological mineralization of bone, the final product is mineralized collagen fibrils (Figure 3a,b). In pathological calcifications, there is a range of mineral-collagen interactions; examples of three different types from breast tissue are shown in Figure 3c–n. A subset of pathological deposits share morphological and compositional characteristics with bone, such as some fibroadenomas in breast tissue[167]. Figure 3(c–f) shows an example case of senescent fibroadenoma (where the epithelial element of the disease is inactive if not absent). In this example, collagen fibril alignment resembles the alignment seen in fetal bone (Figure 3a,b). At the periphery of the calcification (Figure 3e,f), the transition between unmineralized and mineralized collagen fibrils is unambiguous, when comparing the backscattered electron (BSE) and the secondary electron (SE) images. In other examples, such as the case of sclerosing adenosis shown in Figure 3g–j, mineral deposits can be in close contact with collagen fibrils but, in this case, the bulk mineral contains no fibrillar collagen, and the interfacial collagen fibrils appear unmineralized as well. Interestingly, a similar juxtaposition of collagen fibrils and mineral

deposits has been described for early stages of kidney stone formation[260]. Finally, in many cases there is no fibrillar collagen in contact with the mineral, as in the example shown within a case of low grade ductal carcinoma *in situ* (Figure 3k–n). Note that as we discuss additional examples of pathological mineralization (Section 3.3), we will see additional examples of a range of collagen-mineral interactions.

3.2.8. Putting it all Together.—As with physiological mineralization, pathological calcification progresses with time, and often we can only observe the pathological minerals as they appear at the end of processes that we did not monitor. Regardless, by drawing on our extensive knowledge of physiological mineralization pathways[2], we can propose possible pathways, as well as identify key features in the mineral and matrix to consider when describing pathological deposits. In contrast to physiological mineralization, which is directly coordinated by cells along a precisely defined pathway, pathological mineralization has a spectrum of possible pathways, ranging from a lack of cellular involvement to various cellularly regulated processes. Regardless of the tissue type, cells often appear in direct contact with mineral, while in other cases, only cellular debris or stroma is evident near the mineral. Even when cells contact mineral, are they passive bystanders or are they taking an active role in the calcification process? We define two theoretical extremes along this spectrum: unregulated mineralization (only rules of physics apply, and we can expect processes similar to abiotic mineralization) and regulated mineralization (cells actively coordinate mineralization, similar to physiological mineralization of bone, but the bone is forming at the wrong place, e.g., heterotopic ossification[22]). In between, we propose the term “dysregulated mineralization”, which comprises a continuum of processes in which the role cells play in mineral formation ranges from apparently passive to overtly active.

Unregulated Mineralization Pathways are defined as theoretical extremes in which pathological mineral formation occurs without the involvement of active cellular processes, i.e., the only pathway components are the ion sources and matrix components, in some cases also including cell debris, already present in the microenvironment. Thermodynamically, for a mineral to precipitate from solution, the solution must be supersaturated with respect to the product. In supersaturated solutions, homogeneous nucleation occurs in the bulk, and heterogeneous nucleation can also occur on favorable substrates at lower supersaturations (Figure 2a). Nucleation is followed by crystal growth via classical ion-by-ion growth[261,262] or non-classical particle attachment[5] mechanisms. As already described, in healthy organisms, the supersaturation in biological fluids is tightly controlled to avoid unwanted spontaneous nucleation, whereas in some pathological conditions this balance is disrupted. Similarly, in healthy tissues, the interfacial tension is designed to induce mineralization only at desired locations. Surface energies can be controlled, for example, by changing the functional groups of substrates to favor nucleation and by utilizing proteins to optimize the surface tension[56,57].

An example of unregulated pathological mineralization is the precipitation of calcium pyrophosphate dihydrate (CPPD) crystals within synovial fluid of patients with pseudogout, in which the balance between pyrophosphate and phosphate ions has been disrupted, leading to supersaturation of the fluid with respect to CPPD (Figure 2a)[147,263]. Another example of unregulated mineralization is thought to occur in chemically-fixed bioprosthetic heart valves

(tissue valves). It is proposed that mineral nucleates within the fixed tissue as calcium from the blood concentrates within the cells, which are unable to pump calcium ions out, as living cells would, and eventually mineral growth spreads into the extracellular matrix (collagen and elastin)[264].

Dysregulated Mineralization Pathways are defined as processes involving cells, which are not osteoblasts. These pathways involve multiple pathway components, in which cells regulate crystal nucleation, growth, and/or aggregation to some extent, via vesicle secretion, intracellular mineralization, and/or an imbalance between proteins that function as mineralization inhibitors and promotors[24]. This type of pathological mineralization is not a result of uncontrolled nucleation on cell debris or ECM, nor regulated osteogenic processes that produce bone-like materials. For example, in cardiovascular tissues, where the extracellular matrix has an active role in inhibiting calcification[20], interference with the inhibition mechanism can lead to pathological calcification[265]. In dysregulated pathways, the pathway components highlighted in Figure 2 will appear in different combinations, and perhaps in different sequences than physiological pathways.

Regulated Mineralization Pathways are processes that look very similar to physiological mineralization, but occur in anatomical locations where bone formation should not occur. The pathway components, i.e., cells and matrix components, as well as the final product, e.g., mineralized collagen fibrils (Figure 3a,b), are all similar to the physiological processes. Such processes produce a bone-like material in a pathological context, e.g., heterotopic bone formation, also called osseous metaplasia[22]. Regulated pathological mineralization requires a change in the cell type, through differentiation or phenotype change of the local cells, to osteoblast-like or chondrocyte-like cells that can deposit collagen and mediate the mineralization process according to a pathway resembling that described in Section 2.2.

3.3 Representative Examples of Pathological Mineralization

Here, we will focus on three examples of pathological calcifications formed in different anatomical locations and as the result of different disease processes- **vascular calcifications, breast microcalcifications, and psammoma bodies** (found in thyroid and ovarian cancer and in meningioma tumors in the brain). We will discuss vascular calcifications, which are a well-studied system where the root cause is unrelated to cancer, and two cancer-related calcifications, breast microcalcifications and, the least studied type, psammoma bodies.

For each example, we discuss evidence for the involvement of different “pathway components” described in Figure 2: cell death (necrosis/apoptosis), an (im)balance of mineralization proteins (inhibitors and promotors), intracellular mineralization, and vesicle secretion. These pathway components are combined in different ways to lead to the final product, i.e., the pathological mineral deposit, and the resulting pathways range from unregulated to dysregulated to regulated. In addition to identifying the pathway components, we also consider the morphology and composition of the final product. We consider the presence of bone-like, mineralized collagen fibrils to be indicative of a more regulated mineralization pathway. We assume that the formation of such structures usually requires a highly coordinated series of events from uptake of ions to matrix production to matrix mineralization. By using pathway components to help define the level of cellular regulation

over pathological mineralization, future studies may focus on identification of these "markers" in pathological calcifications to better understand the mineralization pathway, and begin to develop interventions to prevent or even reverse pathological mineralization.

3.3.1 Vascular Calcifications.—Cardiovascular calcifications are associated with higher risk of mortality and cardiovascular events[266] and often cause organ dysfunction[24,119]. These pathological calcium phosphate deposits are found in blood vessels in atherosclerotic plaques (intima), in the medial layer of blood vessels (Figure 4a), and in aortic valves[24,267]. Atherosclerotic plaques contain calcifications in addition to cholesterol crystals, and calcification formation increases with high low-density lipoprotein (LDL) cholesterol levels[268]. Coronary artery calcification is related to elevated hard coronary events and mortality[269–271] and the extent of arterial calcification is correlated with atherosclerotic plaque burden[224,267,272–278]. Also, blood vessel calcification can lead to stiffening of the vessels, which may contribute to vessel rupture[153]. Medial vascular calcifications are associated with diabetes and renal disease[28,279–283] and increase with aging[284].

The mineral phase of intimal and medial vascular calcifications is usually apatite or whitlockite, and in some cases octacalcium phosphate (see Table 1). The calcification sizes range from submicron[197] to larger than 0.5 mm[225] and histology is often used to characterize their morphology and microenvironment[285] (Figure 4a,b). The different calcium phosphate phases can be in close proximity to each other and are in contact with an organic matrix of structural proteins, collagen and elastin (Figure 4 c,d). It was recently shown that there is an interplay between intimal (atherosclerosis-related) and medial calcifications, and that the ratio of apatite to whitlockite increases in the aortic media directly underlying an atherosclerosis plaque[200]. Vascular calcifications present in aortic and valve tissues can appear as mineralized dense fibers, compact mineralized material, and spherical mineral particles[197]. The apatite and whitlockite mineral particles show distinct morphologies, including spheres, plates, and aggregates (Figure 4 e–h).

The types of cells involved in cardiovascular calcification, especially in atherosclerotic plaques, are under debate. In studies of human arteries, medial and intimal calcifications were shown to be associated with vascular smooth muscle cells (VSMCs) activity. *In vitro* studies showed that in certain conditions, VSMCs and macrophages[286] can differentiate into osteoblasts[287–289] and chondrocytes[250]. In atherosclerotic plaques, heterotopic ossification is also known to occur in which cells resembling osteoblasts, osteoclasts, and osteocytes are observed in proximity to bone-like material[285,290,291]. VSMCs can differentiate into osteoblasts, and mesenchymal stem cells can also be recruited to blood vessels and later become osteoblasts[292]. In addition to osteogenic cell-types, during early-stages of atherosclerosis, as part of an inflammatory response, there is an accumulation of macrophages and other immune cells near the lesions[293].

Medial calcifications most likely form through a pathway different from intimal calcification formation[29]. To describe the mineralization pathway and extent of cellular regulation in medial and intimal vascular calcifications, we will use the "pathway components" from Figure 2. In some conditions, VSMC **apoptosis** induces medial vascular calcification

in clinical samples[173] and *in vitro*[223,294]. In fixed VSMCs, mineralization increases as compared to live cells and the resulting mineral is less crystalline in live cells as compared to lysed cells[295]. VSMC apoptosis is accelerated by dialysis and induces medial vascular calcification in children with chronic kidney disease[294]. In atherosclerosis (intimal calcification), apoptotic bodies are found in close proximity to calcifications and VSMC apoptosis may promote mineral deposition (demonstrated *in vitro* and in mouse models) [285,296–299].

There is also a large body of evidence for the involvement of imbalances of **mineralization proteins** in vascular calcifications. Using immunohistochemistry and reverse transcription-polymerase chain reactions (PCR), calcified blood vessels are correlated with high expression of mineralization proteins such as ALP, BSP, osteocalcin, and collagen I compared to vessels with no medial [265,282,289,300] and intimal calcifications. In patients with end-stage renal disease, calcifications are associated with osteopontin[198,282,301], type I [282] and type II[265] collagen, bone sialoprotein[265,282], alkaline phosphatase[265,282], Cbfa1 (a transcription factor involved in osteoblast differentiation)[301], fetuin-A[198], and matrix GLA protein[198] (also reported to be downregulated in some cases[265]). Loss of mineralization inhibition may also play a role in medial calcification[267], where matrix GLA and osteopontin most likely inhibit vascular calcification[152,265], and mice lacking the protein developed extensive medial calcifications[20]. VSMCs from bovine and mice that undergo osteogenic differentiation can contribute to bone-like medial calcification[222,302,303]. The calcification of VSMCs incudes vesicle secretion and expression of mineralization proteins, as was reported in various in vitro models[151,154,223,256,267,288,304].

Vesicle secretion also takes part in the mineralization process, as is evident from the abundance of vesicles, sometimes mineralized themselves, observed by TEM (Figure 2e) in the extracellular matrix near medial calcifications[198,256] and from *in vitro* studies[223,299]. In patients with renal disorders, electron-dense vesicular bodies mixed with collagen are present in the extracellular matrix[255] and mineralized vesicles were only abundant in the extracellular matrix at sites of vascular calcification, and not at healthy arteries[256]. Calcifications were also observed near vesicular structures in cases of renal disorders[198] and in calcified atherosclerotic plaque[298]. In atherosclerosis, macrophages secrete vesicles that induce calcification in chronic renal disease[305], and extracellular vesicles secreted by macrophages and VSMCs can either promote or inhibit cardiovascular calcification depending on the tissue pathology[306,307]. It has been suggested that vesicle secretion by macrophages contributes more than osteogenic differentiation of VSMCs in inducing intimal calcifications and that these extracellular vesicles are different in origin and cargo than matrix vesicles secreted by osteoblasts[305].

Intracellular mineralization is not common in vascular calcifications, while increased intracellular calcium concentration associated with hypertension was reported in calcifying atherosclerotic plaques[286].

In human lesions with medial calcifications, the extracellular matrix likely has a role in mineral nucleation and growth and there are several examples of medial calcifications

involving **mineralized collagen**. Medial calcifications are associated with elastin[225,256], collagen II[265] and collagen IV, the vascular wall collagen, which is replaced with collagen I in some areas in patients with end-stage renal disease[282]. Calcifications were observed by TEM near collagen fibrils in cases of patients with kidney disorders[198]. Calcified vesicles associated with elastin and collagen are observed in minimally calcified arteries from patients with atherosclerosis and chronic kidney disease. In more calcified vessels from diabetic patients, mineralization extends into the extracellular matrix fibers[256]. Structurally, in medial calcifications the mineral is not aligned along collagen fibers, its structure does not resemble bone platelets, and calcified spherical particles are involved in the calcification[197,308].

Clearly, there are similarities between vascular calcification and osteogenic processes, and on the spectrum of the extent of cellular regulation, vascular calcification is considered to be a regulated pathological mineralization pathway[51,291]. Though most medial cardiovascular calcifications share some morphological and compositional features with bone[273] (e.g., apatite, mineralized collagen), they are not the same, with the exception of some clear examples of heterotopic ossification. Some differences from bone are that the organic matrix contains a large amount of elastin, whitlockite is often present, mineral density can be heterogeneous, and mineral crystals are not crystallography aligned with collagen fibers[267]. These differences imply that vascular calcifications do not form by the same mechanism bones do, but through a similar mineralization processes in which vesicle secretion, expression of mineralization proteins and in some cases collagen mineralization play important roles.

3.3.2. Microcalcifications in Breast Cancer.—Breast microcalcifications are calcium-containing mineral deposits that form in the breast in association with benign and malignant processes, and serve as an important screening tool for breast cancer based on their appearance in mammography[309,310]. Benign, or non-cancerous, conditions in which microcalcifications have been found include trauma-induced fat necrosis, glandular atrophy with aging, and benign proliferative lesions such as fibroadenoma (Figure 3c–f) or sclerosing adenosis (Figure 3g–j), among others[311]. Malignant microcalcifications are those associated with precancer (*e.g.* ductal carcinoma *in situ*: DCIS) or cancerous processes (*e.g.* invasive ductal or invasive lobular carcinomas). The differences in surrounding tissue architecture, frequency, density, and size of mineral (or mineral aggregates) between benign and malignant calcifications, though comprising a complex diversity of conditions to be sure, provide a morphological basis for diagnosis (and in some cases prognosis[312,313]) by mammography; *i.e.* the projected x-ray images of microcalcifications (and dense tissue) can be indicative of the conditions or severity of conditions in which they are found. For example, microcalcifications that form due to benign fat necrosis and associated inflammation often have an egg-shell like morphology, while microcalcifications with rod-shaped, linear or branching morphology are thought to form in necrotic areas of malignant high-grade DCIS.[310].

Broadly, breast cancers with associated calcifications have worse prognosis than lesions without calcifications[314–317]. The reasons for this difference are not clear. Some suggest that calcification formation is indicative of higher cell turn over[318], though there is

evidence to suggest that it goes beyond this, and that calcification properties may play a role in cancer progression. *In vitro* breast tumor model studies suggest that cancer behavior and malignancy are related to microcalcification mineral characteristics. Mitogenesis of human breast epithelial cells is enhanced by hydroxyapatite presence[319]. Hydroxyapatite mineral morphology and the extent of carbonate substitution in the crystal affect the breast cancer cell behavior in terms of cell growth and secretion of tumorigenic interleukin-8 (IL-8)[130]. Also, in 3D mineralized tumor models, hydroxyapatite promotes features associated with breast cancer metastasis to bone and enhances malignant potential[131,320].

Multiple phases of breast microcalcifications have been identified, *ex vivo*: calcium phosphates including substituted carbonated apatite, whitlockite[158,161,166], and amorphous calcium phosphate[159,165] and, less commonly, calcium oxalate dihydrate bipyramidal crystals[159,160] (Table 1). Calcium phosphate calcifications occur within benign or malignant tissue, while calcium oxalate dihydrate calcifications have only been found associated with benign tissue[321]. The calcium phosphate calcifications are extremely heterogenous in terms of their size, ranging from submicron[166] to a few millimeters[158,160,164], morphology, including needles, punctate particles, aggregates, and laminate calcifications[160,164,166], and local tissue environment (Figure 5). It has been shown that the chemical composition of the apatite microcalcifications, namely the amount of carbonate substitution, may decrease with tissue malignancy as well as cancer grade[167–170]. Trace substitutions (e.g., Na and Mg), and apatite lattice parameters may also relate to malignancy, as well as amount of organic matrix. The presence of whitlockite may also relate to malignancy, though there are conflicting reports[161,162,168,322].

The most well-known calcification environment for breast cancer, perhaps due to diagnostic value, is cancer-related **necrosis**[158–160,166,311,323–329]. Necrosis can occur in DCIS as well as invasive breast cancers. Calcifications are more common in DCIS with necrosis than in DCIS without necrosis[325] and frequency of occurrence has been observed as high as 96% of DCIS cases with associated necrosis[310,325,330,331]. Calcifications associated with necrosis may have markedly differing histological appearances than those formed in non-necrotic cancerous ducts[158,310,327,328,331–333]. One study found organic matrices within necrosis-located granular calcifications stained positive for fragmented nuclear material and weakly positive for acidic mucosubstances[327] while laminate calcifications, found in well-differentiated DCIS, stained strongly for acidic mucosubtances (e.g. glycoproteins or proteoglycans). DCIS often harbors an abundance of cancer cells that undergo **apoptosis**, which could contribute to the presence of necrosis and/or calcifications[311]. Necrosis is a categorization requirement for a particular high grade subtype of DCIS: comedo-type DCIS. Within comedo-type DCIS (Figure 5a) a necrotic region is thought to arise due to hypoxia and a diffusion-based lack of metabolites for certain regions within the expanding duct, where there is a correlation between the duct diameter and the presence of necrosis[311]. Distinct regions of viable and dead cells (necrosis) are often observed.

DCIS necrotic calcifications frequently present as large, tissue-mineral aggregate masses that span hundreds of microns[311,334]. Often, regions of mineralization are detected within the same duct (and neighboring ducts) by mammography, or cross sectionally in H&E[328,334], perhaps suggesting a systemic microenvironment in which multiple, necrosis-

based nucleation events can occur. This could be interpreted microscopically as well; Figure 5a–c shows an area of necrotic duct tissue that contains dispersed punctate particles, as though arising by independent nucleation events[166], though the transition to the large mammographically-detectable calcifications is unclear. Nor is it even clear if the timing of their nucleation coincided with the absence of living cells (though this is generally assumed to be the case). The location of large calcifications within necrotic DCIS also varies from appearing contained within the necrotic region[334], to spanning outward into the viable cells, to existing solely at the periphery of necrosis or even adjoining the collagenous ductal membrane[311,328], though it is unclear where these initially nucleated.

In addition to apatite particles found in necrotic regions of DCIS[166], calcium phosphate deposits have been observed in invasive ductal carcinoma surrounded by cellular debris[159], or in contact with stroma and/or cancer cells[166,167]. Frequency of calcifications within invasive cancer appear to be lower than DCIS, around 50%, though the exact occurrence may depend on patient age[318]. An example of invasive ductal carcinoma is shown in Figure 5d. Particles were similarly sized to those in Figure 5 b,c yet were very different. In this case they were whitlockite particles embedded in (but not mineralizing) a fibrillar collagen organic matrix (Figure 5 e,f). Even within their respective lesion, calcifications can have differing properties, including the presence of multiple phases in which size and morphology can also differ[166]. This heterogeneity of mineral and microenvironment could simply represent the power of degenerative cellular processes to broadly induce calcification[219], but, in cases where living cells interface with microcalcifications, might also suggest varying levels of cellular regulation at work.

In support of a more cellularly regulated process, in addition to necrosis, **vesicle** involvement in microcalcification formation has been suggested[164], and **intracellular calcifications** (non-mitochondrial) in living cells were observed within breast carcinoma cells using TEM (Figure 2d)[163,164,251]. One study specifically focused on intra- and intercellular lumina, structures unique to certain types of tumor cells, and found apatite and amorphous calcium phosphate within these structures in one third of the breast cancer patients analyzed[163]. Furthermore, in addition to colocalized mucosubstances, specific **mineralization proteins** such as osteopontin[162,331,335,336], BMP[162], BSP[337] and osteonectin[336] have been identified in association with breast microcalcifications. Breast carcinomas have significant increase in bone sialoprotein compared to benign lesions[337] and tumors that express osteopontin are more likely to be associated with calcifications[335]. Osteopontin is observed in von Kossa-stained regions of carcinomas and atypical cystic lobules[331] and osteopontin and BMP-2 overexpression is observed in infiltrating carcinomas with microcalcifications and not in those with no microcalcifications[162]. Recently, we showed in a 3D multicellular spheroid model of breast epithelial cells that calcification formation increases with malignancy, is increased in the viable cell region compared to the necrotic region of the spheroids, and is associated with an overexpression of OPN and a decrease in ALP expression[156].

There is an ongoing debate in the literature about the implications of the relationship between microcalcifications and mineralization proteins. The co-localization of mineralization proteins and microcalcifications has led some authors to hypothesize

that microcalcification formation is a bone-like process, which was supported by *in vitro* models[162,227]. Unlike bone, however, no mineralized collagen has been shown to be associated with microcalcifications in the case of malignant calcifications[164,166,338] (though this does not preclude nucleation in a collagenous environment) and there are many other compositional and morphological differences between bones and microcalcifications as described above. Histologically, cases of heterotopic ossification or chondroid metaplasia are known to occur within breast tissue, but are rare in both benign tumors and carcinomas[311,339]. Fibroadenoma (a benign condition) is a special case in which fibrillar collagen can be mineralized (Figure 3c–f), and can even have fibrillar alignment, meeting many more of the morphological criteria to be considered bone-like.

As with vascular calcification, there is difficulty of defining a mineralization pathway within cancers, and there are multiple competing theories in this arena. For breast calcifications the amount of heterogeneity surrounding microcalcifications - their local malignancy, cellular microenvironments, and extracellular matrix components –suggest that one pathway (or combination of pathway components) does not fit all. Based upon our assessment of the literature, most microcalcification formation is likely not the product of a regulated bone-like mineralization pathway, but rather a family of dysregulated pathways that combine several pathway components, including cell death, out-of-balance mineralization proteins, and in some cases intracellular mineralization and/or an active vesicle-based mineralization process. Mechanistic insight is currently limited to individual patient snapshots, typically in an advanced disease state, and with the added complication of cancer heterogeneity, where cancer cells are highly mutated and have often shed their original identities and differ significantly between individuals. Future work in this area should look to expand the combination of biological and materials science techniques to unearth the cancer-hijacked cellular mechanisms that could be associated with mineral formation, e.g., combining mineral properties data with proteomic or lipidomic data sets to identify mineral-matrix “fingerprints” associated with cancer severity.

3.3.3. Psammoma Bodies.—Psammoma bodies are mineral deposits usually found in papillary thyroid carcinoma, meningioma, and papillary serous cystadenocarcinoma of ovary[340], and are sometimes also called microcalcifications. Some breast microcalcifications have been likened to psammoma bodies as well[327]. Approximately one third of papillary thyroid carcinoma and ovarian cancer cases are associated with calcifications, which are often detectable using ultrasound, CT, and MRI[340–342]. Unlike the generally negative response of the tissue to vascular and breast calcifications, it was suggested that psammoma bodies may be the result of a controlled biological process in which tumor cells are being degenerated, serving as a barrier against cancer spread[340].

The mineral of psammoma bodies is calcium phosphate in the form of apatite (see Table 1). Compared to breast microcalcifications, the microstructure and chemical composition of psammoma bodies are poorly characterized. Psammoma bodies are round-shaped, concentric lamellar calcifications, tens of microns in diameter[340]. They can be identified within the tissue using histopathology and are often observed in close proximity to cancer and stromal cells (Figure 6a). In meningioma, psammoma bodies are formed inside concentric whorls of tumor cells[257,343].

Psammoma bodies are often associated with cell debris and **necrotic** tumor regions[171,172], suggesting, at least in part, an unregulated mineralization pathway. TEM of papillary thyroid carcinoma shows that psammoma bodies located in the stalks of the papillae are associated with cellular debris[171] and in fibroblastic meningioma, micron-size bodies containing hydroxyapatite are associated with necrotic regions[257,344]. In a TEM study of serous cystadenocarcinoma of the ovary, calcium deposits are associated with necrotic areas in the tumor[172].

Carcinoma cells that show overexpression of **mineralization proteins** such as osteopontin and bone sialoprotein[345–347] are correlated with calcification presence as shown using immunohistochemistry and reverse transcription PCR. In papillary thyroid carcinoma, the levels of bone sialoprotein and osteopontin are elevated in specimens containing histological calcifications[348]. In ovarian tumors psammoma bodies are associated with BMP-2 and collagen IV[347].

Psammoma bodies show key pathway components of dysregulated mineralization, such as **vesicle secretion**[172,257,344] and **intracellular mineral formation**[340,344,345]. TEM of psammoma bodies in meningioma shows hydroxyapatite-containing extracellular vesicles[257] and in a TEM study of serous cystadenocarcinoma of the ovary, mineralization occurs on lipid-rich vesicles[172]. In cystadenocarcinoma of the ovary, mineralized intracellular vesicles were observed in epithelial and stromal cells[172].

Collagen and elastin fibers seem to have a role in psammoma body formation, and they are often found associated with the calcifications[120,173,257,343,344]. Calcifications in ovarian tumors are surrounded by collagen fibers (Figure 6 b)[173]. In fibroblastic meningioma, mineral-containing bodies are found around collagen fibers[257,344] and collagen fibers are embedded in the mineralized bodies[120] (Figure 6c,d). In meningioma, psammoma bodies show an internal structure of strands and fibers oriented in a circular manner[343]. There is no current evidence, however, that the psammoma bodies share the hierarchical bone structure or even that aligned apatite crystals reside within the collagen fibrils. Future studies could better assess whether mineral is truly intrafibrillar or interfibrillar within psammoma bodies. Also, it is not clear whether the cancer cells deposit the collagen fibers or the psammoma bodies are simply associated with stromal collagen[340].

4. Concluding remarks and future directions

In this Progress Report, we have provided a framework within which to compare and contrast biomineralization pathways in a representative physiological system, bone remodeling, and in three diseases in which pathological calcification is prevalent. In all cases, we evaluated evidence for different types of “pathway components” such as necrosis, apoptosis, expression of mineralization proteins, vesicle secretion, and intracellular calcification. The varied combinations of these components manifested within mineralization pathways encompass a spectrum of cellular regulation, in which we define the terms unregulated, dysregulated, and regulated pathways for easier classification of the extent of cellular involvement in pathological calcification. Physiological bone remodeling is used as a quintessential example of a regulated mineralization pathway, which under

strict cellular control yields a hierarchically-structured, mineralized tissue whose primary building blocks are apatite nanocrystals embedded within type I collagen fibrils. In contrast, most pathological calcifications appear to be the product of unregulated and dysregulated pathways, which result in heterogeneous deposits in terms of organic and inorganic composition, structure, and morphology.

By using a common set of terms and concepts throughout this Progress Report to discuss pathological mineralization pathways, we hope to frame the ongoing debates within the literature to enable the identification of common (and disparate) features across these systems. There are multiple unresolved questions in the literature surrounding the nature of pathological biominerals, including their chemical compositions and/or crystal properties, and the degree to which these properties are impacted by, or impact, cellular behavior and/or disease state. We have highlight several of these debates throughout the Progress Report, and there are certainly many more in the literature. For example, increased whitlockite presence in breast microcalcifications has been linked to both cancerous and benign pathologies [161,168], raising questions of how specific pathologies contribute to environments in which whitlockite preferentially precipitates, and whether, analogous to apatite, there may be a range of cellular involvement and/or mineral compositions. In intimal cardiovascular calcifications, there is a lack of consensus regarding the types of cells, e.g., macrophages or vascular smooth muscle cells, that majorly contribute to the mineral deposition[305–307]. A similar debate is ongoing in cancer-related calcifications as to whether cancer cells undergo osteogenic differentiation and subsequently deposit mineral[349]; if they are otherwise participating in mineral deposition via a dysregulated secretory process of mineral and/or protein[150,164,350]; or if the mineralization process stems from a necrosis-based pathway[159,166], with the caveat that the mechanisms themselves are likely context-dependent.

One of the main mechanistic questions we have asked regarding pathological calcification formation concerns the extent of cellular regulation over the processes. Namely, do the cells somehow control the formation of calcium deposits in soft tissues or is pathological calcification primarily a chemically-controlled crystallization process, a byproduct, for example, of cell death? This question is essential in the context of biomedicine because of the interest in using pathological calcifications and their properties to diagnosis disease progression and to identify possible treatments or interventions. Understanding the degree and kind of cellular involvement in the mineralization pathway will provide insight into how the calcifications are related to disease progression and if and how the processes can be therapeutically disrupted. In the case of cardiovascular calcifications, the mineral deposit itself is a risk factor, leading to poor health outcomes. A better understanding of the details of these mineralization pathways could lead to the development of therapeutic approaches to prevent, limit, or even reverse formation of cardiovascular calcifications. For example, recent advances in understanding the crystal growth mechanisms of kidney stones has lead to the development of small molecule inhibitors to treat cystinuria and other types of urolithiasis[351–355]. In the case of tumor-associated microcalcifications and psammoma bodies, the presence of these deposits are often linked to more aggressive cancers, but it is less clear if the calcifications are a by-product and/or a trigger of disease progression. Here, the efforts to elucidate the calcification pathways can lead to fundamental insights at the

biochemical level about the cellular mechanisms that are at work in that individual cancer, and perhaps yield improved diagnostic capabilities based upon the chemical composition and ultrastructural details of individual calcifications. A better understanding of the formation mechanism of malignant calcifications as opposed to benign calcifications may be used to improve disease diagnosis and prognosis based on the calcification characteristics. For example, the potential of using the crystal properties of pathological calcifications for diagnosis was already demonstrated for kidney stones[356–359].

As highlighted throughout this Progress Report, a key to advancements in this field are linked to the application of high-resolution, materials science characterization techniques to advance our understanding of the crystallization pathways by determining the crystal structure, chemical composition, morphology, and organic microenvironment. By coupling these types of techniques with traditional methods from pathology, such as specific and non-specific staining and light microscopy, we can more fully describe both the biological and materials characteristics of pathological calcifications. In particular, the emerging high-resolution imaging techniques are well-suited to examining initial stages of calcification, which often hold important clues to mineralization pathways. For example, the source of the ions that comprise the calcifications, how the ions are concentrated and/or transferred to the site of nucleation, the nature of the nucleation events leading to calcification, and the substrates on which nucleation occurs are still unknown for most pathological calcifications. Answering these types of questions requires characterization of the chemistry and ultrastructure of the microenvironment surrounding the microcalcifications. Similarly, the nature of the interactions of the calcifications with their microenvironment and how these interactions affect disease progression are also not yet understood. For example, by combining histology, Raman microscopy, electron microscopy, and elemental analysis, we have begun to investigate the relationships among the local malignancy, tissue type, ECM chemistry, and microcalcification mineral properties (including phase, morphology and elemental composition)[166]. Future studies of this type have the potential to unravel the diversity of mineralization pathways as a function of cancer severity.

While much of this Progress Report has focused on understanding the pathways via which pathological calcifications form, there is a related set of questions regarding the influence of the calcifications and their physical properties on the surrounding cells and tissues. For example, *in vitro* studies using synthetic HA nanoparticles and mineralized collagen platforms have suggested that tumor cell adhesion, growth, and secretory functions are regulated by collagen mineralization and the mineral properties, including size, crystallinity, and carbonate content[130,131,320,360]. Questions can also be asked about how other cell types, e.g., cancer cells, respond to and alter the properties of physiologically formed mineral, e.g., bone[361]. For example, a recent mouse study using high-resolution X-ray scattering methods and Raman imaging, found that skeletal sites prone to tumor cell dissemination, e.g., bone metastasis, contain less-mature HA (i.e., smaller, less-perfect, and less-oriented crystals)[362]. As our understanding of the interplay among mineral formation, mineral properties, disease progression, and immune response evolves, so will our ability to develop appropriate interventions.

We hope that the approach of identifying mineralization pathway components to better understand the commonalities and differences among pathological calcifications will be more broadly applied to a diversity of pathological minerals. By leveraging decades of study of physiological biomineralization pathways, we ask: how does the mineral form in pathological conditions and what role do the biological components play along the pathway leading to the final mineralized tissue? By asking these types of questions, we believe the entire field will move forward to gain both fundamental insight into these diseases, as well as to improve disease diagnosis, prognosis, and treatment.

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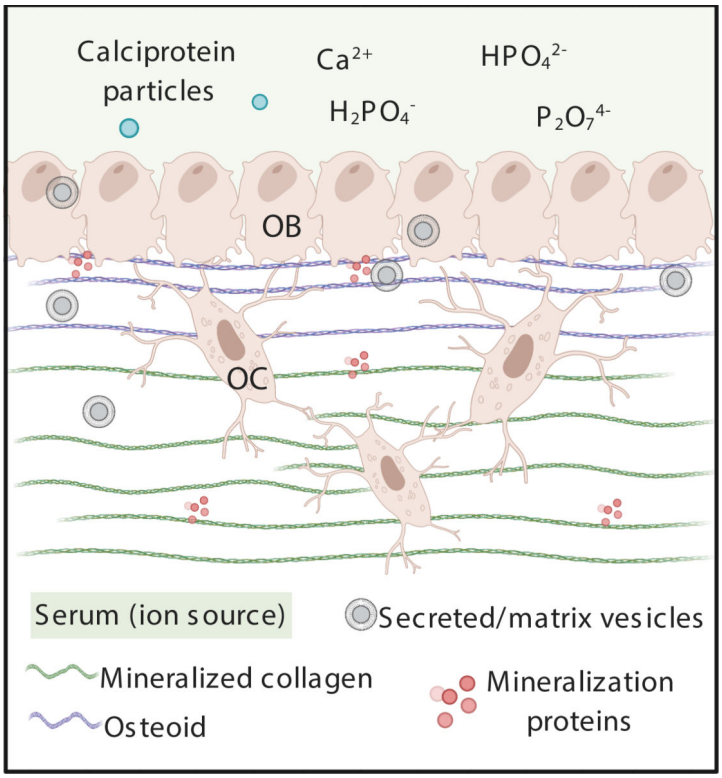

Figure 1.

Schematic showing mineral deposition during bone remodeling. See text for further details regarding the sequence of events. Ion sources in solution (including calciprotein particles), mineralization proteins, cells involved in the mineralization process, vesicle secretion, and mineralized collagen are components of this mineralization pathway. Osteoblasts (OB) uptake mineral precursors from the serum, deposit the bone extracellular matrix (purple; osteoid), as well as release vesicles and mineralization proteins into the osteoid. Mineral nucleation and growth occur within the collagen fibers that make up the osteoid, resulting in mineralized collagen fibers (green). Osteocytes (OC) remain buried within the mineralized matrix.

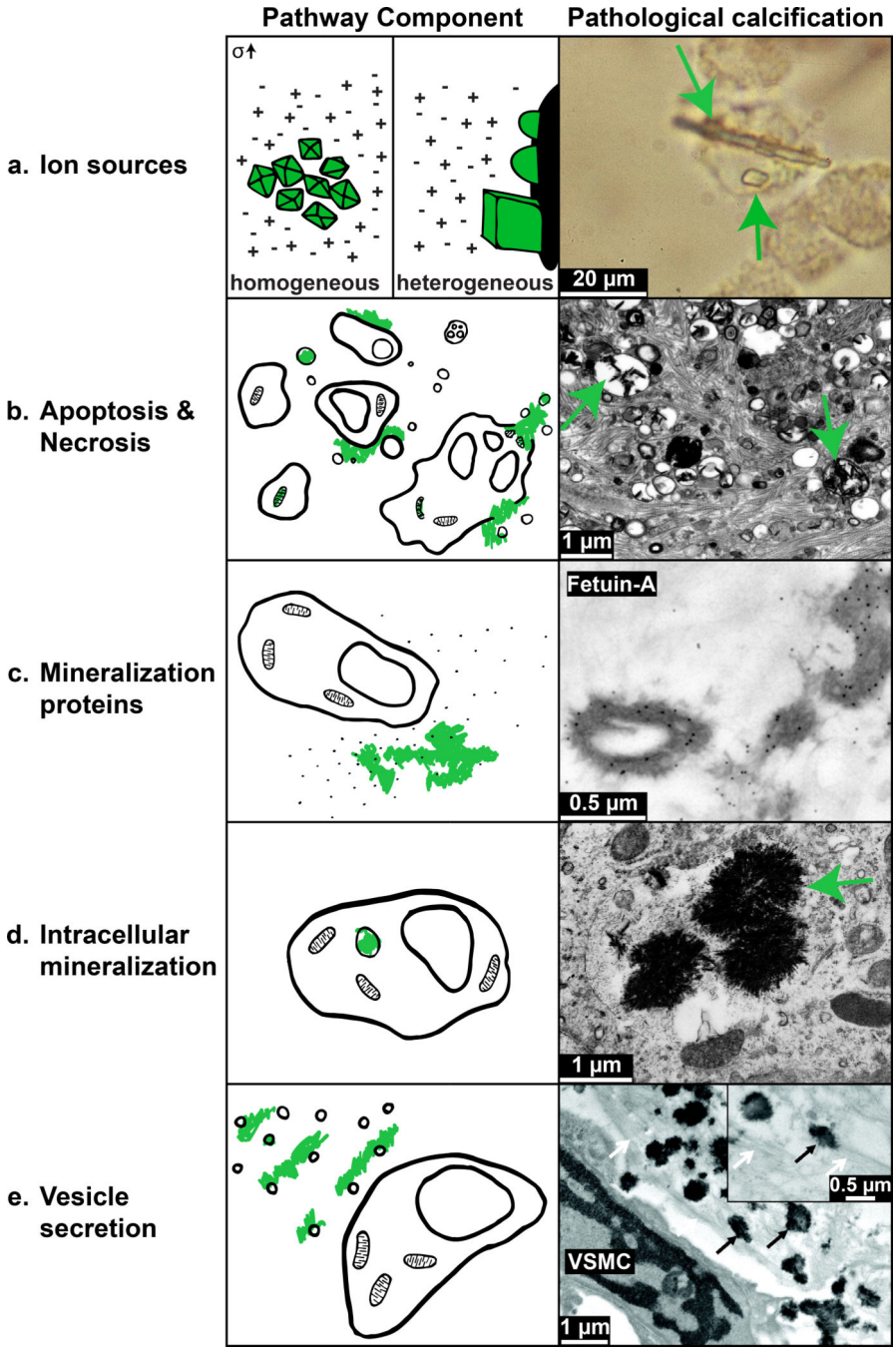

Figure 2.

Pathway components identified in pathological mineralization, see text for more details about each component. Left: Schematic representations (green – mineral) and Right: examples of pathological calcifications where the corresponding pathway component is observed. Though a single cell is shown schematically (left), much pathological mineralization occurs in a multicellular environment: (a) **Ion sources** in solution, i.e., serum and other biological fluids. Homogeneous nucleation can occur in bulk solution when the local supersaturation (σ) increases and heterogeneous nucleation can occur on cellular

components or debris or on foreign bodies at lower supersaturations due to a reduction in the energetic barrier to nucleation. Light microscope image of calcium pyrophosphate dihydrate crystals extracted from the synovial fluid of a patient with joint disease. Rhomboidal and needle-shaped crystals are observed (arrows)[143]. (b) **Apoptosis/necrosis**. TEM image shows vascular calcification, calcium deposits are observed in cell debris areas, some within vesicles (arrows)[120]. (c) **Mineralization proteins**. High-resolution TEM image of immunogold labeling of calcifications in human arteries shows Fetuin-A, a known mineralization inhibitor, associated with calcifications[198]. (d) **Intracellular mineralization.** TEM image of human breast carcinoma, clusters of needle-shaped crystals are observed (arrow) in an intracytoplasmic lumen[164]. (e) **Vesicle secretion**. TEM of human arteries showing a vascular smooth muscle cell (VSMC) surrounded by vesicles containing calcifications (black arrows) in the extracellular matrix. White arrows indicate collagen fibrils. Inset: higher magnification of the calcification and collagen[198].

2a: Adapted from *Best Practice & Research Clinical Rheumatology,* 19, 3, E Pascual, V Jovani, Synovial fluid analysis, 371–386, Copyright 2005, with permission from Elsevier

2b: Adapted from *Ultrastructural Pathology*, 25, 3, 243–267, FN Ghadially, As You Like It, Part 3: A Critique and Historical Review of Calcification as Seen with the Electron Microscope, Copyright 2001 by permission of the publisher (Taylor & Francis Ltd, http://www.tandfonline.com).

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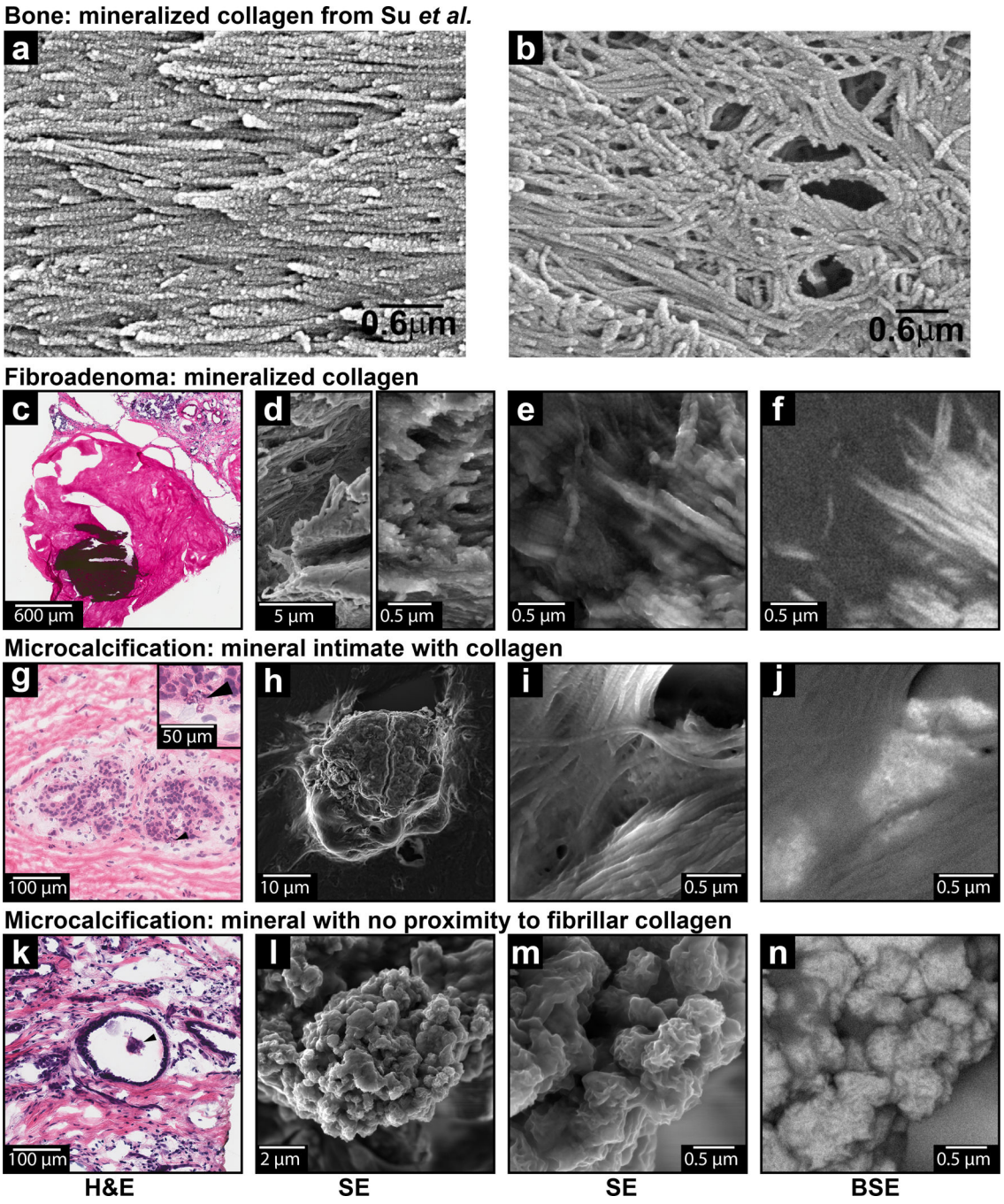

Figure 3.

Diversity of collagen mineral arrangements in physiological and pathological mineralization. (a) SEM images of mineralized collagen in endosteal and (b) periosteal surface layers from longitudinally fractured femoral mid-diaphyseal human fetal bone[44]. Mineralized collagen fibrils are parallel in the endosteal layer and interwoven in the periosteal layer. (c-n) Example breast cancer microcalcifications (human). For each calcification an H&E stained neighboring section (c, g, k), a contextual lower magnification secondary electron (SE) image (d,h,l), a higher magnification SE image (e,i,m), and a corresponding backscattered

electron (BSE) image (f,j,n), taken at the interface between matrix and mineral, are shown. (c-f) Fibroadenoma. (c) The large calcification (black-purple) is amidst connective tissue (pink) within senescent fibroadenoma. (d) Left: morphologies of mineralized collagen within the bulk mineral including packed fibrils and blocky “chunks”. Right: a higher magnification of the packed fibrils. (e,f) a transition from unmineralized to mineralized collagen fibrils at the periphery. (f) The BSE image shows mineral that reproduces exactly the shape and pattern of the collagen fibrils. (g-j) Sclerosing adenosis. (g) A microcalcification is indicated (arrowhead) at the intersection of lobular cells and connective tissue (Inset: higher magnification). (h) From SE, the calcification appears embedded in connective tissue. (i,j) At the interface, mineral and collagen appear colocalized. The BSE image (j) shows a lack of the unambiguous mineral-collagen matching, as in (f). (k-l) Low grade micropapillary pattern DCIS. (k) The section shows some freeze artifact but mineral can be seen within a cancerous duct (arrowhead). (l) The calcification shows no sign of fibrillar collagen. (m,n) The morphology at this magnification appears as aggregate particles set within an organic matrix. Bone images (a,b) were reprinted from *Bone*, 32,2, X Su, K Sun, F.Z Cui,W.J Landis, Organization of apatite crystals in human woven bone, 150–162, Copyright 2003, with permission from Elsevier. Breast calcification images (c-n) were acquired and processed following methods from Kunitake et al[166].

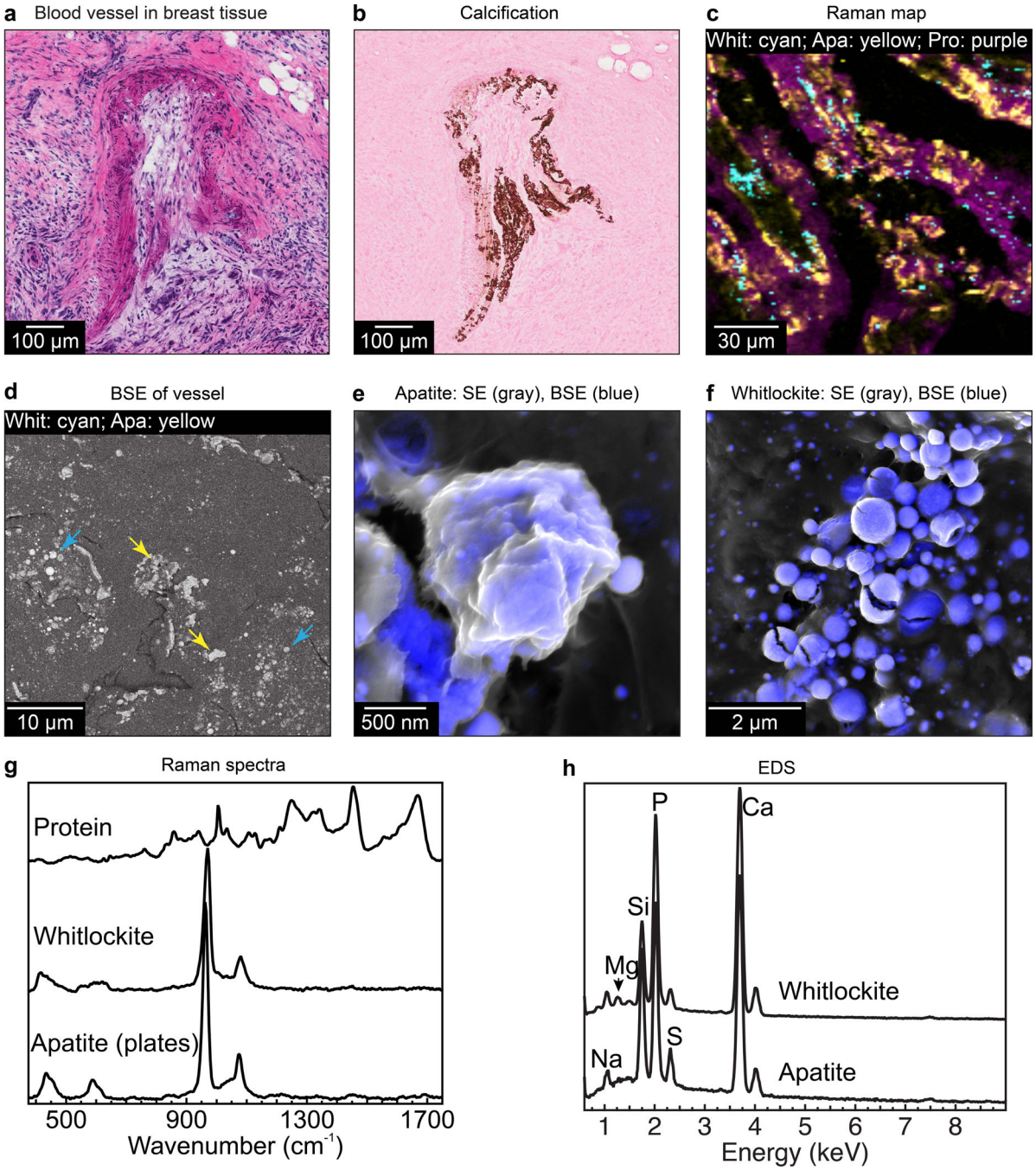

Figure 4.

Histopathology, Raman and SEM coupled with EDS characterization of medial blood vessel calcifications. (a) Histopathology (H&E staining) of calcification-containing blood vessel in human breast tissue. (b) Von Kossa staining of the same blood vessel shown in (a). Calcifications are in brown. (c) Raman mapping of an area within the same blood vessel showing mineral (whitlockite and apatite) and proteins (collagen and elastin). (d) BSE image showing mineral particles embedded within the organic matrix (arrows). (e) and (f) SEM and BSE composite images of the mineral particles. Apatite (e) and

whitlockite (f) particles show distinct mineral morphology. (g) Raman spectra corresponding to components shown in (c). For each component, high intensity pixels were thresholded and other components were subtracted manually. (h) Average EDS spectra (normalized to calcium) corresponding to morphologies shown in (e) and (f). Due to heterogeneity of the matrix and particle sizes smaller than the electron beam spot size, the spectra are qualitative, yet the presence of magnesium in the spheres, and the Raman spectrum (consistent with β-TCP), suggests a Mg-substituted β-TCP, i.e. whitlockite. Whit=whitlockite, Apa= apatite, Pro=protein, BSE=Back-scattered electrons, EDS= Energy dispersive X-Ray spectroscopy). These images and data were acquired and processed following methods from Kunitake et al[166].

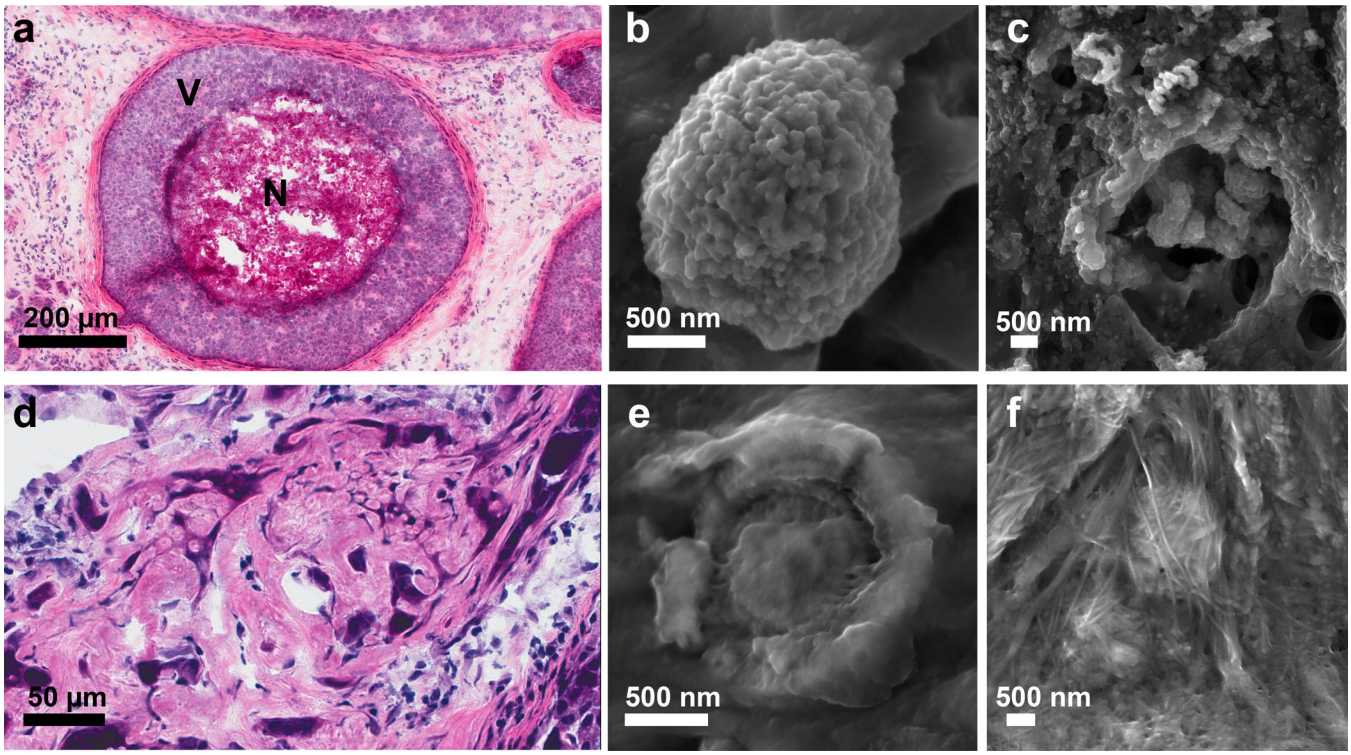

Figure 5.

Example of intertumor heterogeneity of breast cancer microcalcification. Histopathology (a, d) and morphology (b, b, e, f) of breast microcalcifications present in a case of comedo-type ductal carcinoma in situ (DCIS) (a-c) and invasive ductal carcinoma within the stroma (d-f) are shown. (a,d) H&E staining of calcification-containing tissues. (a) The duct cross section is shown, with separate areas of viable epithelial cells (V) and necrosis (N) indicated. (b,c,e,f) Scanning electron microscopy images showing calcification morphology. (b,c) punctate particles dispersed throughout the necrotic region. (e,f) particles within the stroma associated with invasive cancer were of a different phase (whitlockite rather than apatite), morphology (these appeared more spherical and some were aggregate in morphology while others showed distinct rings), and organic matrix (fibrillar collagen rather than cellular debris). The ring-like morphology may be a different morphology entirely or a cross sectional cut. Note that these images are meant to provide an illustrative example and should be taken as representative morphologies for either type of lesion. Adapted from the *Journal of Structural Biology*, 202, 1, JAMR Kunitake et al, Correlative imaging reveals physiochemical heterogeneity of microcalcifications in human breast carcinomas, 25–34, Copyright 2018, with permission from Elsevier.

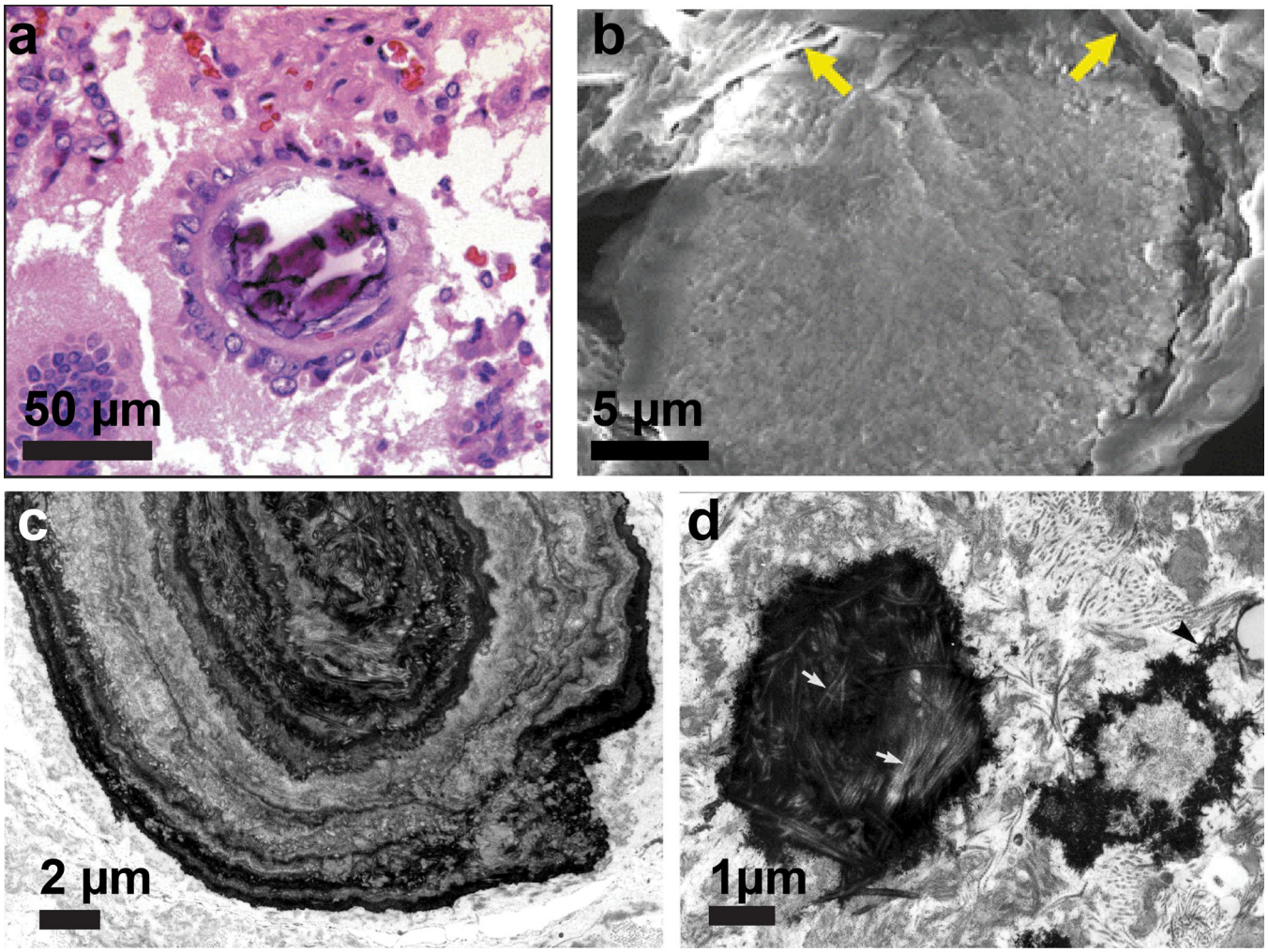

Figure 6.

Morphology of psammoma bodies in cancer. (a) Histopathology staining (H&E) of a psammoma body (stained dark purple) within a papillary thyroid carcinoma[363]. (b) SEM image of a psammoma body and the surrounding collagen matrix (yellow arrows) in a human ovarian tumor[173]. (c, d) TEM images of psammoma bodies found in human meningiocytic meningioma. (c) Calcification with laminated appearance. Laminae with an increased calcium content are identified in electron-dense zones and collagen fibrils are observed in the central electron-dense mass. (d) Calcium-containing mineral deposited on collagen fibrils (arrows), as well as needle shaped crystals (arrowhead)[120].

6a: Adapted from the *American Journal of Clinical Pathology*, JL Hunt, EL Barnes, Non–Tumor-Associated Psammoma Bodies in the Thyroid, 2003, 119, 1, 90–94, by permission of Oxford University Press.

6b: Adapted by permission from *Mineralogy and Petrology*: Springer Nature, Psammoma bodies in two types of human ovarian tumours: a mineralogical study, M Fanlu et al, 109, 3, 357–365, 2015, https://doi.org/10.1007/s00710–014-0342–6, COPYRIGHT 2014.

6c,d: Adapted from *Ultrastructural Pathology*, 25, 3, 243–267, FN Ghadially, As You Like It, Part 3: A Critique and Historical Review of Calcification as Seen with the Electron

Table 1.

Examples of Pathological Calcifications Found in the Human Body

Mineral type		Medical name of calcification	Location	Technique used for mineral identification
Calcium phosphate	**Apatite** $(Ca)_{10}(PO_4)_6(OH)_2$ Commonly known ion substituents: $(Ca, Na, Mg)_{10}(PO_4, CO_3)_6(OH, Cl, F)_2$	Microcalcifications	Breast	XRD[158–161], EDS[159,160,162–164], TEM imaging[160,162–164], Electron diffraction[164], SEM[160,165,166], Raman[166–169], FTIR[165,170], Von Kossa staining[164,166]
		Psammoma bodies	Thyroid	TEM[171]
			Ovaries	TEM[172], EDS[173], XRF[173], FTIR[173], Von Kossa staining[172], XRD[173]
			Brain (meningioma)	XRD[174], TEM[120], TEM lattice fringes and electron diffraction[175], SEM[175], EDS[175], Raman[176,177]
			Brain (pineal gland)	XRD[174,178,179], SEM[178,180], TEM[178,180,181], EDS[178,180], Electron diffraction[178], FTIR[181]
		Microliths	Testicles	XRD[182], SEM[182], TEM[182], Raman[183]
		Kidney stones (Urinary Calculi)	Kidney, Urinary system	XRD[184,185], FTIR[185–187], SEM[184], EDS[184], Raman[188,189]
		Calcifications (macular degeneration)	Eye (nodules, Bruch's membrane plaques)	XRD[190], SEM[121], EDS[121,191–193], TEM[121,191,192], Electron diffraction[121,192]
		Calcifications	Vascular tissue (not including heart valves)	SEM[122,194–196], EDS[122,195,197,198], TEM[122,197,198], Electron diffraction[197–199], FTIR[122,196], Raman[200], XRF[198], XRD[122,195,196,198,199,201]
		Calcifications	Vascular tissue (heart valves)	Raman[202], FTIR imaging[203], XRD[202], X-Ray absorption[202], SEM[197,202], EDS[197,202], TEM[197], Electron diffraction[197,204]
		Calcifications	Bioprosthetic heart valves	TEM[205,206], SEM[205,207,208], EDS[205,207], XRD[207,208], FTIR[207,208] Raman[207]
	Beta-tricalcium phosphate $Ca_3(PO_4)_2$ **Whitlockite** $(Ca, Mg)_3(PO_4)_2$	Microcalcifications	Breast	XRD[158,161], TEM[158], Raman[166,167], EDS[166]
		Calcifications	Testicles	XRD[201]
		Calcifications	Vascular tissue (not including heart valves)	TEM[199], Raman[200], XRD[166,175], XRF[198], Electron diffraction[199]
		Calcifications (macular degeneration)	Eye (retinal drusen)	SEM[121], TEM[121], Electron diffraction[121], EDS[121]
	Octacalcium phosphate $Ca_8H_2(PO_4)_6{\cdot}5H_2O$	Calcifications	Vascular tissue (not including heart valves)	XRD[167]
	Amorphous calcium phosphate	Microcalcifications	Breast	XRD[159], FTIR[165], SEM[165], EDS[159,165]

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Mineral type		Medical name of calcification	Location	Technique used for mineral identification
Calcium oxalate	**Weddellite (Dihydrate)** $CaC_2O_4{\cdot}2H_2O$	Microcalcifications	Breast	XRD[159,160], EDS[160,162]
		Kidney stones (Urinary Calculi)	Kidney, Urinary system	XRD[23,185,210–213], FTIR[185,186,213], Raman[189], SEM[23], AFM[23], EDS[185]
	Whewellite (Monohydrate) $CaC_2O_4{\cdot}H_2O$	Kidney stones (Urinary Calculi)(major component)		