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BIOMINERALIZATION IN HEALTH AND DISEASE:

FROM THE OCEAN TO THE STARS TO THE LAND

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CONTENTS

Crystallopathies

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Deposits of crystals, misfolded proteins or airborne particulate matter at nano-or microparticle size cause diverse medical disorders that can present either as acute or chronic organ injuries. Recent discoveries on crystal biology suggest unifying pathophysiological mechanisms and molecular targets for innovative therapies.

In nature organisms catalyze the aggregation of atoms and ions into amorphous crystals to build these into complex structures like corals, shells, bones, and teeth providing structural stiffness and durability. In the wrong place, the same process can be injurious, e.g. calcifications of vascular walls or tendons. Atoms or ion aggregation in a perfect periodic manner creates self-perpetuating growth of regular-shaped crystals. Single crystals sticking together or glued together by cement-like amorphous crystals can build polycrystalline masses such as calculi.

Local supersaturation of minerals, dietary metabolites, or drug metabolites occurs preferably in excretory organs such as the biliary and urinary tract where concentration and supersaturation is thought of a common initiator of the crystallization process and stone formation. Also endogenous proteins or paraproteins can undergo self-aggregation to polycrystalline-like microparticles. For example, the process of beta-sheet fibrils self-perpetuating fibrillation to plaque-like amyloid deposits in amyloidosis or Alzheimer disease resembles mineral crystallization.

Such particles mostly enter the lungs from airborne occupational, environmental or cigarette smoking dusts[1]. Other sources of extrinsic particles are metallic, plastic or silicone implants, cosmetics or nanoparticle carriers for drugs.

Particle size is a critical determinant of the tissue response. Macrophages and other phagocytes are usually the first to engulf particles for phagocytosis, which is possible for nanoparticles and microparticles of few μm in diameter[2]. Phagosomes fuse with lysosomes containing lytic proteases. The inability to digest crystalline nano- or microparticle cargo destabilizes lysosomes and induces cell stress, autophagy, and leakage of lysosomal proteases into the cytosol. Massive loads of particles may give macrophages a foam cell appearance[3]. Crystal needles and other larger particles that exceed the size of macrophages may iwnduce giant cell formation, which may internalize even larger particles[4]. Calculi or implants imply frustrated phagocytosis and persistent release of phagocytic enzymes attempting digestion[2].

Non-aggregating crystal masses remain liquid, e.g. monosodium urate crystals in bird droppings or gout tophi. In contrast, polycrystalline aggregates may solidify and grow into cavity-filling calculi that can cause tissue injury by mechanical obstruction causing either, colic and organ failure or vascular obstruction. Mineral concentration in excretory fluids promotes supersaturation and crystallization, hence the biliary and urogenital tracts are susceptible to stone formation. Bile is rich in electrolytes, bile acids, cholesterol, phospholipids, and conjugated bilirubin frequently forming stones inside the gall bladder. Mobilization of calculi into extrahepatic bile ducts causes biliary colic. In the urinary tract, crystallization usually starts in the renal tubules where supersaturation is a consequence of stepwise concentrating the glomerular filtrate and of the active secretion of calcium, uric acid, oxalate, phosphate or drug metabolites[5]. Once single crystals adhere to the luminal membrane of renal tubules by attaching to annexin II, CD44 or osteopontin, they serve as a nucleus for building larger polycrystalline plugs obstructing tubules[6]. Diffuse crystal plug formation can cause acute kidney injury, e.g. in polyethylene glycol intoxication-induced acute oxalate nephropathy or in myeloma cast nephropathy[7]. In genetic forms of hyperoxaluria or hypercalciuria persistent crystallization

leads to progressive nephrocalcinosis and chronic kidney disease (Table 1)[8]. Such "stony kidneys" have a white appearance on ultrasound or radiograph exams[8]. More space to form larger crystal aggregates is available in the renal pelvis, where calcified Randall plaques (mineral concentrations on renal papillae) are sites of stone formation[5]. Dislocation of such calculi, cause transient or persistent obstruction of urine flow manifesting clinically as renal colic. Also drug- or diet-related crystalluria can lead to unilateral or bilateral renal colic, and even acute renal failure[9,10]. Gallstones blocking the pancreatic outflow cause acute pancreatitis. Hydroxyapatite calculi in ducts of salivary glands (sialoliths) cause sialdenitis (Table 1).

Crystal masses may also cause vascular obstruction but through different mechanisms. Atherosclerosis is caused by accumulation of cholesterol crystals in the intima of the arterial wall, the atheroma[11]. Atheromatous plaques eventually obstruct the vascular lumen and lead to tissue ischemia, but also plaque cap rupture and subsequent thrombotic vascular occlusion and tissue infarction[3]. Plaque rupture in the aorta or its major branches can launch cholesterol emboli causing ischemic necrosis (Figure 2)[12]. Cholesterol crystals present as spindle-shaped luminal clefts on tissue biopsies or upon funduscopic inspection in retinal arteries[12]. Finally, vascular calcifications, i.e. calcium phosphate deposits in the medial layer of muscular arteries, are common in the elderly, in uremic patients or in primary hyperparathyroidism[13]. This medial calcific sclerosis confers loss of vascular compliance (sclerosis=stiffness) and eventually causes peripheral artery disease or calciphylaxis, and possible ischemic tissue necrosis associated with high mortality. Together, crystal caused obstructions cause colic, inflammation, tissue necrosis, and sometimes, fatal organ failure.

The lecture will discuss the latest discoveries on the shared mechanisms of crystal biology in terms of crystal-induced inflammation, cell death, and tissue remodeling.

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See also

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Calcitropic Hormones

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Because of the essential and evolutionarily-ancient role for calcium in many of biology's most fundamental processes, including neurotransmitter release, stimulus-secretion coupling, and muscle contraction, the transition of life from aquatic to terrestrial environments required and selected for major adaptations. These were needed to address the problem of how to maintain extracellular calcium levels within a very tight range compatible with normal physiologic demands, when it was no longer possible to simply take it up from the surrounding ocean. In the tetrapod transition, bone took on increasing importance for support, resisting gravity, and locomotion, but also crucially as a storage depot for calcium. But new systems were needed to interact with this storage depot and regulate the exchange of calcium with the extracellular fluid to maintain tight homeostasis, leading to the evolutionary emergence of the parathyroid glands in tetrapods. Interestingly, this emergence appears to have its tissue origins in the gills of fish, which express GCM2 (parathyroid-specific in mice and humans), a form of parathyroid hormone and the extracellular calcium receptor.

Phosphorus is the other major mineral component of bone, and similarly possesses essential and concentration-dependent functions in fundamental cellular physiology. While these latter roles for intracellular phosphate are quite distinct from the roles for calcium, they notably overlap as partners in the processes of bone mineralization. Further, the circulating concentrations of calcium and phosphorus are under, but quite close, to levels that would cause their damaging precipitation in soft tissues. Thus, it should not be surprising that systems evolved to regulate extracellular calcium, including "calcitropic hormones" like parathyroid hormone, would also have effects on phosphate handling.

The key role of the parathyroid glands, and parathyroid hormone, in regulating calcium (and/or phosphate) homeostasis is accompanied by important and interacting actions of the vitamin D system and Fibroblast Growth Factor 23 (FGF23). As vitamin D and FGF23 will be addressed separately in this conference, this presentation will focus on the parathyroids. The parathyroid glands develop embryologically from endoderm, specifically the third and fourth pharyngeal pouches in humans. Cells destined to form the thymus are found in close proximity to the some of the parathyroid primordia, but the latter's distinct cell fates are marked very specifically by expression of the GCM2 transcription factor. An essential role for GCM2 is evidenced by the hypoparathyroid phenotype found in GCM2-null mice and humans.

Parathyroid hormone (PTH) regulates the level of extracellular calcium in a rapid, minute-tominute, time frame. An increase in its release acts in a coordinated fashion on target tissues increase calcium, and vice versa. The main targets for PTH action are the kidneys, where PTH increases fractional calcium reabsorption and induces the enzyme which produces the bioactive form of vitamin D; bone, where PTH increases bone turnover and the entry of calcium to the circulation; and the gut, where PTH-induced active vitamin D acts to increase absorption of dietary calcium. Of note, PTH also has a marked phosphaturic effect on the kidney; and 1,25-OH vitamin D increases levels of FGF23, a potent phosphaturic hormone. The parathyroid glands'ability to release PTH in a fashion that precisely maintains homeostasis depends heavily on the extracellular calcium-sensing receptor, a G protein-coupled receptor whose function and downstream signaling determines the "calcium-PTH setpoint". The pivotal role for this molecule is underscored by clinical features and outcomes in individuals with activating or inactivating germline mutations in the encoding gene, CASR. Similarly, the phenotypes of patients with parathyroid tumors that release excessive amounts of PTH, and of those unable to make sufficient PTH, highlight the crucial role for proper

parathyroid function. The molecular basis of selected human disorders, especially parathyroid tumors, that result in abnormal serum calcium concentrations and can cause abnormalities in biomineralization, will be a particular focus of this presentation.

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*denotes key reference

Congenital mineralization of the joint

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Heterotopic ossification (HO) involves the formation and accumulation of extraskeletal bone tissue at the expense of local tissues including muscles, connective tissues and joints. There are common forms of HO that are triggered by extensive trauma, burns and other bodily insults, and there are also rare congenital severe forms of HO that occur in children[1]. Genetically determined forms of ectopic calcifications are rare, and include mainly fibrodysplasia ossificans progressiva (FOP), progressive osseous heteroplasia (POH), pseudo hypo parathyroidism IA, Albright syndrome (PHP1A, AHO), and tumoral calcinosis (TC)[2].

Fibrodysplasia ossificans progressiva (FOP) is a severe rare disease, characterized by the progressive heterotopic endochondral ossification (HOE) of skeletal muscle, fascia, tendons, and ligaments[3]. The range of joint motion in FOP patients, becomes gradually and progressively limited by HOE. Indeed, this ossification is so diffuse that it is commonly referred as a second skeleton[4]. HOE occurs in flare-ups and is most commonly triggered by muscle injury. FOP is not the result of a deficiency but rather a gain of function mutation. All patients with the typical FOP phenotype have a substitution of the Arg206 residue for a His residue in Activin A receptor type I (ACVR1) which is a type 1 Bone Morphogenetic Protein Receptor (BMPR)[5]. In this disorder, common sites of early heterotopic calcification are the neck, spine, and shoulder girdle. Postnatal heterotopic ossification in FOP usually begins before the age of 5 years, and proceeds in predictable temporal and spatial patterns. In the absence of trauma, which alters the natural progression of the disease, episodes of heterotopic bone formation occur in a pattern that parallels the sequence of formation of skeletal elements during

embryonic development. Typically, the upper back and neck are the first parts of the skeleton to be affected; the physiological basis of this progression pattern has not been identified. Over time, ectopic bone formation in FOP is progressive, cumulative and extensive, bridging the joints of the axial and appendicular skeletons and causing near- complete immobilization of the body[6].

Similar to patients with FOP, patients with POH experience extensive formation of bone within soft connective tissues. Like FOP, this bone formation is episodic and progressive. POH heterotopic ossification progresses from the dermis to the underlying deep connective tissues; bone forms within skeletal muscle, sometimes fusing with skeletal bone. Distinct from FOP, POH is not associated with inflammation, predictable regional patterns of heterotopic ossification, or FOP-like big toe malformations. Heterotopic bone formation in POH patients occurs in an asymmetric mosaic distribution and is mainly intramembranous [7, 8, 9]. The age of onset in POH is usually before 1 year of age, although much later does occur as well[10].

POH is a rare disorder, with fewer than 60 clinically-confirmed cases worldwide. The rarity of the disease seems to be a function, at least in part, of the incomplete penetrance of inactivating GNAS mutations and the wide and variable range of clinical phenotypes that are associated with these mutations. POH is among a number of related genetic disorders that are associated with heterozygous inactivating mutations in GNAS, including Albright hereditary osteodystrophy (AHO) and pseudo hypo parathyroidism (PHP). However, the term PHP refers to a spectrum of rare disorders of mineral metabolism, characterized by features due to end-organ resistance to PTH. The phenotypes of Albright hereditary osteodystrophy (AHO), originally described as associated to the disease, and POH, can be associated to the endocrine manifestations of hormonal resistance. Genetic or epigenetic alterations in the complex imprinted GNAS locus, encoding the alpha-subunit of

the stimulatory G protein (GSα) and several other transcripts, give rise to the different forms oh PHP, which can be differentiated according to the phenotype, the response to PTH infusion and in vitro assays testing Gsα activity. Since PHP-related phenotypes are overlapping and other non GNAS-dependent disorders mimicking AHO, the term PHP is today considered obsolete and better referred to the more comprehensive "inactivating PTH/PTHrP signaling disorder (iPPSD)" as proposed in a recent classification. This broad term include all the congenital rare disorders due to impaired PTH/PTHrP cAMP pathway.[11]

Tumoral calcinosis causes widespread ectopic calcifications, which however do not usually involve joints in the distal limbs. It might occur in infancy, although its onset is more commonly observed later, during childhood. The genetically determined forms can be caused by mutations in GALNT3[12], FGF23[13], KL[14] and SAMD9[15] for which functionally relevant sequence variants were excluded by targeted resequencing. This makes the diagnosis unlikely. TC presents with massive periarticular, and seldom visceral, calcified deposits, and usually starts in childhood (2–5 years). In this condition, calcification commonly occurs around or in the shoulders, hip joints, knees, and elbows, while involvement of small joints, such as hands and wrists, is less common. It can occur as primary disorder but also secondary to other disorders, such as renal insufficiency and hyperparathyroidism[16].

In conclusion, congenital mineralization of the joint are an heterogeneous group of rare and severe diseases, affecting childhood, difficult to treat and with multidisciplinary approach.

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How to measure bone quality and quantity in metabolic bone diseases

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Biomechanical principles dictate that bone strength is determined by bone mass, bone structure and the intrinsic properties of the bone matrix. Bone mineral density (BMD) measurements by dual-energy X-ray absorptiometry (DXA) mainly reflect bone mass and are the current clinical standard for assessing fracture risk. Yet, many fractures occur in those who are not identified as high risk by DXA-BMD. Accordingly, several methods have been developed for non-invasive assessment of bone structure, including DXA-based trabecular bone score (TBS) and 3D image techniques, such as computed tomography (CT) and high-resolution peripheral computed tomography (HR-pQCT). Moreover, application of finite element analysis (FEA) to 3D imaging provides accurate estimates of whole bone strength. In contrast, non- or minimally invasive assessment of bone matrix properties has proven more challenging, though several approaches are currently being investigated. This presentation will review the ability of these techniques to go "beyond BMD" in terms of predicting fracture risk in osteoporosis and other metabolic bone disorders.

A. SIGNFICANCE

Fractures are a major and growing cause of disability and health care costs in older men. Two million men in the United States have osteoporosis, ~1/3 of hip fractures in the US occur in men, and men are twice as likely to die after hip fracture as women.[9, 10] Still, men are much less likely to undergo screening or receive treatment for osteoporosis, even after a hip fracture,[11-15] and the US PSTF recommends against osteoporosis screening and treatment in men because of insufficient data. While there have been longterm

studies of changes in bone mass and structure in women, there have been none in men, who are likely to have different patterns of skeletal changes in late life than women.

There is a particular knowledge gap in the oldest old. The rate of bone loss increases exponentially after age 65 and the incidence of fractures is highest in late life.[16, 17] In particular, in the US, the rate of hip fracture is ten-fold higher in those aged >80 than those aged 65-74 years and mortality due to hip fracture also substantially increases, particularly in men. The excess mortality associated with a hip fracture is 7% in those aged 70-74 and a startling 48% in men aged >90 years.[10] *Yet there are no large, longitudinal studies that focus on bone health in men during these critical decades.*

Scientific premise: Several observations provide strong rationale for the proposed aims.

1. Many fractures occur in those who are not identified as being at high risk for fracture by DXA-BMD.
2. Biomechanical principles dictate that fractures occur when the loads applied to a bone exceed its strength. Thus, understanding both the loading (e.g. circumstances of fractures) as well as bone strength are essential to improve identification of those at risk and develop strategies for fracture prevention.
3. Bone strength is determined by bone mass, bone structure and the intrinsic properties of the bone matrix.[20] Notably, declines in bone strength exceed declines in bone mass[22, 24, 25] because of structural deterioration, providing strong rationale to move beyond DXa-BMD to examine changes in bone structure and strength among older adults.
4. CT-based finite element analysis (FEA) integrates bone geometry and the heterogeneous distribution of density to provide robust estimates of whole bone strength, validated

against experimental measures of cadaveric bone strength. Moreover, CT-FEA predicts short term risk of hip and vertebral fracture, providing strong rationale to test how to best utilize CT-FEA to predict short- and long-term risk of all fractures.

5. MrOS has contributed greatly to understanding bone loss and fractures in men.[27-39] Yet, knowledge about changes in bone mass, structure and strength that contribute to the exponential increase in fracture among older men is still lacking. In light of unprecedented measurements and long-term follow-up, MrOS has the unique opportunity to better predict those who will be at highest fracture risk by defining the long term changes in bone density, structure, and strength, and assessing their contributions to the exponential increased risk of fracture in men in late life.

The fractures that occur in 80-100 yr old men are poorly understood. Most large cohorts devoted to bone health have few participants in these advanced ages, and the spectrum of fractures that affect these men is not well defined. *Most critically, the various circumstances that lead to those fractures are unknown*. Falls or other trauma certainly lead to most fractures, but studies of activity in the elderly show that there are important sex differences that may influence the causes and types of falls. Older women engage in more regular but less vigorous activity, while longer periods of inactivity in men are punctuated by bouts of strenuous endeavors. While frailty certainly increases fall and fracture risk in some men, recent studies in MrOS have shown that most falls actually occur in active men (ref), and more falls occur outside the house in men (ref). Understanding the types of fractures that occur in older men and the circumstances of the fracture (Aim 1) would help to design better preventive strategies. Furthermore, improved knowledge of the type of falls (or other activity) that lead to fracture would inform and improve biomechanical approaches to estimate fracture risk (below).
Whereas DXA-BMD is a strong predictor of fracture risk, many

fractures occur in those not identified as at risk by an initial DXA-BMD measurement[3,4] Fall propensity undoubtedly contributes to the risk not predicted by DXA BMD, but we hypothesize that other image-based measures of bone structure and/or strength can significantly improve the ability to identify those at highest risk of fracture.

Novel uses of DXA for fracture risk prediction (Aim 2a). Over a short follow-up interval (~5 yrs), we and others have reported that in men >65 yrs baseline DXA BMD predicts fracture risk.[27] However, we also showed that *loss of DXA hip BMD accelerates with age*[45], and that there is substantial heterogeneity in the rate of BMD loss. Although BMD at an early stage of bone loss (e.g. age 65 yrs) retains some fracture risk prediction into later life, at least in women,[53] the associations of baseline BMD to fracture risk appear to become considerably less robust with longer follow-up. In MrOS, when limiting follow-up time to the first 5 yrs after enrollment, the HR for hip fracture is 3.5 (95% CI: 2.6, 4.6) for each SD decrement in femoral neck BMD; but is reduced to 2.6 (95% C: 2.2, 3.0) with longer follow-up time (maximum=17 yrs), highlighting the limitations of long-term fracture prediction using baseline DXA. This is very consistent with the hypothesis that change in bone measures alters their relationships to fracture. In fact, we also showed that the rate of DXA BMD decline predicts hip fracture[45-51] but the latter finding has been controversial[52] because these studies have been short. *We hypothesize that greater long-term DXA BMD loss predicts increased long term (~20 yrs) fracture risk independent of baseline DXA BMD*. If true, this would be especially germane for the oldest old and would shift emphasis from static measurements of density to "dynamic" measures of change to identify "big losers" who may have the greatest benefit from pharmacologic treatment. *Resolving this issue is critical for understanding how to interpret baseline measures, utilize follow-up measures, and design preventive and therapeutic interventions*. In addition, bone size measures by DXA may improve the ability

to identify older men at highest fracture risk. We recently reported that a DXA estimate *of baseline femoral neck (FN) size exerts important influences on subsequent bone mineral loss in peri-menopausal women*.[46] Women with larger FN width had more rapid bone loss and, because of simultaneous changes in structure, these losses *are not apparent by BMD measures*. In MrOS men (Prelim Data), we show similar trends. In fact, our preliminary data suggest that larger bone size predicts higher fracture risk *independent of BMD*. Although a small number of previous small, cross-sectional studies hint at this finding, these longitudinal data are unique and clearly suggest that complex relationships exist between baseline structure, subsequent bone loss, structure change and fracture risk. *If true, this finding will have important implications for how we can leverage DXA (BMD + structure) to identify men at higher fracture risk in late life*.

CT-FEA may provide independent value in identifying older men at high fracture risk (Aim 2b). Biomechanical principles dictate that bone strength is determined by bone mass, bone structure and the intrinsic properties of the bone matrix.[20] In vitro studies demonstrate that bone structure contributes to bone biomechanical properties independent of bone mass,[21,23] and that declines in bone strength exceed declines in bone mass,[22, 24,25] because of structural deterioration, including trabecular thinning and perforation, increased cortical porosity and reduced cortical thickness. Cadaver studies show that experimentally measured femoral and vertebral strength are better predicted by CT-FEA than by DXA-BMD.[47-49] In MrOS, femoral strength by CT-FEA is only modestly correlated with total hip DXA BMD[44]. *These finding suggest that CT-FEA could provide a DXA-independent measure of fracture risk*. In fact, we and others reported that femoral and vertebral strength from CT-FEA predicts short-term risk of hip and spine fracture as well as DXA BMD.[44, 54, 55] However, bone structure and density change rapidly with advancing age, and our data may not be true for long-term fracture risk and the *ability of femoral and vertebral strength*

by CT-FEA to predict long-term fracture risk is unknown. Other important questions remain, for example: 1) does CT-FEA strength predict risk of non-vertebral and all clinical fractures over decades and into the last decades of life?; 2) how does the predictive value of CT-FEA compare to BMD over the long-term?; 3) does hip + spine FEA strength improve risk prediction over hip strength alone? *Given the large number of baseline CT scans (>3500) and fracture ascertainment over 20 yrs, MrOS is uniquely suited to address these questions*.

Potential for CT-FEA (or BCT) to improve underdiagnosis of osteoporosis, but methods for optimal use and the ultimate clinical utility remain unanswered. Current evaluations of CT-FEA for the prediction of hip fracture risk utilize a biomechanical model of bone strength that assumes a sideways fall. However, it's clear that 1) not all falls that result in fracture are to the side and 2) there is large inter-individual variation in hip structure that suggest there is similar variation in the fall direction most vulnerable for fracture. For instance, we examined a small series of QCT scans from MrOS and demonstrated marked variations in femoral neck structure that yielded large differences in the angle of force most likely to cause fracture (Carpenter). *Thus, we hypothesize that CT-FEA models that identify the "weakest" direction of fall should improve fracture prediction*. Second, we and others have shown that larger amounts of soft tissue over the trochanter reduces fracture risk, apparently because of a diffusion of fall forces in a fall. We can measure soft tissue thinkness in a manner specific for the direction of fall and incorporate those measures in the CT-FEA models above. We hypothesize that CT-FEA *models that include fall-specific soft tissue thickness will further improve fracture prediction*.

If CT-FEA – particularly using the more sophisticated biomechanical models above - predicts long term fracture risk, there are important clinical implications. Phantomless calibration, whereby air and adipose tissue are used as calibration materials,[56]

now allows CT-FEA and BMD assessments on CT scans obtained for other clinical purposes.[57-59] Notably, >10% of the Medicare population had abdominal or pelvic CT scans in 2007,[60] >5X the number (1.7%/yr) of Medicare-eligible men who are screened with DXA.[61] We recently showed that hip CT-FEA from these clinical scans predicts short term hip fracture risk at least as well as DXA.[62-63] A CT-FEA CPT code was recently approved. *Thus, clinical translation of our findings is now possible. Routine CT scans present a huge opportunity to increase the number of older men identified at risk of fracture.*

The large burden of long-bone fractures in older adults raises the important questions of whether peripheral bone density, structure and strength predict fracture; how they change in the oldest old and what are the factors that influence their deterioration (Aim 3).

While often overlooked,[18] peripheral fractures (e.g., non-hip, non-spine) represent a huge medical and societal burden among older adults.[6,8,19] For example, the population-attributable risk for mortality for peripheral fracture is similar to that of cardiovascular disease and diabetes.[8] Despite this, there are no data on the nature of or contributors to changes in bone microstructure among the oldest adults, in whom these fractures are most likely to occur. Preliminary data from MrOS and others[64,66] indicate that HR-pQCT measures of bone density, structure and strength are robustly and independently associated with risk of all clinical fractures. Strikingly, our preliminary data also suggest that HR-pQCT measures predict hip fracture risk independent of hip DXA BMD. However, these provocative preliminary results are based on very few hip fractures and require further evaluation. *If confirmed, our preliminary results showing robust associations of HR-pQCT measures with both hip and non-hip fractures will have major implications for the use and interpretation of HR-pQCT, and the possible clinical and research indications for its use.* Furthermore, identification of the structural features most robustly associated

with fracture will undoubtedly spur the development of novel non-invasive imaging methods amenable to widespread use.

The few previous longitudinal studies of age-related changes in peripheral bone density, structure and strength using HR-pQCT were limited by small sample sizes, inclusion of few older participants (when change is most rapid and fracture risk high) and reliance on the first generation HR-pQCT scanners with lower resolution, less ideal structure measures and more problems from motion artifact.[67] Unlike first generation device, the resolution of 2nd generation HR-pQCT scans allows independent assessment of cortical and trabecular density and microstructure.[67] and provides more robust assessment of cortical porosity, improved precision and reduced motion artifact. Our preliminary data in men studied over 18 months with 2nd generation HR-pQCT show that radial and tibial bone loss is rapid (0.5-2%) and quite variable. *Repeating HR-pQCT scans will enable us to determine compartment-specific changes in bone mass and structure, and to understand the physiologic contributors to the variation in skeletal deterioration among individuals (see below).*

The predictors of change in peripheral bone structure and strength in older men are not known (Aim 3c). Cross-sectional studies suggest that markers of inflammation,[68,69] vitamin D,[70-72] sex steroids,[73,74] and muscle mass or physical function[75,78] may be associated with peripheral bone microstructure. Each may be important, particularly in combination, but longitudinal data are not available. We will test the hypothesis that these factors are major contributors to the exponential late-life declines in bone. In light of the importance of fractures of the peripheral skeleton, understanding the determinants of peripheral bone deterioration is important. *It will provide insight into the biological bases of loss of bone with aging, will inform the potential clinical utility of these potential risk factors for assessing skeletal fragility and will guide the use and development of therapeutics. Stored serum (Visit 4) provides a unique opportunity to test these hypotheses.*

- *Increasing with age, inflammation has an important deleterious influence on bone*. Hip or spine BMD loss, and fractures, are greater in younger men with higher levels of inflammatory markers (especially when multiple markers are higher).[62,79-81] Using innovative large-scale proteomics we showed several novel members of the inflammatory cascade are associated with hip bone loss and fractures[82] and in new Prelim Data we also show serum metabolites are associated with HR-pQCT measures. Yet, there are no data concerning the association of these factors to peripheral bone loss. *We hypothesize that men with higher concentrations of key markers of inflammation will exhibit higher rates of peripheral bone loss and microstructural deterioration*.

- Low 25OHD is associated with greater hip DXA BMD loss and hip fracture risk in men[29,30] but there are no data concerning 25OHD and change in peripheral bone in older men in whom low vitamin D levels are common. Similarly, low sex steroid (especially estradiol) and high SHBG levels are associated with greater DXA BMD loss and fracture risk in men,[32, 83] but again there are few data about the oldest old in whom sex steroid concentrations are lowest,[84] and no studies of the association of these concentrations and peripheral bone loss. Importantly, we observed that simultaneous sex steroid and 25OHD deficiencies are particularly deleterious to hip BMD.[85] *We hypothesize that very elderly men with these perturbations, and especially those with combined deficits, are likely to have the most rapid declines in peripheral bone mass and microstructure*.

- Skeletal health depends on mechanical loading; accordingly, strength and physical activity are associated

with bone structure, strength and fracture risk.[86,87] Yet, the influence of muscle phenotypes on the peripheral skeleton has not been assessed in longitudinal studies. In addition, while DXA measures of lean mass have been largely unrelated to fracture risk[88] (likely because DXA measures lean mass, not muscle mass per se), our preliminary results show that the novel D3Cr dilution measure of muscle mass is strongly related to fractures in MrOS. We have also derived muscle volume and density measures in the calf (at the proximal tibia HR-pQCTsite) using newly developed HR-pQCT methods.[89] Given the rich muscle phenotyping data in MrOS, we have the unique opportunity to determine the influence of muscle health on changes at the peripheral skeleton. *We hypothesize that low activity, low muscle mass by D3Cr dilution, and low physical performance substantially contribute to declines in peripheral bone density and structure assessed by HR-pQCT.*

MrOS has unprecedented resources to address these hypotheses. The current MrOS participants (age >84) represent a unique cohort for the study of fractures in very old men. Almost 20 years ago, we began to obtain serial DXA measures and complete clinical phenotyping. At baseline, hip and spine CT scans (N~3800) were obtained to assess bone structure and strength. Similarly, HR-pQCT (XtremeCTII) scans were obtained in a large number of men (N=1831) in 2014-6. Its large size, rich phenotyping, excellent retention and long follow-up time into the 9th and 10th decades when bone loss is fastest and fracture risk the highest, are ideally suited for this work.

In summary, we have an opportunity to address critical knowledge gaps concerning skeletal health in very elderly men. We can

characterize the fractures that occur in men in the later stages of life, examine novel approaches to identify those at highest risk of fracture, and expand the understanding of peripheral bone loss. No similar studies have been performed, and these data are currently unavailable. If our hypotheses are correct, our findings would have clear implications for the early identification of those with high fracture risk, development of strategies to prevent fractures and ultimate reduction in the burden of fractures in older men.

B. INNOVATION

- MrOS is the only cohort of men in the world with such comprehensive skeletal assessments over two decades, when the risk of fracture is rising exponentially. MrOS has the best phenotyped cohort of the oldest men - in the 9th and 10th decades of life - when fractures are a major threat to maintaining independence. Thus, MrOS remains the unique source of data and discoveries about musculoskeletal aging in men
- There are no previous studies of the circumstances of fracture in very old men. The data from the proposed study will be unprecedented.
- MrOS is exceptional for having three unique types of imaging to assess the usefulness of bone density, structure and strength over very long periods of follow-up: DXA, CT, and HR-pQCT.
- We propose novel applications of CT-FEA that recognize individual variation in vulnerable fall directions and soft tissue thickness to improve fracture prediction.
- Second generation HR-pQCT scans already performed on a large number of men in MrOS and the proposed repeat scans, combined with comprehensive muscle phenotyping (strength, power, and the novel D_3Cr muscle mass measure) and an extensive biobank, allow us a unique opportunity to

understand how the combination of muscle characteristics, vitamin D nutrition, sex steroid concentrations and inflammation contribute to deterioration of peripheral bone structure and strength.

- We will use a novel analysis pipeline that uses 3D image registration to co-localize conserved architectural features across visits and newly developed HR-pQCT-based assessments of muscle volume and density to assess relationships between muscle and bone in the tibia.
- The study takes advantage of unique proteomics and metabolomics to uncover novel biological factors leading to decreased peripheral bone structure, density and strength.

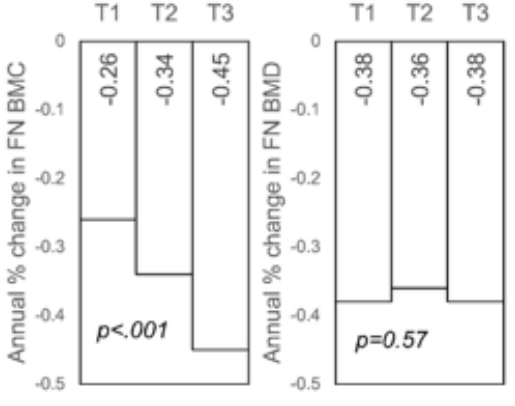

Figure 1. Association between tertiles of baseline FN area and change in FN BMC (left) and BMD (right)

C. APPROACH
C1. Preliminary data
C1a. Anything about fractures in oldest old?

Baseline femoral neck size is associated with subsequent change in BMC but not BMD (Aim 1a). To test the notion that baseline structure is related to subsequent changes in bone mass, density and structure, we used baseline femoral neck (FN) DXA and change over ~14 yrs (from baseline to V4). Men *with the largest FN area lost BMC about twice as rapidly ($p<0.001$, Fig. 1), whereas BMD declined at similar rates regardless of baseline area*. Results were identical at the total hip. These findings are similar to those we recently reported in peri-menopausal women,[46] and support the contention that baseline bone structure influences skeletal biology and subsequent structural change. Since bone size and BMC have important effects on strength, these results *support our hypothesis that change in hip strength is determined in part by baseline structure*. It is remarkable that the *changes in area and BMC were not related to BMD change, and lends support to our hypothesis that long-term BMD change does not adequately reveal critical alterations in structure and strength*. However, these observations were based on DXA. Assessment of bone structure over 20 yrs via CT will allow a much more comprehensive and mechanistic analysis of the association between baseline structure and subsequent changes in bone mass, structure and strength.

Table 1. Distal radius HR-pQCT measures & risk of clinical fracture

Parameter	HR per SD (95% CI)
Failure load	1.8 (1.4, 2.4)
Total vBMD	1.8 (1.3, 2.3)
Trabecular vBMD	1.6 (1.3, 2.1)
Trabecular #	1.4 (1.1, 1.8)
Trabecular thickness	1.3 (1.01, 1.6)
Trabecular area	0.8 (0.6, 0.9)
Cortical vBMD	1.4 (1.1, 1.7)
Cortical thickness	1.4 (1.1, 1.9)
Cortical porosity	1.0 (0.8, 1.3)
Cortical bone area	1.4 (1.03, 1.8)

**adjusted for clinical site, limb length, age, total hip DXA BMD, race, falls and history of fracture; 95 fx in 1655 men*

CT-FEA loading direction or tissue thickness

C1c. HR-pQCT parameters predict incident fracture (Aim 2a). HR-pQCT scans and complete covariate information were available in 1655 men at Visit 4 (follow-up time: 1.7 years; 95 clinical fractures). We showed that 1 SD decrement in most HR-pQCT distal radial measures was associated with a 30-80% increase in risk of fracture, even after adjustment for age, clinical factors and total hip DXA BMD (Table 1).[90] Results were similar at the distal tibia. Also, despite relatively few hip fractures (N=20), our preliminary data show that lower total vBMD and failure load at the distal radius were strongly associated with higher risk for hip fracture after adjustment for age and FN BMD (total vBMD HR per SD: 2.5, 95%CI 1.4,4.6; failure load HR per SD: 2.8, 95% CI: 1.4, 5.6); we saw similar results at the tibia. Notably, there is a suggestion of a non-linear association between the HR-pQCT parameters and risk of hip fracture. Nearly all of the 20 incident hip fractures since Visit 4 occurred in men in the lowest tertile of distal radius failure load (n=17), while only 3 occurred in the higher two tertiles ($p<0.001$). Men in the lowest tertile of distal radius failure load had a 17-fold higher risk of hip fracture than men in the highest tertile (HR:17.5, 95% CI: 2.3-131.1). Similar results were observed for tertiles of distal radial vBMD; and failure load and vBMD at the distal tibia. However, with the small number of events the CIs for the HR are wide. Additional follow-up time to accrue more events will allow us to fully describe this relationship. *These results suggest that HR-pQCT may have clinical utility, and justify our proposal to extend the observation period to establish the associations of HR-pQCT measures to fracture risk, and characterize the change in these HR-pQCT parameters in older men.*

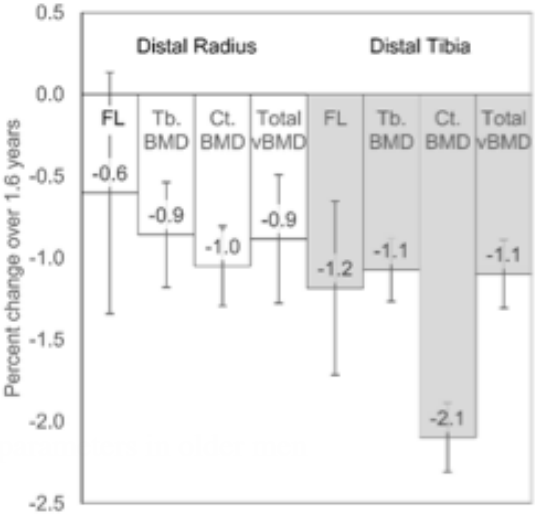

Figure 2. Mean (±CI) change in HR-pQCT measures in 268 MrOS men over 1.6 yrs. (FL= failure load.)

Table 2. Correlations (r) between change in DXA BMD and change in distal radius HR-pQCT BMD and strength over 1.6 years

	Δ Failure load	Δ Total vBMD
Δ Total hip DXA BMD	0.04 p=0.56	0.14 p=0.02
Δ Femoral neck DXA BMD	0.00 p=0.98	0.08 p=0.21

C1d. Rapid and variable loss in radial and tibial structure, density and strength by HR-pQCT (Aim 2b). We obtained repeat HR-pQCT measures in 268 men (mean age 85 yrs) ~1.6 years after the measures at V4. Even after that brief follow-up time, we found significant declines in cortical and trabecular vBMD at both the distal radius and tibia, and in failure load at the distal tibia (Fig. 2). DXA BMD also declined during this period: -0.9% at the total

hip (95%CI: -1.2, -0.5) and –0.5% at the femoral neck (95% CI: -1.0, -0.02), *but changes in DXA BMD were poorly correlated with changes in HR-pQCT indices* (Table 2). Similar results were seen for cortical and trabecular vBMD, and at the distal tibia. These data further support that change in DXA BMD at the hip is distinct from change in peripheral bone density and strength. Furthermore there was large inter-individual variation in the rate of loss. While most men had declines in BMD, a proportion did not lose bone. In those who had repeat HR-pQCT and DXA scans over 1.6 years, ~25% maintained or slightly increased BMD at the FN, total hip, distal radius or distal tibia. The reasons for this variation are not known, and MrOS is uniquely positioned to identify them. Identifying predictors of change in the peripheral skeleton will provide new insight into how bone changes during the time of highest fracture risk.

C1e. Muscle and bone phenotypes (Aim 2c). DXA measures of lean mass have been largely unrelated to fracture risk likely because DXA measures lean mass, not muscle mass per se.[88] At Visit 4 in MrOS, we have shown that low muscle mass assessed with the deuterated creatine (D_3Cr) dilution method is strongly related to poor physical performance and risk of subsequent falls while measures of DXA lean mass are not.[91] In addition, our preliminary results show that the novel D3Cr dilution measure of muscle mass is strongly related to fractures, in particular hip fractures, in MrOS men (Table 3) while there were no significant associations with DXA lean mass (appendicular lean mass/height^2, ALM/ht^2) and fracture. In fact, men in the lowest quartile of D_3Cr muscle mass/wgt had a 10-fold greater risk of hip fractures and a 2-fold greater risk of non-spine fractures than men in the highest quartile after adjusted for confounders. These results suggest that the role of muscle in the etiology of fractures may be underappreciated. Furthermore, given the additional muscle phenotyping in MrOS in Visit 4 (jumping mechanography for lower extremity power; calf muscle volume and density from HR-pQCT[89]), we have the

unique ability to evaluate the influence of muscle characteristics on changes at the peripheral skeleton.

C1f. Proteomic and metabolomic markers of inflammation are linked to bone. As noted above, we and others have linked general markers of inflammation to bone loss. Moreover, in a novel high-throughput serum proteomics study in MrOS, we recently identified multiple markers of incident hip BMD loss and fracture.[82] BMD-loss proteins were significantly enriched in biological processes related to innate immune response and inflammation, including CD14,[92] complement factors CO7 and CO9,[93] and beta-2-microglobulin (B2MG).[94] Even more recently, using state-of-the-art mass spectrometry-based metabolomics (West Coast Metabolomics Center, NIH Regional Resource Core, UC Davis) we examined a panel of serum oxylipins – metabolites enriched in modulators of inflammation and cell proliferation – and found a number that were significantly associated with HR-pQCT parameters ($p < 0.001$ after stringent FDR correction). For instance, the fatty acid ketones/alcohols/diols 5-KETE, 8S-HETE, 15-HETE and 5,6-DiHETrE were associated with both distal radial trabecular and cortical BMD. Cumulatively, these data strongly support the role of inflammation in age-related bone loss. However, these associations have never been examined in very old people, previous studies have used general markers of inflammation, and the role of inflammation in peripheral bone loss has not been studied. We will use complete assessments of inflammation (OLink proteomics) and more comprehensive analyses of metabolites in serum from Visit 4 to more comprehensively delineate these relationships.

C2. MrOS visit structure/overview; proposed Visit 5

In MrOS, 5994 men >65 yrs were recruited and followed at 6 clinical centers (Birmingham, Minneapolis, Palo Alto, Pittsburg, Portland, San Diego). After baseline, there have been 3 subsequent clinic visits with complete phenotyping, including hip and spine

BMD, objective activity and physical performance (Table 4). Men in two sub-studies have also completed additional visits with similar assessments in large subgroups. Incident fractures have been ascertained with 3x/year mailed questionnaires with >95% follow-up rates and validation of reported fractures via medical record review. Retention has been outstanding: at each of the clinic visits we have collected at least partial data on >90% of all survivors and have complete clinic visit data on 70-85% of survivors. *At Visit 5, MrOS men will be ≥81 years old, and we expect the mean age to be ~88 years old*. Since enrollment in MrOS, 22.6% of the participants have experienced at least one fracture, including 305 men with hip fractures. We will continue to follow all surviving MrOS participants, which we project to be N=1767.

Table 3. Risk (HR, 95% CI) of fracture by quartiles of D_3Cr muscle mass/wgt or ALM/ht^2 in 1363 men (101 non-vert fx, 25 hip fx)

	D_3Cr muscle mass/wgt		ALM/ht^2	
	Hip	Non-vert	Hip	Non-vert
Q1 (low)	10.4 (1.3, 84.3)	2.0 (1.1, 3.6)	2.4 (0.5, 11.7)	0.7 (0.4,1.2)
Q2	7.6 (0.9, 60.9)	1.2 (0.6, 2.3)	3.3 (0.7, 15.7)	0.7 (0.4,1.3)
Q3	3.7 (0.4, 33.3)	1.5 (0.8, 2.8)	3.5 (0.7, 16.7)	0.9 (0.5,1.6)
Q4 (high)	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
p-trend	0.005	0.057	0.462	0.157

Adjusted for age, BMD, WHO FRAX probability

Table 4. Schedule of key assessments in MrOS

	Visit 1 2000-2	Visit 2 2005-6	Visit 3 2007-9	Visit 4 2014-6	Visit 5 2019-20
DXA (hip, spine)	X 5994	X 4495	X 3764	X 1833	X 564
CT-based FE analysis (hip, spine)	X 250 3786				X 330
HR-pQCT (radius, tibia)				X 1801	X 564
Weight, Height	X	X	X	X	X
Co-morbidities	X	X	X	X	X
Objective activity			X	X	
Physical performance	X	X	X	X	X
DXA (whole body)	X	X	X	X	
D_3Cr muscle mass				X	
Serum, urine	X	X	X	X	X

**ancillary measures in subsect of men not shown; proposed assessments highlighted in grey*

Baseline CT scan analysis. Hip and lumbar spine CT scans were obtained in ~3800 men at the MrOS baseline visit (2000-01). A random subset of these participants and all incident hip fractures through February 2008 had CT FEA measures completed on the baseline scans (N=250).[44] We will expand the CT FEA analysis to *all archived baseline CT scans (N~3800)*. This sample provides sufficient power to examine risk of any clinical fracture; any non-vertebral fracture; and any non-hip non-vertebral fracture, and determine whether the associations are independent of DXA BMD (Aim 2). We will also determine whether the *combination* of hip and spine strength (by FEA) is more effective in fracture prediction than either

alone, and compared to DXA BMD and whether the "weakest" femoral strength (determined by testing multiple fall directions) predicts fracture better than the standard sideways fall direction.

Participant Selection for Visit 5. At Visit 5 we will repeat DXA and HR-pQCT scans in participants at Portland, Palo Alto and Minneapolis. We project that at Visit 5 in Fall 2019, there will be 869 men alive at these 3 clinical centers. Of these survivors, we conservatively estimate that 65% will agree to a clinic visit (N=564) and have repeat DXA and HRpQCT data. Our estimate of 65% response takes into account the number of surviving participants who reside in nursing homes or assisted living and is substantially lower than response rates at previous visits. At those 3 centers at Visit 5, we will also repeat CT scans in all men who had a baseline CT (we project that 58.5%of those with clinical assessment at Visit 5 will have had a baseline CT). Thus, we estimate that ~ 330 men will have a repeat CT scan. All these men were <70 yrs at baseline, so we can *identify characteristics at an early stage that predict long term deleterious change by late life*. We have carefully considered recruitment feasibility given the age of the MrOS men. Our estimates were informed by previous visits in the oldest old, which has exceeded 70% in those >85 yrs. We also queried a convenience sample of 53 men at the Minnesota site to determine their willingness to attend Visit 5, including a CT scan (if eligible); 74% were willing to participate in such an exam, and 58.5% of those who agreed had a CT scan at baseline. Finally we selected these clinical centers for Visit 5 because they had a high proportion of valid baseline CT scans; and maintained the same CT manufacturer, making long-term comparisons feasible (see CT methods below).

C3. CT Methods

CT scan acquisition and processing. Volumetric spine and hip CT scans were acquired at 120 kVp and 80 kVp, respectively. CT images were reconstructed at 1 mm (spine) and 3 mm (hip) slice

thickness using a large field of view and a standard (soft tissue type) kernel. An external hydroxyapatite calibration phantom (Image Analysis) was scanned simultaneously with each subject to allow conversion of CT-Houndsfield units to equivalent mineral density (mg-HA/cm^3).

CT image analysis. CT image analyses will be performed at O.N. Diagnostics using the FDA-approved VirtuOst software to measure femoral and vertebral strength, as well as several densitometric and structural variables. Cadaver studies have consistently shown that experimentally measured values of femoral and vertebral strength are better predicted by CT-FEA than by DXA-BMD;[47,49] and fracture-outcome studies have validated CT-FEA for fracture risk assessment.[42,44,54, 55, 97,106] Detailed methods for FEA of the lumbar vertebra[99,107] and proximal femur[44,108,109] have been previously published. In brief, the L1 vertebral body and proximal femur will be segmented from the CT scan, rotated into a standard coordinate system, and resampled respectively into 1.0-mm and 1.5-mm cube-shaped voxels. The scan will then be calibrated using the hydroxyapatite phantom; the finite element mesh generated by converting each voxel into an 8-noded brick element; and non-linear material properties assigned based on the calibrated density and empirical relations, accounting for plastic yielding, material anisotropy, and tension-compression asymmetry;[107,110] and then virtual loads are applied. Axial compression and a sideways fall are simulated for the spine and femur, respectively. These approaches have been well validated ($R^2 = 0.78–0.97$) against direct mechanical strength testing for cadaver vertebrae[99,107] and femurs.[44, 108, 109] We will also derive trabecular, peripheral, and integral mass and volumetric density proximal femur (FN and total hip) and vertebral body, and measure structural features of the proximal femur (e.g., FN width and CSA) and vertebral body (e.g., height and CSA). This implementation has been used by O.N. Diagnostics for the past 10 years in trials of major osteoporosis treatments,[50, 96, 108, 109, 111,121] with an inter-operator repeatability precision of from 0.4–0.8%.[111]

C4. HR-pQCT Methods

HR-pQCT overview and participant selection. At V4, 1801 men had valid HR-pQCT XtremeCT II data for at least one of the three scan sites. The same scanner model will be used to acquire follow-up scans.[122] To assess change in HR-pQCT measures, men who had a HR-pQCT scan at V4 will be invited to return for repeat scans at V5. We estimate 869 men will be available and at least 564 will agree to the V5 HRpQCT scans.

HR-pQCT scan acquisition and processing. With a nominal voxel size of 61 μm and images spanning a 1-cm section of bone in forearm and lower limb, the XtremeCT II is a significant advance over the first generation scanner, permitting direct measurement of trabecular structure, more proximal coverage of the distal limbs, and faster scan time.[123] This project leverages the substantial data management, QA, and analytical resources developed by UCSF to conduct highly standardized multicenter HR-pQCT studies, including the previous MrOS V4. *We will use protocols identical to V4*. The distal radius will be scanned beginning 9.0 mm proximal to the mid-point of the distal articular surface. The tibia will be scanned at two positions: a) the standard distal position, starting 22.0 mm proximal to the distal articular surface; and b) with the scan volume centered at an offset corresponding to 30% of the tibial length, with respect to the distal articular surface.[124] Scan data will be transferred by internet to the Coordinating Center for centralized quality assurance and image analysis.

HR-pQCT quality control. Our online training procedure for QC certification reduces inter-operator measurement error for bone outcomes by 50%.[125] Operators at each clinic will complete a standard 2-day training session, and be certified to perform the imaging protocol. A central experienced observer will read all images for motion artifacts using an established semi-quantitative 5-point grading system (1=superior, 5=poor). Images graded 4 or 5 will be excluded.[126] At MrOS Visit 4, <3% of scans were excluded

due to poor image quality. The manufacturer's QC phantom will be scanned daily to monitor for density deviations exceeding a nominal threshold (8 mg HA/cm3). These data will be used to derive correction factors to account for longitudinal drift or deviations, as needed. A density cross-calibration phantom will be circulated between study sites and scanned at the initiation and termination of Visit 5. The phantom will be the same as that used at Visit 4, allowing inter-visit cross calibration. If inter-scanner differences in the reference densities measured in our cross-calibration phantom exceed 1%, correction factors will be applied to harmonize the density measures.[127]

HR-pQCT image analysis: The analysis pipeline begins with an automatic segmentation of the radius and tibia to quantitate volumetric density and structure.[128,129] Based on the compartment and fine-structure segmentation, standard bone density and structure measures are calculated, including BMD (Ct.BMD, Tb.BMD), cortical thickness and porosity (Ct.Th, Ct.Po), trabecular thickness, number, and heterogeneity (Tb.Th, Tb.N, Tb.Sp.SD). Novel measures of calf muscle volume (MV), fraction (MV/TV), and density (MD) will be derived at both tibial scan positions.[89] Linear elastic micro-finite element analysis (μFEA, Scanco FE Software v1.12) will be used to estimate bone strength, as previously published.[130, 131] Analyses will be performed at the UCSF/QB3 Shared Computing Facility – a 7000-node high performance computing cluster.

HR-pQCT considerations for longitudinal analyses: Periosteal expansion and trabecularization derived from endo- and intra-cortical bone loss can confound the standard approach for longitudinal scans. Thus, we will employ a custom automatic pipeline developed at UCSF that uses 3D image registration to co-localize conserved architectural features across visits, and spatially match volumes of interest. Unique to our approach is the transformation of the baseline endocortical contour onto the

corresponding follow-up scan. This unique approach ensures truly matched volumes are measured, and provides consistency in the definition of the cortical and trabecular compartments. For instance, a previous study132 showed that standard areal matching and individual endocortical segmentations underestimated cortical bone loss by more than 50%. Our approach represents a key improvement to the standard approach, and will provide rigor and reliability to our data.

C5. DXA Methods

DXA overview. With the proposed V5 assessment, MrOS men will have up to 7 hip and spine DXA scans over >20 years. Each participant had all previous scans completed on the Hologic 4500 (fan beam) scanner. Hologic does not support for 4500 scanners, so MrOS clinical centers will use new Hologic Horizon scanners at V5, with robust longitudinal calibration procedures to ensure cross-machine equivalency (described below).

DXA scan acquisition and processing. The same hip will be scanned as at all previous MrOS visits. If the same hip is not available (e.g. hip replacement, fracture), then all previous scans will be reanalyzed to match the scans to the same hip at all visits. If a participant is unable to have either hip scanned (e.g., due to bilateral hip replacements), scans after the second hip becomes ineligible will be set to missing.

DXA quality control. MrOS has well developed QC procedures and certification of DXA operators. Standardized procedures were used at all visits to ensure reproducibility of DXA measurements.[45] At baseline, a set of whole body, spine, hip and linearity phantoms were circulated and measured at all MrOS sites. These same phantoms will be circulated prior to V5. We will assess scanner variability across clinics and apply cross-calibration correction factors if required. To adjust for inter-clinic differences, statistical models include indicator variables for the individual scanners.

Each clinic will scan a local spine and hip phantom throughout the study, and correction factors applied as appropriate. The precision of DXA scans of the spine and hip is 1-2%.[133]

C6. Key predictors of change in HR-pQCT parameters

Muscle phenotypes. TTo support the analysis for predictors of change in HRpQCT measurements (Aim 1c), we will make use of the extensive MrOS database. For example, at Visits 3 and 4 we used the SenseWear Armband to derive objective assessments of activity,[134] and men at Visit 4 completed an extensive physical performance battery including the SPPB, grip strength, a long distance corridor walk,[135] and jumping mechanography assessments of lower extremity power. At most previous visits, including Visit 4, whole body DXA scans were used to assess lean mass, fat, and visceral adipose tissue. In addition, at Visit 4, we measured muscle mass using the novel deuterated creatine dilution (D3Cr dilution) method.[136] Finally, we have assessed calf muscle volume (MV), fraction (MV/TV), and density (MD) in the Visit 4 HR-pQCT scans.[89] These previously collected data are integral to understanding the relation between muscle, physical activity and change in bone microarchitecture in the peripheral skeleton.

Biochemical assays from Visit 4 archived serum will be used to examine associations with subsequent HR-pQCT change. Measures will be done in the 564 men who will have HR-pQCT scans at Visits 4 and 5 using fasting serum at Visit 4 stored at -80°C. 25OHD concentrations will be measured by LC-MS (Mayo Laboratories) as done previously in MrOS.[29] Sex steroids including total testosterone and estradiol will be measured by LC-MS/MS, and sex steroid binding globulin by 2-site chemiluminescent immunoassay (Mayo Laboratories) as done previously.[137] Inflammation will be comprehensively assessed with 1) proteomics (OLink): a comprehensive panel of 92 inflammation-related cytolines/proteins will be measured with proximity extension assay technology,[138] where oligonucleotide labeled antibody probe

pairs are allowed to bind to their respective target in the sample. A PCR reporter sequence is formed by a proximity dependent DNA polymerization event and is then detected and quantified using real-time PCR. The assays are highly precise and sensitive (pg-fg/ml). Analytical performance has been carefully validated; and 2) metabolomics - The UC Davis West Coast Metabolomics Center, a NIH-funded core facility, will perform the measures (see Fiehn letter and Resources). The majority of metabolites of interest will be lipids/bile acids/steroids or biogenic amines. We will use a) combined multi-targeted assay (HILIC-QTOF MS, LC-QTRAP MSMS) of bioactive oxylipins, steroids and bile acids, and b) a combined targeted and untargeted biogenic amine assay in HILIC-QTOF MS, including branched and unbranched acylcarnitines, TMAO, choline and amino acids. Data will be acquired for up to 100 identified compounds through HILIC-QTOF MS/MS on biogenic amines and up to 60 compounds on oxylipins and steroids through LC-QTRAP MSMS. The oxylipin/steroid samples will also be injected into an accurate mass LC-MS platform for untargeted analyses. Data will be normalized by pooled QC samples.

C7. Clinical covariate assessment (factors to be repeated at Visit 5) MrOS men have been extensively characterized. We will use identical protocols and data collection forms as previous visits. Critical measures likely to be important time-varying factors will be included in the Visit 5 assessment. As with previous visits, we will measure weight with calibrated balance beam or digital scales and height with wall-mounted stadiometers. Physical activity at Visit 5 will be self-reported on the Physical Activity Scale for the Elderly (PASE).[139] Men will again self-report a physician diagnosis of medical conditions including diabetes, heart failure, chronic obstructive lung disease, cancer, atherosclerotic cardiovascular disease, osteoarthritis and a variety of other conditions that are common in old age. We will repeat assessments the Short Physical Performance Battery[140] (balance, chair stands, walking speed) and grip strength.

C8. Fracture outcomes

We will continue follow-up for incident fractures in all survivors using well established MrOS protocols. Participants will be sent questionnaires 3x/year; those who report fractures will be contacted by clinical center staff to provide information about the circumstances of the fracture and obtain consent for medical record collection. Clinical center staff will forward radiology reports or image to the Coordinating Center for review. Vital status will be updated by death certificate review.

C9. Statistical Analyses

Data Analysis will be overseen by Drs. Peggy Cawthon (Coordinating Center) and Jodi Lapidus (Portland), and conducted by analysts with many years of experience with MrOS data. All analyses will begin with assumption-free plots of associations and models appropriate for the nature of the variables and data distributions; e.g. we will transform variables that are not normally distributed prior to including them in regression models. We will decide a priori whether we consider covariates to be confounders or mediators when we include them in multivariate models. We are testing a series of distinct pre-specified hypotheses. Thus, we will control for multiple comparisons within each aim, but not across aims: e.g., the analysis that raise the biggest concern about type I errors are those at that evaluate metabolic and proteomic markers of inflammation as predictors of peripheral bone loss. Our approach to multiple comparisons is below (Aim 2a.) In all models, we will consider prior history of a fracture as a co-variate and explore interactions with fracture history as appropriate.

Table 5. Summary of analysis approaches

Aim	Approach
1a. Characterize long term change in density, structure, strength	Linear regression (mixed effects reg. secondary)
1b. Long term BMD change & incident fracture	Proportional hazards; (joint models secondary)
1c. CT-FEA & incident fracture	Proportional hazards
2a. HRpQCT & incident fracture	Proportional hazards
2b, 2c Change in HR-pQCT	Linear regression

Table 6. Summary of sample size by aim

Aim	N	Data	Inclusion
Aim 1a (CT)	330	V1 & V5	Men at PO, PA, MN w/repeat CT (must have V1 CT)
Aim 1a (DXA)	4652	V1-5	Men with at least one repeat DXA scan during follow-up
Aim 1b	1832 (262 fx)	V1-4, fxs	Men with Visit 4 DXA & incident fracture data
Aim 1c	3786 (856 fx)	V1	Men with baseline CT & incident fracture
Aim 2a	1801 (58 hip fx)	V4, fxs	Men with Visit 4 HRpQCT scan & incident fracture data
Aim 2b, 2c	564	V4 & V5	PO, PA, MN men w/repeat V5 HRpQCT (& V4 HRpQCT)

Aim 1a. Characterize long-term change in hip and spine bone density, structure and strength over 20 years. For analyses of continuous predictors and outcomes, such as baseline hip structure, bone area and change in bone strength, we will use linear models. We will carefully consider the role of other determinants of change in strength (such as weight change), both as potential confounders, time-varying and interacting variables. These models will allow us to identify predictors of long term deleterious change in bone, for instance large bone area. To characterize change in CT-based measures, we will analyze simple linear change, as values will be available from only two time points (baseline and Visit 5). For assessing change in DXA BMD, our primary approach will be to use mixed effects linear regression models, as we have done previously.[45] These models accommodate the correlation of outcomes within subjects by including random intercept and slope terms. This method provides subject-specific predicted slopes (changes) and intercepts (initial values). We will use the predicted slope (change), and the intercept (initial value) to define favorable vs. unfavorable trajectories. We will also test for non-linear change by including age^2 and age^3 terms in the models. We will first model the trajectories as continuous variables, and we will evaluate thresholds using splines. If no clear thresholds are evidenced after examining the spline analyses, trajectories will be categorized into

favorable vs. unfavorable groupings using distributional methods, such as using quartiles or standard deviations above/below the mean change as previously employed.[45] An alternative approach will be to develop trajectories using shared parameter models[141] with PROC NLMIXED in SAS. These models include a random effect that links two trajectories together (for example, in DXA BMD and area), and will allow for estimating the correlation between the trajectories. Both approaches can accommodate unequal numbers and timing of measurements (including missing data at interim and follow-up visits), reflecting the data in MrOS. To compare the change in DXA BMD and CT-FEA will compare the magnitude of change in standardized variables.

Aim 1b. Determine whether baseline and long-term change in DXA BMD predict fracture. We will examine change in DXA BMD from baseline to Visit 4 (as described for Aim 1a), and its relation to fracture after Visit 4. Our primary outcome of this aim is any clinical fracture, but we will also examine any non-vertebral, hip and non-hip non vertebral fractures as secondary outcomes. Our primary approach will be proportional hazards models with time to first event after Visit 4; we will verify the proportionality assumption. We will consider the competing risk of mortality by using the approach of Fine and Gray.[142] Finally, in this and other analyses we will include reported falls (data collected every 4 months throughout the study) as a time-dependent covariates subsequent to Visit 4. We will also explore the use of joint models.[143] Briefly, this method simultaneously models longitudinal process and event time outcome to estimate associations between hazards of an event and a time-varying covariates (such as DXA BMD, and change in DXA BMD). Given the endogenous nature of the bone measures (i.e. a fracture could directly influence the future trajectory of bone loss), this provides flexibility in expressing relationship between the two processes (e.g. trajectory slope, cumulative effects, etc.), and ability to estimate predictions for individual participants. We posit that fracture risk depends on

both current value of DXA BMD as well as its slope as it changes over 15 years. We will parametrize the longitudinal process in the joint models to estimate these associations. Also, we will fit joint models that estimate associations between the cumulative effects of the bone measures (area under the trajectory curve) and fracture. We will fit all models using Bayesian approaches as implemented in the jmbayes package in R.[144] Competing risks of mortality and covariates (e.g. change in weight) will be incorporated into the models. Our primary DXA BMD measurement site will be femoral neck BMD, but in this and other BMD analyses we will also explore total hip BMD. We will explore the relative clinical utility of change in BMD as a predictor of facture (vs. Visit 4 BMD) in a number of ways. First, we will evaluate discrimination using Harrell's C-index[145, 146] (a discrimination measure analogous to the area under the ROC curve). Second, we will calculate overall and clinical net reclassification improvement[147, 148] for the final model (change in BMD and Visit 4 BMD) relative to reduced models (Visit BMD alone). Finally, we will evaluate model calibration by comparing predicted vs. observed values.

Aim 1c. Baseline femoral bone strength from CT-FEA and fracture. We will analyze all baseline scans for CT-FEA (N=3786). Our primary outcome will be any clinical fracture, and we will run secondary analyses with time to non-vertebral fracture, and non-hip non-vertebral fracture as outcomes. We will also confirm associations with hip fracture we previously published using a case-cohort design and relatively short follow-up time (5.6 years).[44] We will examine the association between femoral strength and incident fracture using proportional hazards models, and test whether this association is independent of DXA hip BMD. As in Aim 1b, we will explore the clinical utility of the CT-FEA measure (vs DXA-BMD) by evaluating the discrimination, reclassification improvement and calibration of models with CT-FEA alone vs models with both CT-FEA and DXA-BMD as predictors.

Aim 2a. HR-pQCT parameters and hip fracture. As in Aims 1b and 1c, we will use Cox models to estimate the association between HR-pQCT parameters (at Visit 4) and fracture. Our primary outcome will be hip fractures, and we will run secondary analyses with time to non-vertebral and non-hip non-vertebral fractures as outcomes. We will confirm associations with any clinical fracture previously published with short follow-up time (1.7 years).[90] In these models we will consider time-varying covariates, the competing risk of mortality, and the independence from DXA BMD. We will standardize HR-pQCT parameters before inclusion in models to allow comparison between these variables. Since the HR-pQCT variables are inter-related and moderately correlated with each other, we will explore the use of penalized regression to identify the few HR-pQCT metrics most highly related to fracture risk. To do this, we will include all significant HR-pQCT metrics that are associated with hip fracture in a single, "elastic net" regression model. These models address the problem of the high variance of regression coefficients in models that occur with high number of correlated predictors, a situation encountered with HR-pQCT variables. This machine learning method obviates the need for subjective model building strategies, leading to models with better predictive ability compared to non-penalized methods.

Aim 2b. Establish the rate and character of peripheral bone loss, and Aim 2c. Key determinants of peripheral bone loss. We will estimate change in HR-pQCT parameters between Visits 4 and 5 using simple linear change. To analyze the relative contribution of cortical vs. trabecular bone in the loss of μFEA-bone strength, we will use models with trabecular and cortical bone measures as separate variables. We will use linear regression models to test the association between measures of D_3Cr muscle mass, physical performance, sex hormones, 25OHD, and markers of inflammation/metabolism with changes in peripheral bone. We will consider confounding and interacting variables such as weight, weight loss, adiposity and comorbidity when constructing all of these models.

For proteomic and metabolomic data (Aim 2c), we will use a modification of our published analytic pipeline,[82] which consists of (a) rigorous methods to robustly estimate and test individual protein- and metabolite-level associations, accounting for multiple comparisons, and (b) generation of signatures and network-level interpretations to data generated to elucidate relationships.

Missing data. Men returning for CT or HR-pQCT scans might be healthier than men who do not return, which may bias estimates from analyses describing change in these measures (i.e., Aims 1a, 2b, 2c). We will implement an inverse probability weighting (IPW) strategy to limit such bias. We will model the probability of missingness at V5 based on previously collected MrOS data (e.g., comorbidities at V1-4.) We will use the inverse of those probabilities as weights for complete records in the models. We will use standard procedures to develop the missingness model including selecting candidate predictors a priori, evaluating transformations and/or interactions to improve model fit, and reducing the influence of excessively large weights.[149]

C10. Sample size and power

Refer to the Recruitment and Retention Plan for figures that depict the sample size for each aim. Aim 1a. Characterize long-term change in hip and spine bone density, structure and strength over 20 years. There are two main sets of analyses for this aim. First, analyses of change in CT-based measures of density, structure and strength will include the 330 surviving men who will have repeat CT (Table 6). With this sample size, at 80% power, we will be able to detect small associations. For example, for each 1 SD increment in a numeric predictor, such baseline bone size, we will be able to detect an increment of change in femoral strength as small as 0.15 SD. Second, analyses of change in DXA BMD will include 4652 men with at least 2 measures of DXA BMD. We will be well powered to detect very small associations. We have calculated minimal detectable effects using linear regression, as standard

software packages do not estimate effect sizes for mixed effects regression models. Thus, for each SD increment in a numerical predictor (such as bone size) we will be able to detect an increment of change in femoral neck BMD as small as 0.04 SD. Given the very small effects we will also consider clinically meaningful changes when interpreting statistically significant findings.

Aim 1b. Determine whether baseline and long-term change in DXA predict fracture. For primary analyses for Aim 1b analyses will be limited to men with Visit 4 DXA BMD (N=1832). Our primary outcome is any clinical fracture. We estimate that between Visit 4 and Year 4 of the currently proposed project, 13% (N=262) of these men will have experienced at least one clinical fracture. This is based on fracture and mortality rates previously experienced in MrOS men, standardized to the age of the men in the proposed phase of the project. With a correlation between covariates at 0.4, for each SD increment in change DXA BMD, at 80% power, we will be able to detect an HR as small as 1.25, which translates to an HR of 1.67 for Q1 vs. Q4.

Aim 1c. Baseline femoral bone strength from CT-FEA and fracture. Our primary outcome is any clinical fracture after V1. Of the men with a baseline CT (N=3786), we anticipate that 856 men will have experienced at least one fracture between baseline through the end of the fourth grant year of the proposed project. At 80% power, we will be to detect an HR as small as 1.13 per SD increment in a continuous predictor (such as CT-FEA based strength), which translates to an HR for Q1 vs. Q4 of 1.32. This accounts for a correlation between covariates of 0.4, allowing us to test whether CT-FEA measures are independent of DXA BMD.

Aim 2a. HR-pQCT parameters and hip fracture. In the 1801 men with HR-pQCT measures at Visit 4, we anticipate that 3.2% (N=58) will have a hip fracture (primary outcome for this aim) by the end of Year 4 of the project. For each SD increment in a Visit

4 HR-pQCT parameter (such as failure load), at 80% power, and a correlation between covariates of 0.4, we will be able to detect an HR as small as 1.61, translating to an HR of 2.99 for Q1 vs. Q4. This effect size is smaller than the associations we report between failure load and total vBMD and any clinical fracture (Table 1), and much smaller than the preliminary association between failure load or vBMD at the distal radius and hip fracture in MrOS (Prelim Data). In addition, we anticipate that 11% (N=198) will experience a non-vertebral, non-hip fracture (secondary outcome for this aim) allowing us to detect an HR as low as of 1.29 per SD in a continuous HR-pQCT predictor for this outcome.

Aim 2b. Establish the rate and character of peripheral bone loss, and Aim 2c Key determinants of peripheral bone loss. 564 men will have repeat HR-pQCT measures. At 80% power, we will be able to detect small associations. For example, for each 1 SD increment in a numeric predictor (such as walking speed), we will be able to detect an increment of change in failure load at the distal radius as small as 0.12 SD. From the metabolomics and proteomics panels, we estimate that ~250 protein and metabolite associations with bone loss will be assessed. Controlling for false discoveries and assuming between 10-30% of the proteins/ metabolites will be correlated with bone loss, we estimate sample sizes between 452 and 592 are required to detect modest correlations (0.15) at an average power of 80%, with FDR=5%.[150]

C11. Rigor and transparency in research
We will ensure rigor and transparency in this study by following best practices. Analyses will be strictly governed by the MrOS Publications Committee. Manuscripts must have an approved analysis plan that details statistical methods and provides justification for the analyses. Statistical analyses in manuscripts from outside the MrOS team are reviewed for errors and omissions; all papers undergo Publication Review. This rigorous process reduces "data dredging" to search for spurious associations and ensures high quality, reliable results.

C12. Study administrative structure

The current MrOS administrative structure will serve as a foundation for the next grant cycle. The project will be led by Principal Investigators Dr. Orwoll (who is the PI for the current MrOS project), Dr. Cawthon, and Dr. Bouxsein (see Multiple PI plan.) These investigators will form the Leadership Committee for the project, and will meet regularly by phone. The clinical site PIs (Drs. Cauley, Ensrud, Stefanick, Shikany and Kado), along with Dr. Cummings (co-I, Coordinating Center), will form the Steering Committee. Proposed publications will be reviewed by the Steering Committee. The MrOS Study is monitored by an independent Observational Study Monitoring Board (OSMB) created by NIA that regularly reviews participant safety and subject burden.

C13. Timeline

Visit 5 participants will be recruited over 12 months after a 6-month start-up period for staff training (by the Coordinating Center, the UCSF HR-pQCT center and O.N. Diagnostics). The CT-FEA will begin in the second half of Year 1 (analysis of baseline scans and receipt/review of the newly acquired Visit 5 scans) and will continue through Year 3. Repeat HR-pQCT imaging, processing and analysis will begin in Year 1 and continue through Year 3. Biochemical measures on the men with repeat HR-pQCT scans will be completed in Year 3. Statistical analyses will begin in Year 1 and continuing throughout the project period with analyses of all specific aims. Datasets will be updated and released every six months and preliminary incident post-Visit 5 fracture analysis will begin when all participants have accrued at least one year of post-Visit 5 follow-up time.

C14. Pitfalls, Alternative Approaches, Future Directions & Implications

- Since MrOS includes only men, direct comparisons between men and women are not possible. To compare our

findings to those in women, we will continue to leverage outside collaborations, including data from cohorts of women (e.g. SWAN) or cohorts with data from both men and women (such as Framingham).

- The CT analyses will study survivors. Nevertheless, these survivors are indeed those at the highest risk of fracture. Moreover, MrOS is the most comprehensive, long-term study of musculoskeletal health in men. Our findings will set the stage for replication in other cohorts.
- We will ask elderly men to have non-invasive but time-consuming procedures, potentially limiting the number willing to volunteer. However, we have an outstanding record of retention in MrOS, have designed protocols to optimize the ease of participation and have conservatively based our estimates of the number willing to participate on extensive experience. Our power estimates suggest we will have quite adequate numbers.

Results from these proposed studies will inform clinical practice by providing new insights into identification of men at high risk for skeletal deterioration and fractures and optimal use of therapies. By identifying the mechanisms that underlie skeletal deterioration, our results will also promote development of novel interventions. Finally, the imaging and biomarker data collected in this unique population will provide many opportunities for further research on musculoskeletal aging.

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Common Pathogenetic Mechanisms in the Association between Low Bone Density and Artery Calcification

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Because of the essential and evolutionarily-ancient role for calcium Cardiovascular disease (CVD) and osteoporosis are both common age-related conditions with major public health impacts since they result in disability, substantial health care use and costs and increased mortality. Epidemiological and biological evidence suggest possible associations between CVD and osteoporosis. Several cohort studies have shown that CVD events are associated with an increased risk of hip fracture: in the large Swedish twin registry of all most 32,000 persons, the multivariable adjusted hazard ratio (HR) (95% confidence intervals (CI)) of hip fracture after a diagnosis of heart failure was HR=4.40 (95% Cl, 3.43-5.63); stroke, HR=5.09 (95% Cl,4.18-6.20); peripheral atherosclerosis, HR=3.20 (95% Cl, 2.28-4.50) and ischemic heart disease, HR=2.32 (95% CI, 1.91-2.84). The greatest risks were observed in the early follow-up period [1]. Results were consistent across several types of CVD events. Vascular calcification is a marker of arteriosclerosis and an independent predictor of CVD and its mortality. In a recent meta-analysis of prospective studies, Wei et al showed that the relative risk (RR) of any fracture in those with the highest abdominal aortic calcifications (AAC) compared with the lowest AAC was RR=1.64 (95% Cl,1.30-2.07)[2]. Results were similar in studies that adjusted for bone mineral density (BMD). Results were especially strong for hip fracture, RR=1.64 (95% Cl,1.22-2.20) but not with vertebral fracture, RR=l.45 (95% Cl,0.81-2.58)[2]. Low BMD has also been linked to CVD mortality, morbidity and subclinical arteriosclerosis.

The link between BMD and osteoporosis may reflect shared risk

factors: Age, premature menopause, estrogen efficiency, sedentary lifestyles, smoking, diabetes, vitamin D deficiency, genetics, glucocorticoid therapy and rheumatoid arthritis[3,4]. Risk factors with the opposite effect on osteoporosis and CVD include gender and obesity. Common pathological mechanisms may involve sex steroid hormones, inflammation, cytokines, oxidative stress and lipids. Atherosclerotic calcification and bone calcification share several common features: mineral in atherosclerotic plaque is similar to hydroxyapatite; calcified plaques express several bone matrix proteins and osteogenic cells have been found in atherosclerotic plaques. Other pathogenetic mechanisms may include a role for parathyroid hormones and fibroblast growth factor 23 (FGF23), sclerostin and osteoprotegerin. Further understanding of the link between the two major diseases may improve our understanding of the pathophysiology of each condition and may help to identify those at higher risk of two of the most important age-related disease outcomes.

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Congenital Dental Disorders

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Tooth development or odontogenesis is the complex process by which teeth form from embryonic cells, grow, and erupt into the mouth. Primary teeth start to form between the sixth and eighth week of prenatal development, and permanent teeth begin to form after birth, therefore reflecting two completely different environments for biomineralization. Tooth is a highly calcified organ associating tissues from various embryonic origins, e.g. the ectomensenchyme which derives from the neural crest for pulp, dentin and cementum and the ectoderm for enamel. Dentin and cementum are similar to bone in several aspects, especially regarding the composition of the extracellular matrix (ECM), which is secreted by well-differentiated odontoblasts, cementoblasts and osteoblasts, respectively. Bone is a repository for stored calcium (about 99% of total body calcium) where it can be quickly mobilized for exchanges with the extracellular space. Bone is richly vascularized and participates in the control of calcium and phosphate metabolism to a large extent (together with the kidney and intestine) through a permanent process of remodeling. In contrast, neither dentin nor cementum are involved in the regulation of the calcium and phosphate homeostasis. In addition, dentin is never remodeled under physiological conditions and only the cellular cementum can undergo remodeling in response to mechanical constraints

mediated by forces of mastication, continual tooth eruption and drifting, parafunctional gnathic activities, and orthodontic tooth movement. Several genetic disorders leading to low phosphatemia disturb bone but also dentin and cementum mineralization[4,8,9]. These mainly include X-linked hyphophosphatemia (XLH) due to inactivating mutations of the *PHEX* gene, but also mutations in the *ENPP1* gene (GACI syndrome), autosomal recessive hypophosphatemia due to *DMP1* mutations, gain of function mutations of *FGF23* and loss of function mutation in the *ALP* gene (hypophosphatasia). Several genetic disorders also result in hyperphosphatemia including loss of function mutations in *FGF23* and *GALNT3*. For these patients, it can also be considered that the dentin, not remodeling during life, reflects the biomineralization process in both physiological and pathological conditions. Dentin can be used as a marker to study the impact of the systemic treatment on the mineralization process. The tooth is therefore a valuable tool to study the biomineralization process, and we had extensively demonstrated this hypothesis in the context of XLH over the years. By studying XLH teeth, we have contributed to the knowledge of the underlying mechanisms of the disease, particularly regarding ECM protein degradation and release of pathological peptides[2,3,5,7]. We have also shown that the conventional treatment, based on phosphorus supplementation and active vitamin D analogs and commonly administrated from early childhood to the end of growth, strongly improves dentin and cementum mineralization. This results in better dental health, with lower occurrence of dental abscesses and pulp necrosis as well as lower susceptibility to periodontitis in adults1,8. Patients with genetic disorders causing hypocalcemia such as hypoparathyroidism (gain of function mutations in the *CaSR* or *GNA11* genes, loss of function mutations in the *GCM2* and PTH genes…) or Pseudohypoparathyroidism10 (mutations or imprinting defects in the *GNAS* locus) may also present dental abnormalities mainly manifesting by abnormal enamel structure that can result in localized enamel defects (enamel hypoplasia or enamel opacities) or in Amelogenesis

imperfecta, which affects the enamel structure of all the teeth. In addition, we have recently reported that loss-of-function mutations in Claudin-16 and-19 (CLDN16 and 19) encoding genes, initially identified to cause Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis (FHHNC), also resulted in *Amelogenesis imperfecta*[11]. In parallel, several congenital disorders mainly affect the tooth structure without obvious clinical manifestations on other organs and especially the skeleton. Both localized *Amelogenesis imperfecta* or *Dentinogenesis imperfecta* are mainly related to genes encoding major components of the enamel (*AMELX, ENAM, MMP-20….*) or the dentin (*DSPP*). These disorders show clinical signs very similar to disorders related to the mineral metabolism and it is sometimes difficult to settle a firm diagnosis without the molecular analysis. The aim of this presentation will be to give an overview on the impact of congenital disorders related to the mineral homeostasis on tooth formation. In all these disorders, patients may develop severe dental manifestations strongly altering their quality of life during childhood as well as adulthood. Therefore, their dental condition should be part of the diagnosis, treatment and follow-up for each patient.

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Congenital Skeletal High Mineralization Disorders

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The establishment and maintenance of bone mass is an exquisitely fine-tuned process. Bone-forming osteoblasts and bone-resorbing osteoclasts play the major roles in determining the degree of mineralization of the skeleton.

Historically, the congenital high bone mass disorders were defined and categorized by their radiographic appearance, anatomical distribution of the lesions, and the mode of inheritance. The identification of the genetic and molecular underpinnings of these disorders over the last two decades offers a new way of categorizing these disorders that complements and refines our understanding.

A logical and informative way to understand the high bone mass disorders is to divide them into disorders of impaired bone formation (osteoblasts), and impaired bone resorption (osteoclasts), and to examine them in light of the molecular pathophysiology caused by the underlying genetic defects.

Osteoblasts are mesenchymal-derived cells that produce the collagen-rich extracellular matrix that is the scaffolding upon which the mineral component of bone, hydroxyapatite, is deposited. Relative to the size of the cell, the amount of matrix produced is massive. Approximately 90% of bone extracellular matrix is type 1 collagen, the rest non-collagenous glycoproteins, hyaluronan and proteoglycans. An additional unique feature of bone extracellular matrix is that it serves as a sort of repository for growth factors, such as TGF-β, that can be released during bone remodeling and have local effects. Defects at any step of this process can lead to bone sclerosing disorders.

The most important and best studied pathway involved in high bone mass-related diseases associated with osteoblastic bone formation is the Wnt pathway. The Wnt pathway is a highly conserved signaling pathway in which activation in osteogenic cells leads to bone formation and an increase in bone mass. The first evidence of the importance of this pathway in humans was the identification of gain-of-function mutations in the low-density lipoprotein receptor-related protein 5 (LRP5), found in families with benign high bone mass. It has been shown that LRP5 (and LRP6) and the G-protein coupled receptor Frizzled are coreceptors for Wnt proteins and activate the canonical Wnt signaling pathway. Downstream Wnt signaling results in β-catenin stabilization, nuclear localization and transcription of β-catenin-regulated genes. The pathway is kept in check by binding of sclerostin (encoded boy SOST) to LRP5/6, which impairs Wnt binding to the LRP5/frizzled complex, and thus blocking Wnt signal transduction.

An important osteoblast-mediated pathway that couples osteoblast and osteoclast activity and functions to control the relative levels of bone formation and resorption is the Receptor Activator of Nuclear factor Kappa-B (RANK)/RANK Ligand (RANKL)/ osteoprotegerin (OPG) pathway. Osteoblast-produced RANKL, either cell membrane associated or secreted, binds to RANK expressed on the cell surface of osteoclasts and promotes osteoclast differentiation and activity. Osteoblast-produced OPG serves as a "decoy receptor" for RANKL and blocks RANKL/RANK interaction and signaling. Mutations in either TNFSF11A or TNFSF11, which code for RANK and RANKL, respectively result in the sclerosing disorder of osteoclast-poor osteopetrosis.

Osteoclasts are cells derived from monocytes of the hematopoietic lineage. Through the combined action of RANK/RANKL and monocyte stimulating factor (M-CSF) preosteoclasts are stimulated to differentiate and are recruited to sites of bone turnover. They form tight junctions on mineralized surfaces and initiate bone resorption by creating an acidic resorption pit into which protons are secreted and demineralization of matrix is enabled. Mutations in key proteins

involved in acid generation include carbonic anhydrase II, which generates protons, chloride voltage-gated channel 7 (CLCN7), which is necessary for maintenance intracellular electroneutrality in the process of proton generation, osteoclastogenesis associated transmembrane protein 1 (OSTM1), which functions as a beta subunit of CLCN7 and is necessary for normal CLCN7 function. Vacuolar-type H+-ATPase (V-ATPase) pumps protons across the plasma membrane into the resorption pit. Osteoclasts also produce a number of lysosomal proteases including cathepsin K (CTSK), a cysteine protease, and matrix metalloproteinases (MMPs), zinc-dependent proteolytic enzymes that function as collagenases and are necessary for bone lysosomal matrix digestion. Mutations in any of these genes can lead to increased bone mass and osteosclerosis. The identification of the genetic variants responsible for the high bone mass diseases have not only elucidated our understanding of these rare diseases, they have led to the development of drugs that treat osteoporosis such as the RANKL inhibitor denosumab and the recently approved sclerostin inhibitor romosozumab.[1,4]

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Effects of Osteo-anabolic Drugs on Bone Formation and Mineralization

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Bone anabolism requires an increase in bone mass that is caused by an increased rate of new bone formation without a simultaneous increase in the rate of bone resorption. This increase in bone mass can occur on trabecular, endocortical, or periosteal surfaces. Histomorphometric data from bone biopsies are necessary, but not sufficient to characterize an agent as having anabolic activity. Likewise, non-invasive measurements of bone mass are necessary, but not sufficient. Both types of measurements are required[1].

Osteoporosis is characterized by reduced bone mass and micro-architectural deterioration leading to increased fracture risk. While paired bone biopsy studies have shown that antiresorptive drugs are able to prevent further deterioration in bone structure, only anabolic drugs agents have been shown to restore microstructure towards normal in both cortical and cancellous skeletal compartments. In other words, only anabolic drugs have the potential to reverse the disease process, rather than simply prevent it from worsening. Therefore, in patients with more severe osteoporosis (by low BMD and/or fracture criteria), it is clear that the best approach is to restore microstructural integrity with a short course (1-2 years) of an anabolic agent and then maintain the improved bone structure with an antiresorptive agent[2].

The initial response of iliac bone to daily, subcutaneous teriparatide (TPTD) treatment, observed as early as 4 weeks, is an increase of osteoblastic bone formation achieved by an increase in the linear rate of mineral apposition and an increase in extent of bone

forming surface, as revealed by increased osteoblast, osteoid, and mineralizing perimeters, mineral apposition rate, and bone formation rate. The results from the study by Lindsay et al.[3] suggest that TPTD is capable of stimulating bone formation in remodeling units that were active before initiation of treatment. This is presumably achieved by stimulating the production rate of preexisting osteoblasts, and/or by enhanced recruitment of osteoblasts into preexisting bone-forming units, and/or by increasing osteoblast life span. One of the most dramatic effects of TPTD treatment is its ability to increase the surface area of individual forming units, which is achieved by extending bone formation to quiescent surfaces adjacent to the original resorption cavity. While most of the new bone formation that is induced by TPTD treatment occurs over scalloped reversal lines (~70%) indicating prior resorption, there is evidence for formation on previously quiescent surfaces with smooth reversal lines, i.e., modeling-based bone formation.

The improvement in cancellous and cortical bone structure with TPTD treatment was confirmed by micro-CT measurements of the iliac bone showing that TPTD treatment increased cancellous bone volume and trabecular connectivity, with a shift towards a more a plate-like structure, and increased cortical thickness[4]. Analysis by quantitative backscattered electron imaging (qBEI) revealed that TPTD treatment increased the percentage of bone matrix with lower matrix mineralization, and mineral crystallinity associated with a larger proportion of newly formed bone[5]. The SHOTZ trial compared the effects of TPTD and zoledronic acid (ZOL) treatment over 6 and 24 months on mineralization density distribution assessed by qBEI. In cancellous bone, ZOL treatment was associated at 6 and 24 months with significantly higher average degree of mineralization, and with lower percentage of low mineralized areas, and reduced heterogeneity of mineralization, indicating higher mineralization density and more homogeneous mineral content versus TPTD. Within the ZOL group, significant changes were found in all parameters from month 6 to 24, indicating

a progressive increase in mineralization density. In sharp contrast, mineralization density did not increase over time with TPTD, reflecting ongoing deposition of new bone[6].

Recently, Cosman et al reported[7] the effect of a brief course of TPTD on the human femoral neck. This was the first histomorphometric evaluation of the effect of any osteoporosis medication at this important site of osteoporosis-related fracture. The bone formation rate was increased in cancellous and endocortical compartments after just 6 weeks of treatment.

Abaloparatide (ABL) is a human parathyroid hormone-related peptide [PTHrP(1-34)] analog. The ACTIVE trial was a randomized, double-blind, placebo controlled study. Iliac bone biopsies were obtained in a subset of patients treated with placebo, ABL or TPTD for between 12 and 18 months. Histomorphometric data[8] showed, that in a standard panel of static and dynamic indices among the three treatment groups, there were only few significant differences, i.e., a higher mineral apposition rate in the TPTD-treated group than in the placebo-treated group, a lower eroded surface in the ABL-treated group than in the placebo-treated group, and a higher cortical porosity in both the ABL and the TPTD-treated groups than in the placebo-treated group.

Romosozumab (ROMO) is a humanized monoclonal antibody that binds to sclerostin, which is a key inhibitor of bone formation. Two phase 3 clinical trials showed that a 12 month treatment with ROMO rapidly increased bone density and reduced vertebral, non-vertebral and clinical fracture risk compared to placebo treatment and also compared to treatment with alendronate. Using quadruple labeling, a bone biopsy study[9] demonstrated that ROMO significantly increased bone formation rate at 2 months in the cancellous and endocortical envelopes. Micro-CT of bone biopsies after 12 months of ROMO treatment revealed superior cancellous and cortical bone mass and microstructure.

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Calcium requirements in bone mineralization

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Calcium is an essential element that plays numerous biological functions in the body, and one of the most important is skeletal mineralization. Calcium is the major component of bone, where it is present at more than 99% as calcium-phosphate complexes, and provides skeletal strength and structure, making the bone a metabolic reservoir to maintain the intra-and extra-cellular calcium pool. Specifically, both bone forming and resorbing cells use calcium signals as regulators of differentiation and activity. A great deal of research has focussed on determining the relationship between calcium intake and bone health.

As discussed above, bones are formed mainly of mineralised connective tissue. They have several roles that include mechanical, synthetic and metabolic functions. The most notable metabolic function of bone is mineral storage, particularly calcium and phosphorus as 99% of body calcium is found in the skeleton. By weight, bone is approximately 70% mineral and 30% organic. The composition of the mineral phase is about 95% hydroxyapatite, a highly organized crystal of calcium and phosphorous, and other ions (such as sodium, magnesium, fluoride, and strontium) while the organic phase (also known as osteoid) is made up of 98% collagen fibres, and by a ground substance formed by glycoproteins and proteoglycans.

Bone can be classified into two types; cortical bone, a dense layer that forms the outer surface of most bone and the shafts of the long bones and cancellous or trabecular bone, which is spongy in nature and it is found at the end of long bones and within flat bones and vertebrae. The calcification of these two types is different. Hence while cortical bone has a predominantly structural function,

as 80%-90% of its volume is calcified, trabecular bone serves a metabolic function and is only 15%–25% calcified.

It is widely recognized that bone mass and density are determined by various concurrent factors, such as genetics, hormones, physical activity, and certainly, nutrition. While genetic factors have a critical role in growth and peak bone development, an adequate intake of bone nutrients represents the main factor for the full expression of a given genetic potential and for bone maintenance during adulthood. Among the various nutrients, calcium and vitamin D have proven their efficacy for normal bone growth and development in children and adolescents and for the maintenance of bone mineral loss in postmenopausal women. An optimal calcium intake is necessary for bone health at all stages of life. Dietary requirements for calcium are determined by the need for bone development and bone maintenance, which vary throughout life, being higher during childhood and adolescence, during pregnancy and lactation, and in the elderly (Table 1).

Calcium can be obtained from the diet and also through supplementation, and the latter is often recommended for patients with osteoporosis along with vitamin D. Despite the acknowledged importance of calcium in bone health, studies examining the effects of calcium supplementation on bone mineral density have been inconsistent. Although some have shown positive effects of calcium on areal BMD, a meta-analysis of calcium supplementation in healthy children showed differing effects depending on pubertal stage and site examined. It has been suggested that calcium supplementation may be more beneficial in those that have lower baseline levels although this has not been confirmed. Furthermore, it is uncertain whether any gains in bone mass remain once the supplementation ceases with evidence, particularly in children, pointing to a lack of sustained effect. Later in life, the benefits of calcium supplementation without adjuvant vitamin D supplementation on fracture risk are not well demonstrated, with

some concerns regarding possible cardiovascular risk associated with calcium supplementation (although this link remains uncertain and no such risk has been demonstrated with dietary supplementation).

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Table 1. IOM 2010 Institute of Medicine of the UA National Academy of Sciences recommendations for daily dietary calcium intake

INFANCY TO ADOLESCENCE	MG/ DAY
0-12 MONTHS	200mg first 6 months; 260mg thereafter
1-3 YEARS	700mg
4-8 YEARS	1000mg
9-13 YEARS	1300mg
14-18 YEARS	1300mg
WOMEN	
19-50 YEARS	1000mg
POST MENOPAUSE	1200mg
MEN	
19-70 YEARS	1000mg
71 YEARS +	1200mg

Defects in mineralisation: Osteoporosis and Atypical Femur Fractures

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Bone formation occurs during bone development as part of bone modelling and during remodelling of the skeleton throughout adult life. Treatments for osteoporosis take advantage of the bone remodelling cycle and either inhibit bone resorption, increase bone formation or do both. During bone formation, osteoblasts deposit a collagen-containing osteoid matrix which is strengthened by accrual of calcium hydroxyapatite crystals. Mineralisation is initially rapid (primary), and continues slowly (secondary) to a maximum, which optimises bone strength. Antiresorptive drugs, such as bisphosphonates, reduce bone remodelling and increase secondary mineralisation of bone matrix. An area of debate is whether prolonged treatment with antiresorptive drugs increases secondary mineralisation above optimal levels, resulting in brittle bone. Anabolic drugs, such as teriparatide and abaloparatide, increase bone remodelling and bone formation, and induce primary mineralisation in newly formed bone. As bone forms, some osteoblasts are embedded in the bone matrix and further differentiate into osteocytes. Recent data show that these osteocytes are the bone cells that promote secondary mineralisation, and this is inhibited by the transmembrane protein EphrinB2 (*gene: Efnb2*). In mice with osteocyte-targeted deletion of *Efnb2*, the healthy level of mineralisation is exceeded, leading to brittle bones.

This preclinical model parallels the high mineralisation seen in bisphosphonate-associated atypical femur fractures (AFF) in humans. These are spontaneous transverse full-width stress fractures originating in lateral femur cortex in the subtrochanteric or diaphyseal regions of the femur at sites of greatest biomechanical

stress during walking. AFF is devastating to the individual and 28% of AFF patients suffer bilateral fractures, and non-healing occurs in 30% of patients. Although AFF location and appearance has been well defined, the underlying cause of AFF is unknown, and susceptibility cannot currently be determined, despite a strong relationship with duration of bisphosphonate use. Evidence is accumulating that this may be an intrinsic defect in the bone material composition: (1) there appears to be underlying genetic susceptibility to AFF, (2) Asian women have a 6-fold higher risk of AFF, and higher underlying tissue mineral density than Caucasian women, and (3) when bone from fracture sites of patients with AFF was assessed by FTIRM and laboratory-based mechanical testing, it was found to have a higher mineral:matrix ratio, and lower fracture toughness, than bone from women with typical osteoporotic fractures. Using high resolution-peripheral quantitative computerised tomography (HR-pQCT), we have also identified a higher matrix mineralization density and lower cortical porosity in postmenopausal women with AFF compared with either healthy or osteoporotic postmenopausal women. The higher mineral:matrix ratio in bone from patients with AFF reveals a striking similarity between AFF bone and the brittle mouse bone due to osteocyte-targeted deletion of Efnb2, and has led us to hypothesise that similar mechanisms cause high mineral:matrix ratios in both patients with AFF and in osteocytic EphrinB2 deficiency. However, further studies of both conditions are now required to define how mineralisation is controlled by osteocytes, and identify pathways that may be targeted in the future to increase bone strength from within the matrix itself, to prevent AFF. The identification of either novel or existing genes associated with AFFs may also lead to further insights into bone mineralisation in health and disease, and new treatment pathways.

Biomineralization of the skeleton in Health and Diseases

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Bone material consists of organic matrix, mineral particles and water. The organic matrix is predominately collagen type I and includes non-collagenous proteins and proteoglycans. Minerals confers rigidity to the material and the amount of mineral per volume of matrix seems to be controlled within a narrow window for a given species[1]. Indeed, it has been shown by quantitative backscattered electron imaging (qBEI) of transiliac human biopsy samples that, irrespectively of age and gender, the distribution of calcium content in adults has an average of 22.2% with a full width at half maximum of 3.3%[2]. Any deviation from this physiological window is potentially deleterious and leading to increased bone fragility: hypermineralization stiffens the material and, likely, makes it more brittle, while hypomineralization leads to softer and weaker bone[3]. However, during growth and in situations of high bone turnover, calcium content can be slightly lower because newly formed bone packets take time to mineralize[4,5].

Bone mineral density (aBMD) as assessed in clinical context by DXA is a combination of bone volume and the calcium content within the bone matrix, which both impact the fracture resistance of bone. This lecture will focus on the latter quantity, which may be assessed in biopsy samples by means of the Bone Mineralization Density Distribution (BMDD). The BMDD histogram provides information on the calcium (and, thus, mineral) distribution within the mineralized bone matrix, reflecting bone turnover, mineralization kinetics and tissue age[5]. Genetic or metabolic bone disorders may affect BMDD in various ways, thereby increasing bone fragility.

Hypomineralization of the bone matrix often results from vitamin D deficiency and/or low calcium intake. At the world-wide level, nutritional rickets in children remain a severe problem. The condition is characterized by defective chondrocyte differentiation and mineralization. At the bone tissue level, there is osteomalacia, a pathologic increase in osteoid (unmineralized bone matrix), quantified by bone histomorphometry. Post-mortem analyzes of a bone sample from an infant that died by cardiac failure with radiographically confirmed rickets, low ionized calcium and low 25(OH) vitamin D levels, showed a shift of the BMDD curve towards lower mineral content and a 6-fold increase of the fraction of poorly mineralized matrix compared to reference values[6]. In healthy adults, the necessity of systematic vitamin D supplementation is still matter of debate. A post-mortem study from northern Germany that examined iliac crest biopsies from 675 individuals revealed osteomalacia in about 25% of the cases independently of age and gender but not when 25(OH) vitamin D exceeded 75 nmol/L[7].

Vitamin D and calcium are generally prescribed in postmenopausal osteoporosis, a systemic disorder, where bone loss results from increased bone turnover which in addition, leads to larger fraction of younger and thus less mineralized bone packets. In a basic multicellular unit (BMU), osteoclasts need about three or four weeks to form a resorption pit while osteoblasts require a couple of months to "refill" the cavity[8]. During menopause (or other alterations in sex hormone levels) the net number of such BMUs is elevated resulting in a predominance of bone resorption over formation. In consequence, there is a decrease of bone volume due to excessive activity of osteoclasts and a decrease of the degree of bone matrix mineralization due to the high abundance of newly formed bone that has not enough time to fully mineralize. Treatment with antiresorptive agents such as bisphosphonate have shown to reduce fracture risk but often do not show a net increase in BMD. By combining measurements of lumbar spine

BMD and qBEI analysis of paired iliac crest biopsies of patients treated with calcium/vitamin D with or without the bisphosphonate risedronate, the effects on bone volume and the calcium content within the bone matrix could be separately evaluated[9]. Calcium/vitamin D supplementation increased bone matrix mineralization in all patients and the increase was higher when mineralization was lower at baseline. This indicates that available mineralization sites are filled with mineral with increased calcium/vitamin D intake. After bisphosphonate treatment there was an additionally increase in bone volume most likely due to the reduction in bone resorption.

Hypermineralization of the bone matrix often mirrors a long-lasting situation of abnormally low bone turnover. This might happen for example in children with chronic inflammatory bowel disorders, such as Crohn´n disease or other illnesses that require treatment with glucocorticoids. These drugs are known to promote osteoblasts, osteocytes apoptosis and to delay growth and pubertal development[10]. A special situation is observed after solid organ transplantation, that has become a successful mode of therapy within the last years. Already before transplantation, children with hepatic and renal osteodystrophy were at high risk of developing osteoporosis, fractures and scoliosis. After transplantation, high fracture incidence often persists due to the long-term glucocorticoid and immunosuppressive medication. Indeed, it was shown in a cohort study of 23 young allograft recipients that the bone matrix was hypermineralized, probably as a consequence of low bone turnover as assessed by histomorphometry in the same biopsy samples[11]. Growth impairment, low bone turnover, and similar BMDD curves reflecting hypermineralization were also found in children with chronic kidney disease on dialysis[12]. Interestingly, after growth hormone therapy, these patients showed a marked increase in height together with a boost in bone turnover and a decrease of bone matrix mineralization towards normal range. This indicates that skeletal growth has not only the potential to improve the bone microarchitecture in juvenile osteoporosis, but also to

normalize mineral content levels in the bone matrix.

An inherently different situation is found in Osteogenesis imperfecta (OI), an extremely heterogenous, inherited collagen-related disorder characterized by low bone mass, high bone fragility, where bone tissue is hypermineralized despite high bone turnover [13]. About 85% of the cases are caused by dominant autosomal mutations in the type I collagen coding genes (*COL1A1* and *COL1A2*), affecting collagen quantity or structure. Very rare forms of OI often result from autosomal recessive mutations in genes encoding proteins involved in type I collagen synthesis, processing, secretion and post-translational modification, or proteins involved in the differentiation of bone-forming cells. OI caused by collagen-gene mutations have the same increase in mean calcium content of the bone matrix independently of the type of mutation (either qualitative or quantitative) and the clinical severity (mild to severe, i.e. type I, IV, III). The same is true for OI with defects in post-translational collagen modification, such as in non-lethal type VII and VIII OI. Moreover, the increased mineral content remains unchanged by bisphosphonate therapy. In bone biopsy samples from children with mild forms of OI, the thickness of bone mineral particles was the same or smaller than in control bone from healthy children. In combination with the increased matrix mineral content this implies that mineral particles are more densely packed in OI, making the bone material stiffer and more prone to fractures. The hypermineralization of the matrix increases its radiodensity, so that DXA measurements often fail to identify patients with mild forms of OI as osteoporotic. Consistently, very rare cases of type XII OI with extremely high bone matrix mineralization were even described as a “high bone mass OI” phenotype by DXA.

A very special case is type V OI, caused by a gain-of-function mutation in *IFITM5*. Phenotypically, the disorder is characterized by interosseous membrane ossification and hyperplastic callus. At the bone tissue level, the mineral content is increased as

in other forms of OI. However, unlike other OI types, there is "mesh-like" appearance of the bone matrix and a highly increased osteocyte lacuna density. This is most likely caused by exuberant overproduction of highly disordered primary woven bone at the expense of a lamellar arrangement[14]. Together with the hypermineralization of the bone matrix, the failure in organizing ordered bone lamellae does certainly contribute to bone fragility in type V OI.

The examples discussed above show the contribution of either hypo- or hypermineralization of the bone matrix to skeletal fragility. Beyond the mineral content, the organization and quality of the bone matrix as well as its microarchitecture at all hierarchical levels play a crucial role for the biomechanical integrity of bone[15].

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Earth's Magnetic Biosphere:
magnetite biomineralization from the archean rise of magnetotactic bacteria to the human geomagnetic sensory system

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Magnetite biomineralization is arguably one of the first true matrix-mediated biomineralization systems to have evolved within the terrestrial biosphere. Although the initial discoveries of the magnetotactic bacteria were in only a few branches of the Proteobacteria, we now know that mag-netotaxis exists in at least five separate phyla within the Bacterial Domain, with estimated diver-gence times that extend back into mid to late Archean time (3.5-3.0 Billion years ago[1,2]). As shown on Fig. 1, this constraint comes from DNA sequences of the homologous "magnetosome island" genes that control magnetosome formation, demonstrating that they follow the RNA phylogeny with no evidence of lateral gene transfer. There is also an inversion in the gene order between the Nitrospirae and other Phyla, which also demonstrates no significant genetic exchange since before these groups diverged[1]. These data show that Earth's most primitive biosphere was magnetic, having the capability to accumulate environmental iron, organize it into vesicles and with precise specificity induce the biomineralization of two ferrimagnetic minerals, magnetite (Fe_3O_4) and Greigite (Fe_3S_4), which have similar genetic controls.

In addition to demonstrating the biochemistry for matrix-mediated biomineralization was present (a.k.a., Lowenstam[3]), these genetic data also support strongly the continuous presence of Earth's geomagnetic field over this time interval, and hence the existence of vigorous convection in the liquid Ni-Fe outer core of Earth needed to generate it.

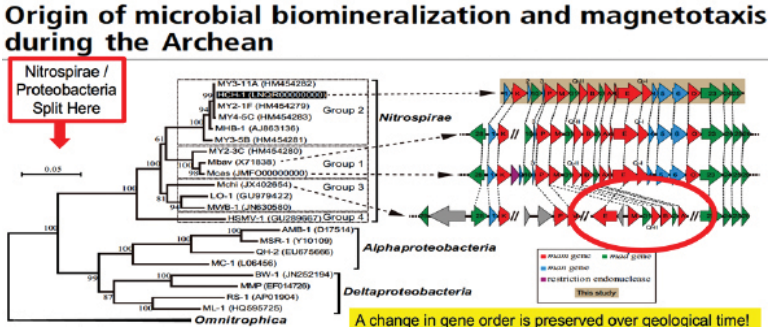


Figure 1. evolutionary relationships and gene order within the magnetosome island operon in divergant bacterial phyla. from Lin et al.[1]

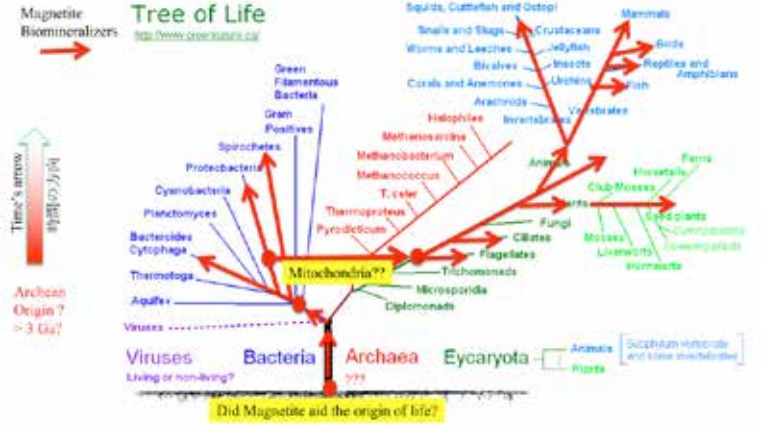
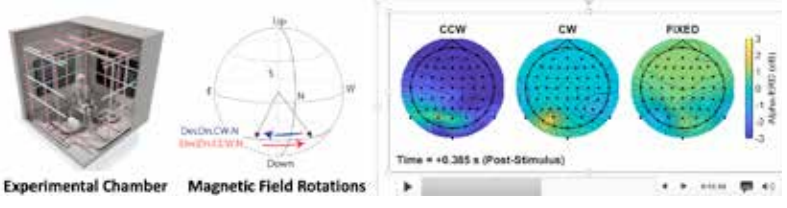
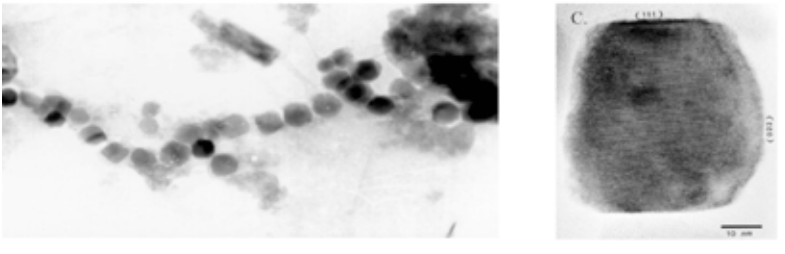


Figure 2. Schematic representation of the 'treeof life', showing with red arrows the know (or presumptive) distribution of magnetite-precipitating organisms.

Magnetite biomineralization is also clearly present in a large variety of Eukaryotes, ranging from: 1) single-celled Protozoans[4] and other protist fossils with gigantic, sculpted crystals[5], 2) grasses[6], 3) its original discovery as a capping material in the radular teeth of the chitons (molluscs of the class Polyplacophora[7], 4) the magnetosome chains present in the frontal tissues of fish[8], and 5) in a variety of human tissues, including the brain[9,12]. Figure 2 shows a simplified evolutionary scheme arguing that magnetotaxis might be an original trait of the Eukaryotic domain, perhaps with inheritance from the α-Proteobacterial endosymbiont that was the ancestor of all mitochondria[13]. Figure 3 compares TEM images of magnetite crystals extracted from the salmon with those from the human brain. It is also possible that the magnetite biomineralization system formed the template for other matrix-mediated biomineralization processes via genetic exaptation[14].





Salmon Magnetosome Chains

Human Brain Magnetite

Figure 3. Comparison of biogenic magnetite crystals extracted from the frontal tissues of salmon[15] (left) with those from tissues of the human brain[9,16] (right). All of thee lie within the single-domain stability field for magnetite, indica-ting that they are uniformly and stably magnetized, and suitable for use in animal's magnetic sense organelles.

Figure 4. Illustration of the Wang et al.[24] discovery of the geomagnetic sensory system in humans, as measured via 64 EEG recordings on a participant's head. Left: schematic diagram of the magnetic stimuation chamber, housed within an isolated Faraday cague. Center: Schematic representation of the 0.1 second field sweeps of a downwards-directed Earth-strength magnetic field, simulating the relative change of the magnetic field with respect to a human head that would be produced by shaking one's head from left to right. (Clockwise = CW; counterclockwise = CCW, add FIXED is the control with no field change; note that participants sit quietly in the dark are are asked to minimize movements during the 7-minute recording sessions.) Right: Screen-shot of a video clip of the relative power in the alpha band of a highly-responsive brain. 385 seconds after the field shift began. The dark blue image of the CCW shifts is a typical whole-brain drop in alpha-power called an alpha event-releated desynchronization, or alpha-ERD.

Biogenic magnetite was suggested many years ago as a possible biophysical transducer for the geomagnetic sensory system in homing and migratory animals[17-19] (see these reviews[20,21]). Magnetite-based receptors can explain both polar and axial compasses responses[22], and uniquely accounts for the effects of strong magnetic pulses such as those that disorient homing bats[23].

We reported recently the discovery that human brainwaves are affected selectively by spe-cific rotations of earth-strength magnetic fields that are designed to mimic the normal motions of nodding or shaking one's head[24], as summarized here in Fig. 4. Our data show

that human brains are receiving information from the geomagnetic field and processing it selectively before triggering specific patterns of neural activity, including pronounced decrease in the amplitude of the ~ 10 Hz alpha wave that dominates EEG signals. This filtering stops the brain from responding when the field is 'unusual' compared to the normal configuration. In particular, no response is seen in the presence of a static vertical magnetic field, eliminating both electrical induction and a quantum compass as the underlying geomagnetic sensor[24]. Biogenic magnetite present in specialized sensory organelles[18] is the only remaing hypothesis that can explain all of these results; these have been characterized in fish[25-27].

A final, surprising, and extraordinarily important role of magnetite in Earth's biosphere con-cerns its remarkable ability to nucleate ice crystals when it is present in supercooled water. Without an ordered surface capable of arranging water molecules to form an ice seed crystal, ultrapure water will cool to below -30˚C before freezing homogeneously. In a recent series of papers, Kobayashi et al.[28-30] demonstrated theoretically and then experimentally that ppb levels of fine-grained magnetite present in plant and animal tissues control the ice nucleation process. Applying a weak ~ 1 mT ro-tating magnetic field to these tissues that is designed to agitate the magnetite particles more than normal Brownian motion was found to suppress this nucleation, allowing tissues to supercool. This is known to minimize the tissue damage from ice crysals, potentially reducing the enormous loss of food between the farm and the kitchen in the human supply chain[30].

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Fetal Development of the Skeleton

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In the adult, the intestines, kidneys, and bone each play important roles in maintaining mineral balance and bone mass. The intestines are the main source of minerals, the kidneys reclaim or excrete minerals, and the skeleton maintains a daily input and output of minerals in response to normal bone turnover. Disturbances in any of these compartments cause disorders, such as impaired intestinal calcium absorption leading to hypocalcemia with compensatory secondary hyperparathyroidism.

But mineral and bone metabolism are regulated quite differently *in utero*. The fetal kidneys, intestines, and skeleton are not dominant sources of mineral supply for the fetus. The kidneys passively filter and excrete minerals into amniotic fluid, which is largely composed of urine. Any swallowed amniotic fluid is absorbed through the intestines, which at this stage of development are capable of only passive absorption. The actions of the renal-amniotic-intestinal loop ensure that any mineral excreted by the kidneys aren't permanently lost to the fetus. The skeleton does provide some minerals to the circulation, especially if secondary hyperparathyroidism develops in response to inadequate supply from the mother. However, for the most part the directional flow of minerals is into the rapidly developing and mineralizing skeleton, with 80% or more of minerals accreted during the third trimester.

The placenta meets the fetal demand by actively transporting calcium, phosphorus, and magnesium from the maternal circulation; it also serves the functions that the kidneys will claim after birth. The fetal circulation contains higher concentrations of minerals than in the mother and normal adult; such high levels appear necessary for the developing skeleton to accrete a normal

amount of minerals by term. The high serum calcium may also protect against life-threatening post-natal hypocalcemia, because there is an obligatory fall in calcium with the onset of breathing and cutting of the umbilical cord.

Parathyroid hormone (PTH), calcitriol, and the sex steroids circulate at low concentrations in the fetal circulation. Intact fibroblast growth factor-23 (FGF23) circulates at half to equal to the maternal concentration. The high fetal serum calcium acts through the known parathyroid calcium sensing receptor (CaSR) to suppress PTH, while an unknown placental or fetal receptor is responsible for setting the high level of fetal calcium. Other receptors may set the levels of phosphorus and magnesium.

Fetal bone development and the regulation of serum minerals are critically dependent upon PTH and PTH-related protein (PTHrP), but not vitamin D/calcitriol, fibroblast growth factor-23 (FGF23), calcitonin, or the sex steroids. Absence of PTH leads to hypocalcemia, hyperphosphatemia, and normal endochondral bone development except for low mineral content. In contrast, absence of PTHrP leads to hypocalcemia and hyperphosphatemia, but accelerated endochondral bone development, shortened limbs, and normal to increased mineral content due to premature and abnormal mineralization of structures. Absence of vitamin D, calcitriol, or the vitamin D receptor do not disturb serum mineral concentrations, PTH, or skeletal development and mineralization. Absence or excess of FGF23 does not disturb mineral or bone homeostasis, in contrast to its dominant role in regulating phosphate metabolism after birth. Loss of calcitonin causes mild hypomagnesemia and reduced skeletal mineral content, but fetal mineral and bone metabolism are otherwise normal. Loss of the sex steroid receptors do not cause any overt abnormalities at birth, but the effect of their absence has not been studied in utero.

After birth, the serum calcium falls and phosphorus rises before

gradually reaching adult values over the subsequent 24-48 hours. The intestines become the main source of minerals for the neonate, the kidneys begin to actively reabsorb minerals, and bone turnover contributes minerals to the circulation. This switch in the regulation of mineral homeostasis is triggered by loss of the placenta and a postnatal fall in serum calcium. It is followed in sequence by a rise in PTH and then an increase in calcitriol. Over hours to days, the kidneys become responsive to the CaSR and FGF23. Intestinal calcium absorption is initially a passive process facilitated by lactose, but later becomes active and calcitriol-dependent. However, calcitriol's role can be bypassed by increasing the calcium content of the diet, or by parenteral administration of calcium.

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Biomineralization and the kidney

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1. Introduction:
The kidney plays a unique role in biomineralization by: 1) synthesizing the biologically active form of vitamin D, 1α,25-dihydroxyvitamin D (1α,25(OH)2D), which regulates calcium (Ca) and phosphorus (P) absorption in the intestine and kidney, and bone resorption and mineralization; 2) regulating the renal excretion of Ca and P through parathyroid hormone (PTH) and vitamin D-dependent and independent processes. Many of these processes are altered in the context of chronic renal failure (CRF) with attendant hyperphosphatemia, hypocalcemia, and secondary hyperparathyroidism (HPT) occurring as a consequence of intestinal Ca malabsorption and alterations in renal tubular P handling. The pathophysiology of secondary HPT in the context of CRF has been extensively described.
The focus of my presentation will be altered biomineralization that occurs in the context of urinary stone disease and recent data demonstrating changes in metabolism of 1α,25(OH)2D.

2.0 Urinary Stone disease and its consequences:
Urinary stone disease (USD) is a painful metabolic disorder with a lifetime prevalence of 7.2 - 7.7%1 and a 10 yr. recurrence rate of 30% or more2-4. Health care expenditures for a person with USD are twice those of an individual without USD5. The cost for treatment of USD exceeds $10.3 billion5. Reduction of morbidity through prevention of stone passage episodes is an important goal. Existing dietary and drug preventative interventions are often ineffective and the identification of high-risk populations through genomic testing and development of drugs based on new pathogenic mechanisms is needed to improve patient care.

2.1 Altered Biomineralization in Urinary Stone Disease: Biomineralization in the kidney is altered in idiopathic Ca stone formers (ICSF), a majority of whom have increases in the supersaturation for Ca driven by increases in urinary Ca as a result of increases in the Ca regulating hormone, 1α,25(OH)2D6,7.

2.2 The Pathophysiology of Altered Biomineralization in USD: A majority of urinary stones contain Ca, specifically Ca oxalate. Among, incident symptomatic kidney stone formers, 94% have purely Ca containing stones (Ca oxalate and/or hydroxyapatite), so called idiopathic calcium stone formers (ICSFs)6. Even among the remaining 6% (mostly uric acid stone formers), some component of Ca is often present in the stone and future episodes are often Ca stones6. Many patients with USD have hypercalciuria or uCa excretion that is higher than that in control subjects and that increases urinary supersaturation for Ca oxalate7. In many of these patients, stone formation begins in Randall's plaques (RPs) - renal medullary interstitial Ca phosphate deposits8, characterized by deposits of Ca in and around the ascending limb of Henle's loop. My colleague, J. Liekse9,10 & others11, have highlighted the importance RPs, in the pathogenesis of Ca stones.

2.3 The heritability of Hypercalciuria:
The heritability of USD risk factors such as hypercalciuria is well established12. In recurrent SF, variations in genes related to vitamin D and Ca metabolism, and genes affecting the urinary milieu are sometimes present13. GWAS studies have associated gene variants of the CYP24A1, CLDN14, SLC34A1, AQ1, and DGKH genes with USD14-16; variants of CYP24A1, CASR, and GATA3 genes with changes in sCa; and variants of the DGKD gene with altered bone density17. Environmental factors also influence stone risk, suggesting epigenomic changes in these pathways may also be important.

2.4 Altered Vitamin D Metabolism in Hypercalciuria and USD:

In an effort to identify the cause of hypercalciuria in USD, we studied 149 1st-episode SF, >90% of whom had Ca stones, and showed that SF had higher serum and urine Ca, s1,25(OH)2D, with lower s24,25(OH)2D/25(OH)D ratios compared to age- and sex-matched controls7. Others have confirmed the presence of higher sCa, UCa, and s1,25(OH)2D in SF18. Our expanded data show that increased sCa and UCa in SF are associated with elevated s1,25(OH)2D as a consequence of impaired 1,25(OH)2D inactivation by the 1,25(OH)2D-24-hydroxylase(OHase) that can be measured by assessing s24,25(OH)2D and its substrate, s25(OH)D (Table 1). These findings in SF are supported by observations in patients with familial hypercalcemia with hypercalciuria and nephrolithiasis due to inactivating mutations of the CYP24A1 gene19-21. Studies suggest that a reset in Ca metabolism, including a subtle increase in serum and urine Ca, associates with the amount of RP and a RP-like stone morphology22,23. In a series of over 345 SF the Lieske group has demonstrated that RP amount correlates with urine Ca excretion and the propensity to form stones10. Lieske et al have suggested that hypercalciuria occurring as a result of elevated 1,25(OH)2D concentrations contributes to the formation of RP by mechanism outlined in Figure 1.

3.0 Conclusions:

Abnormal biomineralization in ICSF is associated with increases in serum and urinary Ca and s1,25(OH)2D, together with decreased s24,25(OH)2D/25(OH)D ratios suggesting impairment in the 24-hydroxylation of 1,25(OH)2D.

Fig.1. The proposed mechanism of RP formation.
↑ 1,25(OH)2D3 leads to ↑ sCa (A) which ↑ Ca in UF. ↑ sCa suppresses thick ascending limb Ca reabsorption (B), while the ↑ 1,25(OH)2D3 stimulates DT Ca reabsorption (C). Ca reabsorbed in the DT is deposited in RP (D). Hypercalciuria (E), favors growth of stones upon RPs. Courtesy J. Lieske MD. Mayo Clinic, Rochester.

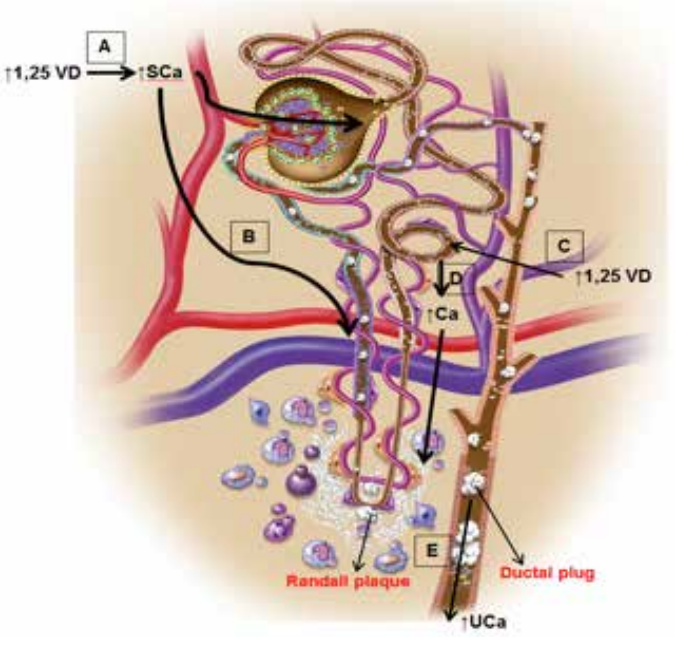

Table 1. Baseline characteristics of first time stone formers (SF) and matched controls in southeastern Minnesota. All values are represented as % or mean ± SEM (N with measurements currently available).			
Biochemical Parameter	Controls (n=460)*	SF (n=444)*	P
Male	48.8%	47.2%	0.25
Age (yrs.)	45.7 ± 14.8	46.6 ± 14.4	0.27
BMI	28.1 ± 5.7	30.6 ± 7.1	<0.001
Hypertension	20.7%	33%	<0.001
Diabetes	8.6%	12.2%	0.14
S Ca, mg/dL	**9.24 ± 0.0.63 (n=400)**	**9.40 ± 0.53 (n=351)**	**0.0009**
S Pi, mg/dL	**3.41 ± 0.51 (n=400)**	**3.73± 0.1.64 (n=460)**	**0.02**
S Uric Acid, mg/dl	5.05 ± 1.50 (n=400)	5.63 ± 1.40 (n=351)	<0.001
S Creatinine, mg/dl	0.84 ± 0.20 (n=400)	0.90 ± 0.51 (n=353)	0.03
S PTH, pg/mL	**38.84 ± 1.21 (n=201)**	**42.05 ± 1.53 (n=149)**	**0.096**
S 25(OH)D, ng/mL	32.12 ± 0.66 (n=201)	33.76 ± 0.91 (n=149)	0.136
S 1,25(OH)2D, pg/mL	**38.11 ± 0.94 (n=201)**	**43.71 ± 1.07 (n=149)**	**0.001**
S 24,25$(OH)_2$D, ng/mL	3.18 ± 0.11 (n=201)	3.094 ± 0.12 (n=149)	0.591
S 24,25(OH)2D/	**0.097 ± 0.03 (n=201)**	**0.087 ± 0.03 (n=149)**	**0.008**
S FGF23 pg/mL	**59.22 ± 1.59 (n=201)**	**62.22 ± 2.38 (n=149)**	**0.278**
U Volume, ml	1850 ±1267 (n=397)	1718 ± 786 (n=349)	0.18
U Ca, mg/24h	**158 ± 154 (n=397)**	**211 ± 127 (n=349)**	**<0.001**
U Pi, mg/24 h	658 ± 556 (n=397)	715 ± 366 (n=349)	0.001
U Citrate, mg/24h	582 ± 444 (n=397)	583 ± 338 (n=349)	0.644
U Oxalate, mmol/24h	0.28 ± 0.20 (n=397)	0.24 ± 0.17 (n=349)	<0.001
U Uric acid, mg/24h	448 ± 434 (n=397)	432 ± 211 (n=349)	0.81

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Bone Morphogenetic Protein: Function, Applications and Complications

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Bone Healing involves a complex interaction of cells, cytokines and growth factors. Marshall Urist in the late 1900's reported from his seminal work a series of investigators have progressively identified a family of discovery of a growth factor he named bone morphogenetic protein that had the capacity to initiate a bone healing cascade. Wang and Wosney purified three different BMP's and identified their amino acid sequences. Today there are more than 15 such proteins all related to the transforming growth factor superfamily. BMP 2, 4, and 7 receptors are heteromeric complexes of type I and II serine/threonine kinase. These specific BMP's (2, 4, and 7) uniquely differentiate mesenchymal stem-cells toward osteoblastic and chondrogenic direction. Following an array of animal studies the BMP's were shown to fill cranial bone defects, enhance healing of critical sized bone segmental defects and produce spine fusions. Based on the successful preclinical animal studies BMP's have progressed to extensive clinical trials and widespread application to fracture healing and spine fusion. Rigorous clinical trials have demonstrated their efficacy in augmenting bone healing, however, the complication profile and large cost have tempered widespread usage.

Around 2002 the European Medicines Agency and the American Food and Drug Administration approved BMP-2 for the treatment of open tibial fractures after stabilization with an intramedullary nail. Soon thereafter BMP-7 (OP-1) was also approved for the treatment of recalcitrant long-bone nonunions. The supporting clinical trials were very specific and the indications narrow.

However, once released, the BMP treatment was broadly applied to fracture healing. In a recent systemic review of BMP augmented bone healing, Krishnakumar reported that BMP-2 was the most frequently used factor. Among RCT and comparative trials, 5/9 reported positive results, 2/9 neutral results/ and2/9 identified significant complications. All noted enhanced tibial healing, less interventions and/or lower infection rates. However in several of the studies there were a higher rate of complications including heterotopic ossification, local edema, and calcinosis. There is minimal data when applied to nonunions. The papers utilized BMP-7 (OP-1) with comparable results to autogenous bone graft. Several series also reported HO. There is only one direct comparison of BMP-2 vs. BMP-7 and that was in a retrospective study of nonunion. In that limited study the BMP-2 was superior but the level of investigation was low and further studies are needed.

The most common application for BMP resides in spine fusion. BMP has been applied to posteriolater fusion, interbody fusion both anteriorly and posteriorly, and cervical fusion. In all of these randomized trials the BMP led to a higher rate of fusion but large numbers were required. Soon after their release, the authors were challenged about the complication profile by Caragee. Two highly academic groups revisited the released and not-released data to independently assess the true complication risks. The major adverse events included ectopic bone formation, osteoclast activation with osteolysis and subsidence, bone cyst formation, seroma formation, radiculitis, and severe spine swelling. This latter finding has led to the nonuse of BMP in anterior cervical spine fusion. Other areas of concern are not resolved and these include an increased risk for cancer and retrograde ejaculation. The Yale analysis (Simmonds) concluded “at 24 months, rhBMP-2 increases fusion rates, reduces pain by a clinically insignificant amount, and increases early postsurgical pain compared with ICBG”. Evidence of increased cancer incidence is inconclusive.

The remaining issue is the large cost of the BMP. How cost effective is this agent in spine fusion and fracture healing. Further studies are needed. Nevertheless, BMP's do offer true enhancement of bone healing and now need a critical examination as to the most appropriate application.

Clinical Lessons and Potential Solutions for XLH

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X-linked hypophosphatemia (XLH), due to PHEX mutations, is the most frequent form of hypophosphatemic rickets/osteomalacia. XLH is a dominant disorder with a prevalence of approximately 1.7/100,000 children to 4.8/100,000 persons[1]. XLH represents approx. 80% of all cases of XLH[2] up to 87% of familial cases and 72% of sporadic cases[3].

XLH is X-linked dominantly inherited, hence there are twice more affected girls than boys. PHEX, the gene responsible for XLH was identified on chromosome Xp22[4]. It codes for a cell surface-bound protein-cleaving enzyme expressed predominantly in bone and teeth[5]. The altered function of this bone-derived endopeptidase causes both the mineralization defect and the renal phenotypic abnormalities of XLH. A large number of inactivating PHEX mutations can cause XLH and there is no obvious correlation between genotype and phenotype. Mutations may lie in intronic regions or in the promotor[2]. In addition, somatic mutations of PHEX may mimic an autosomal dominant trait in XLH patients[6]. Strangely, there is no major gender-difference in the phenotype[7].

The molecular defect in PHEX leads to an increase production of circulating FGF23. FGF23 is critical in controlling serum phosphate level through the reabsorption of phosphate in the renal proximal tubule. In situations where FGF23 is produced in excess, phosphate leaks through the kidney in the urine, hence serum phosphate is below the normal range. In addition, FGF23 has a strong inhibitory effect on 1,25(OH)2D synthesis, hence reduces calcium absorption through the gut. Therefore, patients with PHEX mutation usually present with low serum phosphate,

mildly decreased serum calcium, phosphate wasting and reduced 1,25(OH)2D levels[8]. The abnormal phosphate level, the defect in 1,25(OH)2D synthesis, and the accumulation of ASARM peptides[9] lead to an impaired mineralization of the squeleton and ultimately, rickets, osteomalacia and insufficient growth.

Clinical manifestations of XLH occur most often around the age of walking, despite an adequate vitamin D supplementation. In children the primary clinical symptoms are skeletal pain and deformity, abnormal gait, decreased growth velocity, dental abscesses and craniosynostosis[10,12]. In the absence of diagnosis and/or treatment, short stature worsens progressively until the age of 5 years and becomes disproportionate. This may lead to extreme short stature with an adult height below -2 SD[13]. Tooth eruption is often delayed, but, when present, teeth display a normal enamel. The impaired mineralization of dentin is the cause of dental abscesses and early decay of lacteal and permanent teeth. Young adults present with increased frequency of periodontitis and altered perialveolar bone[14,16]. In adults, osteomalacia, bone pain, stiffness and enthesopathy (calcification of tendons, ligaments, and joint capsules) are typical findings[17]. Hypertension, left ventricular hypertrophy and cardiac insufficiency have been sporadically described in children and young adults[18,19]. Dizziness and deafness due to abnormalities of the inner ear may develop towards adulthood[20]. Many patients may have partial synostosis of the sagittal sutures leading to a dolichocephalic shape of the head[21]. This may be accompanied by intracranial hypertension[10,21,23]. Type 1 Chiari malformation is a complication of XLH in about 25% of the patients, triggering the search for headaches and neck pain[24].

XLH is characterized by elevated ALP, low serum phosphate, phosphate wasting and elevated levels of circulating FGF23[25]. Normal FGF23 levels do not exclude XLH, since FGF23 levels depend on other factors, e.g. phosphate intake[26].

On radiographs, the metaphyseal signs are those of common rickets. In contrast, the bone has a mesh-like appearance with gross bone trabeculations and the cortices are thick. There is no signs of bone resorption, as PTH is within the normal range. As a consequence, the Thatcher score of patients with XLH is usually graded below 3 on a scale of 10.

The diagnosis of XLH is based on clinical, radiological, and biochemical findings[27]. The x-linked inheritance strongly argues for the diagnosis. FGF23 is not available everywhere and is not fully mandatory for the diagnosis. In a subset of patients without familial history, i.e. one third of the patients, mutational analysis of PHEX is recommended[2]. In case of atypical features and/or lack of PHEX mutation, further work-up is recommended.

The current conventional treatment of XLH associates vitamin D analogues and repeated doses of phosphate supplements[27]. Active vitamin D analogues (calcitriol 0.5-0.75 ug/d or alfacalcidol 0.75-1.5 ug/d) are given to counter calcitriol deficiency, prevent secondary hyperparathyroidism, and increase phosphate absorption from the gut[28]. The dose of phosphate supplements ranges 20-60 mg/kg/day of elemental phosphorus.

The human anti-FGF23 monoclonal antibody, burosumab, is now an alternative to the conventional therapy as it was approved by The Food Drug Administration (FDA) for adults and children and by the European Medicines Agency (EMA) in children and is now available in some countries[27]. Administered subcutaneously to XLH children, burosumab demonstrated favorable clinical and biochemical effects, i.e. radiographic improvement of rickets, improved distance during the 6-minute walk test, increase in serum phosphate, increase in TmP/GFR, and increase in 1,25(OH)2D[29]. Patients with severe rickets show greater rickets, growth, and biochemical improvement under burosumab than upon conventional therapy[30]. Most side effects are reactions at the site

of injection[31]. Burosumab is therefore an alternative in children refractory or do not respond adequately to the conventional therapy or in patients with severe rickets.

The administration of growth hormone (rhGH) improves growth in prepubertal children with XLH[32] but no clear indication exist to support systematic treatment of patients with XLH.

Surgery is indicated for severe bowing or tibial torsion unlikely to improve with medical management alone. Except in extremely deformed limbs, corrective osteotomies (a surgical procedure in which a bone is cut to straighten it) are usually not performed in children before puberty, as medical therapy improves bow deformities until this age. Eight plates guided growth surgery have been used in young children, yet seems to give better results in valgus deformities and have not been evaluated until the end of growth[33].

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Phosphate and Pyrophosphate in Biomineralization

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The restriction of mineralization to specific tissues (skeleton, teeth) and the absence of mineralization elsewhere (vessels, tendons, skin) is essential for life. About 60 years ago, early pioneers such as Herbert Fleisch and Melvin Glimcher found that native collagen, supersaturated calcium, and inorganic phosphate (Pi) were all that was needed to induce mineralization (hydroxyapatite formation) in vitro, even when collagens from tissues that usually do not mineralize were used. The addition of plasma inhibited this process by an activity that was destroyed by alkaline phosphatase; pyrophosphate (PPi) was identified as the essential inhibitor. With insight provided by modern molecular biology, we now have a much more detailed picture of the delicate balance between the activators and inhibitors of mineralization. Mineralization requires multiple factors, including an adequate supply of mineral ions (Pi and calcium), the regulated removal of inhibitors of mineralization, and the presence of fibrillar collagen; all at a specific pH.

As one of the key activators of mineralization, Pi is kept within a relatively tight concentration range in the serum. Its primary hormonal regulators are parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and 1,25(OH)2-vitamin D[1]. The main regulation occurs through renal excretion of Pi. Regulation of intestinal absorption and release from bone also contribute, although to a lesser degree. Conditions of low serum Pi can be inherited, such as XLH, or acquired, such as tumor-induced osteomalacia. Low serum Pi leads to osteomalacia (undermineralized bone), or to rickets when present during growth. When Pi (and calcium phosphate product) is elevated, extraskeletal calcifications occur. For example, basal ganglia calcifications are common in hypoparathyroidism, which is a condition that often leads to high serum Pi concentrations.

PPi is one of the most potent inhibitors of mineralization [2]. Insides cells, large amounts are produced during protein synthesis. Only a tiny fraction is transported (through ANK) to the extracellular space, but even this small amount has a clear function, as mice deficient in ANK develop arterial calcification on a high-phosphate diet. Most extracellular PPi, however, is derived from ATP secreted from cells through vesicular exocytosis. ATP (and other nucleotide triphosphates) is hydrolyzed by extracellular pyrophosphatases (eg, ENPP1) to ADP and PPi. In addition to inhibiting mineralization locally, circulating PPi has also been shown to play an important role. ENPP1 null mice have very low plasma PPi and develop aortic calcifications. When aortas from ENPP1 mice are transplanted into normal mice, the calcifications of the aorta are completely arrested, showing that systemic levels of PPi are sufficient to prevent further vascular calcifications even when local production of PPi is curtailed. Normal aortas transplanted into ENPP1 null mice calcified despite the presence of normal localized ENPP1 activity, demonstrating an essential role for circulating PPi levels to prevent mineralization [3].

The role of the ectoenzyme tissue non-specific alkaline phosphatase (TNAP), which hydrolyzes PPi and is critical for mineralization of bone in the presence of circulating levels of PPi, will be discussed in more detail elsewhere.

The ratio of Pi (activator) to PPi (inhibitor) within bone is fundamental for regulation of mineralization. Diseases that change this ratio demonstrate its importance, both for ectopic mineralization, as well as for adequate skeletal mineralization. For example, inactivating mutations of NPP1, the enzyme that increases PPi by hydrolyzing ATP, lead to extraskeletal calcifications; loss-of-function of TNAP, the enzyme that hydrolyzes and decreases PPi, has the opposite effect.

While enormous progress has been made over the last few decades in the identification of key players in mineralization, a sensor for

serum Pi has not yet been identified, nor has a receptor (or sensor) for PPi - an important agenda for future research.

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The distribution of Biogenic Chemical elements in the Cosmos

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The year 2019 is the international year of the Periodic Table of Elements (Mendeleev, Principles of Chemistry, 1868-1870).

This table illustrates the atomic structure of the elements which compose the baryonic matter in the Universe. The baryonic Universe is composed mainly by hydrogen (roughly 71%), by helium (roughly 27%) and the reamaining roughly 2% contains all the elements from carbon to uranium and beyond.

During the first three minutes of the Big Bang, the event which gave origin to our Universe, only the light elements formed, namely hydrogen, deuterium, helium and lithium. All the other elements, with a larger number of baryons (protons and neutrons) in the nucleus, were built inside the stars through a sequence of nuclear reactions of fusion. Therefore, all the so-called biogenic elements have formed inside the stars which have restored them into the interstellar medium at their death , so that their percentage in the gas ,out of which subsequent stellar generations formed, has increased up to the actual value of roughly 2%, which corresponds to the concentration of heavy elements as measured in the atmosphere of the Sun (Asplund et al. 2009; Lodders et al. 2003).

The origin of life is strongly linked to the formation of biogenic elements, such as hydrogen, carbon, nitrogen, oxygen, magnesium, sulphur, calcium and iron (H, C, N, O, Mg, S, Ca and Fe) plus others, which are parts of many living organisms.

In my talk I will explain how all the chemical elements and in particular the biogenic elements did form. To this aim, I will describe the main phases of the evolution of stars of different initial

mass, and their death as supernovae or white dwarfs. The main nuclear reactions responsible for the formation of heavy elements will be also described.

I will show in a simple way how do we measure chemical abundances in stars and interstellar gas and model the chemical evolution of galaxies, and in particular our Milky Way. The results of models predicting the temporal evolution of the abundances of some key element in the solar vicinity will be shown and interpreted (see Matteucci 2012, for a Monograph on the subject).

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Tooth Development and Biomineralization

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Mature teeth consist of an elaborate bio-architectural design of an outer enamel layer, the dentin body of the tooth, a central pulp cavity, and cementum covering the roots and anchoring the tooth in the alveolar bone. Tooth development involves reciprocal interactions between the surface epithelium (ectoderm) and the underlying neural crest-derived mesenchyme (ectomesenchyme). Spatial and temporal cues provided by cellular, molecular, and matrix-mediated signaling interactions govern the intricate programs directing tooth initiation, morphogenesis, organogenesis, four distinct mineralization processes, and eventual tooth eruption. It is not surprising, therefore, that a host of well-conserved signaling pathways involving WNT, bone morphogenetic protein (BMP), fibroblast growth factors (FGFs), SHH (sonic hedgehog), and EDA (ectodysplasin) are required to orchestrate tooth morphogenesis and organogenesis. In addition, key transcriptional regulatory signals, such as Msx-1/2, Pax9, Runx2, Pitx, GEP, and Epiprofin, are among those that play important roles during tooth development and maturation.

As embryonic tooth development proceeds, soft tissues of the dental germ layers begin to mineralize the enamel, dentin, and cementum layers until such time as the tooth erupts into the oral cavity. These coordinated activities are conducted by the odontoblasts (dentinogenesis), ameloblasts (ameliogenesis), and cementoblasts, (cementogenesis) which develop and are responsible for the interactive mineralization processes associated with dentin, enamel, and cementum formation, respectively. Whereas mesenchymal-derived cells produce dentin (and bone), epithelial-derived ameloblasts are primarily responsible for enamel formation and mineralization. Enamel is the hardest material in

the human body and it provides a highly wear-resistant, protective insulating barrier for the tooth that does not remodel (unlike bone) because once mineralized, enamel is acellular. Mineral disorders of teeth include a heterogeneous array of amelogenesis and dentinogenesis imperfecta conditions that underscore the essential roles of specific regulatory factors and pathways associated with normal tooth mineralization.

Enamel consists almost entirely of an ordered arrangement of densely packed hydroxyapatite-based inorganic mineral crystals. Enamel formation and mineralization rely on specific enamel matrix proteins (EMPs) that serve as a crystallization template and modulate the processes of crystal secretion, orientation, and maturation, however, these organic EMPs are subsequently removed and only trace amounts remain in the fully mature enamel. Amelogenesis generation of a mature mineralized enamel crown takes place in three well-defined stages known as the secretory, transitional, and maturational phases. The secretory phase produces an initial gel-like material representing the entire thickness and volume of enamel, which is composed of similar amounts of EMPs, water, and mineral. The positioning and orientation of ameloblasts (connected by intercellular junctional complexes) is critical for the proper formation and deposition of the enamel. Secretory ameloblasts are highly polarized cells that develop a unique secretory structure, known as the Tomes process, for the exocytosis of secretory vesicles containing structural EMPs (amelogenin, ameloblastin, enamelin, and tuftelin). The Tomes process ultimately determines the orientation and formation of hydroxyapatite crystals, and defines boundaries between rod and interrod regions. Protons released during hydroxyapatite formation acidify the space and require active pH regulation by the ameloblasts.

A brief transition phase ensues following the secretory phase, during which elongated ameloblasts become shorter, lose their

secretory Tomes' processes, downregulate EMP gene expression, and upregulate ion transport, proteolytic, and pH regulatory gene expression. Nearly 25% of ameloblasts undergo apoptosis (possibly due to calcium overload) at this point. The maturation stage is marked by shorter ameloblasts (compared to secretory stage ameloblasts) that engage in ion transport, acid-base balance, proteolysis, removal of EMP debris, and further apoptosis. An array of proteolytic enzymes (cathepsins, MMPs notably MMP20 [enamelysin], and kallikrein-related peptidase [KLK4] are critical for clearing the temporary proteinaceous organic matrix that enabled enamel mineralization. In this phase, ameloblasts alternate between ruffle-ended (RA) and smooth-ended (SA) cell morphologies. RA cells have increased capacity for endocytosis of EMP debris, anion exchange, pH control (AE2), and local bicarbonate production (via carbonic anhydrase). SA ameloblasts, which loosely adhere to one another, permit diffusion of intercellular fluids and small molecules into and out of the maturing enamel, and help neutralize local pH. Ameloblasts, like most other odontogenic epithelial cells (dental lamina, HERS root cells) are fated to degenerate following the completion of tooth formation, leaving the mature enamel acellular and avascular. Owing to its low solubility, apatite is the most chemically stable phase of calcium phosphate mineral. The crystal lattice of hydroxyapatite exhibits hexagonal symmetry with channel positions available for anions (OH-, Cl-, F-, carbonate) to bind, either conforming to the space or deforming the lattice and thereby potentially increasing or decreasing its solubility and strength.

Unlike ameloblasts which apoptose, odontoblasts of ectomesenchymal origin remain and continue to function to generate the tooth body and periodontal structures. Studies of odontogenesis, mostly using mouse teeth as models, indicate that the position, number, size and shape of different teeth are under genetic control. Odontoblasts are highly differentiated, post-mitotic cells originating from the neural crest, which are organized

at the periphery of the pulp as a cellular palisade. Odontoblasts synthesize the components of the predentin (type I collagen, glycoproteins, and other non-collagenous proteins), and are responsible for its mineralization. In addition to producing the primary dentin during organogenesis, odontoblasts also synthesize secondary dentin throughout the life of the tooth and tertiary dentin if the tooth undergoes pathological damage. Like dentin, cementum is synthesized during the entire lifetime of the teeth. Dentin is a less mineralized tissue than enamel and contains a higher amount of proteins. However, like enamel, dentin mineralization requires the participation of certain proteins and highly intricate cellular and molecular processes to form mature dentin.

Our growing understanding of the elaborate processes underlying tooth development and biomineralization has inspired new and evolving innovative approaches in tissue engineering and regenerative medicine aimed at the biological repair, regeneration, or replacement of teeth and their surrounding structures.

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Methods to Quantify Biomineralization in Bone

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The study of biomineralization is undertaken to understand this process in health, with the aim to use this knowledge to either mimic it, and/or employ in treating diseased biomineral.

Bone is a hierarchically structured material, combining a soft (organic matrix) and a brittle (apatite mineral), along with water, into a composite with extraordinary mechanical competence. Moreover, it is widely accepted that its mechanical properties are dependent on both the amount and the quality of its three major constituents: organic matrix, mineral, and water. An extra obstacle to be circumvented when studying bone is that both the quantity and the quality of all three constituents are not uniform but rather change as a function of tissue age and health status.

The only analytical technique that directly and simultaneously measures the amount of all three bone constituents is thermogravimetric analysis, yet its use is very rare as bone tissue is destroyed during measurement. On the other hand, plenty of techniques estimate mineral content based on either attenuation of x-rays (Dual x-ray absorptiometry, DXA; micro–computed tomography, microCT) or a mineral surrogate (a chemical constituent of the apatite crystallites that make up the mineral portion of bone) such as quantitative backscattered electron imaging (qBEI; directly measures greyscale levels which are then converted to calcium concentration and subsequently mineral density distribution), and vibrational spectroscopic techniques such as Fourier Transform Infrared (FTIR) and Raman spectroscopic methods (both directly measure the phosphate concentration).

Although DXA is the clinical mainstay in estimating fracture risk, its limitations are well-recognized (e.g. areal expansion, patient age), and recent reports have cast doubt on its ability to identify patients who are truly at elevated risk of sustaining fragility fractures even when complemented with algorithms such as FRAX [1,2]. On the other hand, microCT analysis of bone provides additional information on the structural properties of bone. When combined with other techniques such as fluorescent microscopy and qBEI, it offers additional information on the distribution of mineral content with respect to various anatomical landmarks. One example of such combinatorial approach would be the investigation of mineral content with respect to the canalicular network, showing, surprisingly, that a positive correlation exists between mineral content and network density in human osteonal bone[3], thus providing new insights in the highly dynamic process of mineral homeostasis.

Techniques that determine mineral content using a surrogate are subject to potential confounding factors such as variable content as a function of tissue age, or variability in mineral maturity / crystallinity. The first may be addressed by analyzing the whole bone volume / surface in which case information on both its content and spatial distribution may be obtained, as is routinely the case with qBEI[4] or vibrational spectroscopic analysis[5]. Combining qBEI with small x-ray scattering (SAXS) analysis offers the opportunity to determine mineral content corrected for variations in mineral crystallite size. Such an approach revealed that in osteogenesis imperfecta, bone consists of smaller crystallites packed tighter than in healthy bone[4]. On the other hand, FTIR and Raman spectroscopic analysis determine mineral content and mineral maturity / crystallinity simultaneously, thus can also readily correct the mineral content for the variable crystal sizes evident in bone. Using such an approach it has been shown that postmenopausal osteoporosis patients form crystallites that are initially bigger than the ones encountered in health[5].

Less investigated, yet just as important as mineral content, is the amount of organic matrix. The stiffness of the bone material is usually believed to be determined by the mineral content, yet recent results indicate that the properties of the organic matrix as well as the spatial arrangement of the mineral / organic matrix composite are also important contributors[6]. There are only two analytical techniques that readily measure organic matrix content: thermogravimetric analysis (directly), and vibrational spectroscopic techniques (using carbonyl and / or N-H groups that are part of the organic matrix molecules as a surrogate). Interestingly, despite the fact that postmenopausal osteoporosis is associated with bone mineral loss as measured by DXA, tissue age-normalized Raman analysis of human bone showed that the first quantitative change immediately following menopause is a reduction in the amount of organic matrix synthesized by the osteoblasts rather than a decline in mineral content[7]. Vibrational spectroscopic techniques can also provide detailed information on the composition (lipids, and glycosaminoglycan content, as well as information on the enzymatic and non-enzymatic collagen cross-links) and organization (collagen fiber orientation) of the organic matrix[5].

Recently, renewed interest in the role of tissue water in the determination of the mechanical properties of bone has been expressed[8]. As the case with the organic matrix, thermogravimetric analysis directly measures tissue water content, while vibrational spectroscopic techniques may estimate it using embedding material content as a surrogate amongst others[5].

In summary, plenty of new analytical techniques offer the capability of measuring all three constituents of the bone composite. When employed in a combinatorial fashion and considering what is measured while guarding against overinterpretation, it is feasible to quantify biomineralization, describe mechanisms controlling it, and determine factors affecting bone stiffness, toughness, and strength.

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Vitamin D and Vitamin K in Biomineralization

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Vitamin D is an important regulator of calcium and phosphorus homeostasis. By promoting optimal extra-cellular calcium and phosphorus concentrations, it ensures mineralization of newly deposited bone and cartilage matrix[1].

Its role in preventing rickets in childhood and osteomalacia in adults is well established. The question arises whether its role in rickets and osteomalacia prevention and treatment is direct or indirect, ie through changes in extracellular ion concentrations. Its active metabolite calcitriol, stimulates transepithelial transport of calcium and phosphate through both genomic and non-genomic mechanisms[2].

Trans-apical membrane transport of calcium through the channel TRüV6 is stimulated by calcitriol, while the extrusion at the basolateral membrane is carried out by calcium ATPase 1b. Calcitriol may also regulate paracellular transport, acting on various tight junction proteins. Large intestine is equipped with a potent vitamin D-dependent calcium transport system, which is usually poorly used, since the substrate calcium is complexed with anions like oxalate preventing it to access to the transport system[3].

Modifying gut microbiota with prebiotics, the metabolism of which decreases large intestine content pH and increases calcium bioavailability. Calcitriol stimulates intestinal phosphate absorption as well[4].

Calcitriol is a potent stimulator of bone resorption, by increasing expression and production of RANKL by osteoblasts. By

mobilizing calcium and phosphate from the intestine and from bone, vitamin D increases calcium and phosphate extracellular concentrations to supersaturated level allowing the mineralization of cartilage and bone[1,2].

A very old observation highlights the important role of extracellular phosphate concentration in the mineralization process. Indeed, by mobilizing phosphate from soft tissue stores, starvation improved rickets in experimental animals[5].

Adequate phosphorus level is necessary for growth plate normal development, because of a phosphate-dependent apoptosis of hypertrophic chondrocytes[6].

Infusion of calcium and phosphate in vitamin D-deficient rats results in normal mineralization[7].

In VDR systemic knock-out model, rickets and osteomalacia can be prevented by a diet rich in calcium and phosphate[2].

However, all bone defects in Cyp27b1 mice, ie animals lacking the 1 alpha hydroxylase, are not totally normalized by providing minerals, suggesting some direct effect of vitamin D as well. A severe impairment of mineralization is observed in VDR selective deletion in the intestine, whilst a rescue can be achieved by selective expression of VDR in the intestine. This underlines the major role of vitamin D-dependent transport systems in the mineralization process. Vitamin K is a cofactor in post-translational modification of protein bound glutamate residues called gamma-glutamylcarboxylation [8].

The gamma-carboxylation of the glutamate residues, ie Gla-residues, is necessary for prothrombin activity by providing a calcium binding capacity. Other proteins contain vitamin K-dependent Gla residues. Among them is osteocalcin. Its undercarboxylated form

seems to play regulatory roles, besides calcification, in energy and reproductive metabolisms. From osteocalcin-deficient mice models, it appears that osteocalcin influences hydroxyapatite crystal growth and structure[8].

The extrahepatic vitamin K-dependent protein matrix gla protein is implicated in the inhibition of ectopic calcification[9].

Its overexpression in the growth plate inhibits cartilage calcification and endochondral ossification. A gla-rich protein is found in bone and calcified cartilage. Coumarins are vitamin K antagonists which inhibit vitamin K recycling and deplete thereby vitamin K tissue stores, leading to undercarboxylation of vitamin K-dependent proteins. Spontaneous precipitation of calcium and phosphate present in supersaturated concentration in extracellular fluids is prevented by inhibitors like matrix gla-protein[10].

Depletion of this protein is associated with massive arterial calcification[11], as does the prevention of matrix gla-protein carboxylation by to vitamin K antagonists. Altogether, this indicates a risk of soft tissue calcification in states of vitamin K deficiency. Whereas vitamin D deficiency is associated with impaired cartilage and bone mineralization, soft tissue calcifications are observed in vitamin K depletion.

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Anti-Resorptives as Bone Therapeutics

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During the past few decades several different types of medicines have been introduced for the treatment of bone diseases. Drugs that inhibit bone resorption ("anti-resorptives") continue to dominate the therapy of bone diseases characterized by enhanced bone destruction. The pharmacological basis for the action of each drug class is different, enabling choices to be made to enable their optimal use in clinical practice.

Bone resorption is accomplished by highly specialized multi-nucleated osteoclasts, which dissolve the bone mineral phase of bone by secreting acid, and degrade collagen and other bone matrix proteins utilizing a battery of secreted or intracellular enzymes. There are many ways in which drugs can influence osteoclast development and action, directly or indirectly. Even though their individual pharmacological actions may involve different biochemical and molecular mechanisms, the net result of reducing bone destruction makes them very useful in the therapy of bone diseases, many of which are characterized by enhanced bone resorption. Indeed the use of drugs that inhibit bone resorption continues to dominate the treatment of not just osteoporosis, but also Paget's disease of bone, myeloma and bone metastases secondary to breast, prostate and other cancers, as well as many less common acquired or inherited skeletal disorders, such as osteogenesis imperfecta, inflammatory bone loss, and glucocorticoid-associated osteoporosis.

There are now several different classes of drugs approved for treating osteoporosis. Historically treatment options have included hormones, such as estrogens, as well as calcitonins. These have

now been replaced by more effective treatments, which have convincingly been shown to reduce the occurrence of fractures in well-designed prospective clinical trials.

The potential adverse effects of oestrogens, especially on breast cancer, led on to the development of SERMs (Selective Estrogen Receptor Modulators. Considerable effort was devoted to developing SERMs (Selective Estrogen Receptor Modulators) as substitutes for classical estrogens, and several entered clinical trials, but among these only raloxifene and bazedoxifene continue to be used.

Other therapies such as strontium salts were introduced in some but not all countries, but side effects have been a problem. Several cathepsin K inhibitors have also been studied. One of these, odanacatib, was evaluated in huge clinical trials and came close to being approved, but adverse cardiovascular events prevented it from being registered for clinical use.

Currently the mainstay of treatment worldwide is still with bisphosphonates, as used clinically for over 40 years. A more recently introduced therapy is the anti-RANK-ligand antibody, denosumab. The therapeutic options have been much enhanced by the introduction of this biological therapy, which, like the best bisphosphonates, can reduce the occurrence of fractures at all major skeletal sites. These include vertebral and non-vertebral fractures, and particularly importantly hip fractures.

It is fascinating to note how the study of rare diseases has led to the identification of potential drug targets, and thence to several of the drugs now used for or being evaluated for treating skeletal diseases. Even the bisphosphonates were first studied as analogues of pyrophosphate for their inhibitory effects on mineralization, as had been revealed in studies of hypophosphatasia, which is now treated by enzyme replacement with alkaline phosphatase.

The development of denosumab and Cathepsin K inhibitors can both be traced back to the study of rare skeletal diseases. Among other potential anti-resorptive agents derived from studies of various osteopetrotic disorders are src inhibitors, chloride channel blockers, and ATP proton pump inhibitors. Similarly new bone forming agents such as anti-sclerostin antibodies and dkk antagonists have their origin in studies of the genetics of wnt pathway modulators. Although the many genetic discoveries underlying the osteopetroses and other high bone mass disorders have inspired many pharmacological approaches, the treatment of osteopetrotic syndromes themselves remains challenging, but marrow transplantation, or interferons, can be helpful in selected cases.

Currently the mainstay of treatment worldwide is still with bisphosphonates (BPs). This year will be the 50th anniversary of the first description of their biological and pharmacological effects (see https://bisphosphonates2019.org). Much is now known about their mechanisms of action and clinical effects. The pharmacological effects of BPs as inhibitors of bone resorption appear to depend upon two key properties; their affinity for bone mineral, and their inhibitory effects on osteoclasts. There are differences in binding affinities for bone mineral (hydroxyapatite) among the clinically used BPs, which may determine their distribution within bone, their biological potency, and their duration of action. Within bone bisphosphonates are internalised selectively by osteoclasts and interfere with specific biochemical processes. The antiresorptive effects on osteoclasts of the nitrogen-containing BPs (including alendronate, risedronate, ibandronate, minodronate and zoledronate) appear to result principally from their inhibition of farnesyl pyrophosphate synthase (FPPS), a key enzyme in the mevalonate pathway, which generates isoprenoid lipids utilized for the post-translational modification of small GTP-binding proteins that are essential for osteoclast function. The accumulation of the upstream metabolite, isopentenyl pyrophosphate (IPP), as a result

of inhibition of FPPS may be responsible for immunomodulatory effects on gamma delta (γδ) T cells, and can also lead to production of another ATP metabolite called ApppI, which has intracellular actions. BPs may have other biologically important cellular effects, on inhibiting osteoclast differentiation, on decreasing tumour cell viability, and on preventing osteocyte apoptosis, the latter through other pathways eg connexin channels.

In the case of osteoporosis some of the currently topical issues include deciding whom to treat and for how long, which BP to use, and how to manage poor compliance. In general BPs have proved to be not only highly effective but also very safe drugs. Nonetheless, issues of side effects and adverse events attract often disproportionate attention, as with osteonecrosis of the jaw (ONJ) and atypical femoral (subtrochanteric) fractures, where the nature of any association with BPs remains unclear.

Several recent studies suggest that BPs may be associated with other clinical benefits outside the field of bone diseases, eg on mortality, cardiovascular disease, and reduction of cancers. The pharmacology underlying these potential effects needs to be understood, and we may be entering an era in which BPs are viewed as modulators of mevalonate metabolism rather than as bone-active drugs. Further examples of intriguing non-skeletal effects include inhibition of several protozoan parasites, increasing longevity in animal progeroid models, and enhancing human stem cell life span, DNA repair and tissue regeneration. BPs are now also being evaluated for their ability to target drugs to bone for local release. BPs have now become largely generic drugs, as key patents have expired, but are likely to remain major drugs for treating bone diseases for some time to come. In looking ahead, there are obvious opportunities for extending the use of BPs to other areas of medicine.

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Congenital skeletal low mineralization disorders

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A decreased mineral density and/or bone fragility in childhood can be related to several conditions.

In the updated Nosology of Genetic Skeletal Disorders these are major features of two group of disorders: "Osteogenesis Imperfecta and decreased bone density group" and "Abnormal mineralization group".

Osteogenesis Imperfecta (OI) indicates a set of hereditary disorders characterized by increased bone fragility. Other clinical features include blue sclerae, dentinogenesis imperfecta and hearing loss. OI phenotypic spectrum is a continuum, ranging from lethal forms with prenatal findings (bone fractures, severe skeletal deformities and short stature) to mild forms with few or no fracture and normal stature.

OI causing genetic alterations alter structure or biosyntesis of collagen type I. COL1A1 and COL1A2 gene alterations account of about 90% of OI cases. In the past years many further genes related to OI phenotype have been identified and, in particular, are related to genes encoding collagen chaperones, proteins involved in type I procollagen assembly, processing and maturation or in the formation and homeostasis of bone tissue. Due to clinical and genetic heterogeneity of the disease an updated classification, including clinical, radiological and genetic information, is needed.

The second group includes Hypophosphatasia, Hypophosphatemic Rickets and other conditions with abnormal mineralization.

Hypophosphatasia is a rare disorder characterized by defective bone and teeth mineralization, and deficiency of serum and bone alkaline phosphatase activity. The clinical expression is variable, from prenatal/

congenital manifestations to adult onset with teeth involvement only (Odontohypophosphatasia).

The disease is caused by mutations in the liver/bone/kidney alkaline phosphatase gene (ALPL) and both autosomal dominant and recessive forms are reported.

Differential diagnosis has to be made with OI, Rickets and some skeletal dysplasias (i.e. Achondrogenesis)

Overlap in clinical presentation of the diseases in these two groups and the genetic heterogeneity of some of these disorders may cause a delay in the diagnosis, negatively affecting an appropriate management, therapy and follow-up and definition of recurrence risks, for which correct diagnosis is mandatory.

An overview of clinical and genetic of the most representative disorders with low bone mineral density is provided.

FGF23: From its Discovery to the Development of a Therapy

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Physiological biomineralization is strictly regulated locally and systemically. One of the key processes which controls biomineralization at systemic level is renal phosphate handling, which by itself is mainly controlled by parathyroid hormone and fibroblast growth factor (FGF) 23 in an endocrine manner. FGF23 was identified in analyses of two distinct diseases, autosomal dominant hypophosphatemic rickets (ADHR)[1] and tumor-induced osteomalacia (TIO)[2]. Both diseases share similar clinical symptoms such as renal phosphate wasting independently of parathyroid hormone and insufficient bone mineralization; excess activity of FGF23 underlies both pathogenic mechanisms. After measurement tools for circulating FGF23 emerged, high or low levels of FGF23 were shown in various disease conditions associated with deranged phosphate metabolism, such as kidney disease and various types of renal phosphate wasting disorders, respectively. In particular, patients with X-linked hypophosphatemic rickets, the most frequently observed genetic hypophosphatemic rickets, were also shown to have normal-high to high levels of FGF23 in circulation; this excess activity of FGF23 was established to be the cause for renal phosphate wasting as judged by genetic and intervention studies in Hyp mouse, a murine homolog of XLH.

Antibody therapy is generally well-tolerated and useful for therapeutic targets which are in circulation or exposed on cellular surfaces but share molecular similarity among family members or its receptors. In this context, an antibody against FGF23 can be a specific approach to inhibit excess activity of FGF23 because both other members of the FGF family and FGF receptors share the core structure and signalling mechanisms. In addition, long-lasting pharmacokinetics of antibody-based drug usually enables less frequent dosing. In contrast, because of the novelty of antibody-based therapeutic approach, detailed and

long-term clinical evaluations for both benefits and limitations are always required.

Hyp mouse is an ideal tool to evaluate the effects of anti-FGF23 antibodies, since it has a deletion in *Phex* gene, and exhibits very high levels of FGF23 in circulation. Neutralization of FGF23 by the antibody resulted in an increase in serum levels of phosphate which were accompanied by upregulation of the sodium phosphate cotransporter 2a; thus, this intervention restored renal phosphate reabsorption[3]. Repeated dosing resulted in almost normalized bone mineralization and length of long bones. Histomorphological analysis demonstrated that antibody treatment restored alignment of hypertrophic chondrocytes in the growth plate of metaphysis, as well as bone formation rate. In adult mice, similarly to the study with juvenile Hyp mice, normalization of renal phosphate reabsorption was also observed and was accompanied by a significant increase in bone mineralization within a short time. Furthermore, treatment with antibody increased grip force levels in adult Hyp mice, which may have contributed to the marked increase in locomotor activity. Despite being preliminary, these observations may suggest that antibody treatment can improve bone quality, muscle function and mobility also in adult XLH patients.

Clearly, animal studies always have limitations and clinical investigations must be conducted. However, combination of clinical and bench studies should provide a better understanding of the mechanism of the new therapeutic approach in XLH as well as its potential in other therapeutic areas associated with increased FGF23 levels.

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The Fossils to Read the History of Biomineralization

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Most of what we know about ancient life comes from the fossils found in sedimentary rocks, and the bulk of fossils are of organisms that possessed biomineralized skeletons. But what can fossils tell us about the history of biomineralization?

Fossils are the remains (or traces) of living organisms preserved through natural processes of burial. Most animal fossils consist of shells, bones or teeth. Coincidentally, the organic nature of fossils was largely established in Florence by Nicolas Steno who worked as a physician to the Medici family during the 17th century. Although the fossil record provides a rich chronicle of life through time, it can be likened to a book from which the great majority of pages have been torn out and lost, and the words and sentences on those pages that do survive are incomplete and cannot be easily deciphered (Taylor & O'Dea 2014). Diverse phyla living today such as Nematoda have left no fossil record. Even among groups with resistant, potentially fossilizable skeletons, only a minority of species may be represented in the fossil record. This can be due to the lack of the right environments at the right times and in the right places, or just the absence of exposed rocks from which to collect fossils. Despite its limitations, the fossil record does provide us with minimum dates for the appearance of biological groups (clades) and particular morphological traits. When interpreted in conjunction with evidence obtained from living organisms – including molecular phylogenetic trees – it offers key insights into the timing and evolution of biomineralized structures. And without fossils we would never have known of the existence of dinosaurs, or the catastrophic mass extinctions that have punctuated life's history.

The single most important time for the evolution of biomineralization was the late Ediacaran to Early Cambrian, between about 550 and 520 million years ago (Ma). In rocks formed during this relatively brief interval, numerous phyla capable of manufacturing biomineralized skeletons make their debuts in the fossil record. These include molluscs, echinoderms, brachiopods, sponges, cnidarians (corals), and arthropods (notably trilobites). The appearance of these phyla in marine sedimentary rocks is an important part of the 'Cambrian Explosion', when the diversity and disparity of animals in the fossil record increased at a very rapid rate. The cause of the Cambrian Explosion has been vigorously debated: was it driven by geological events, a key evolutionary innovation, or ecological interactions that precipitated an arms race? Whatever the cause, phylogenetic trees imply that biomineralized skeletons evolved de-novo independently in several biological groups (clades), the majority acquiring skeletons of calcium carbonate (calcite and aragonite) but others of calcium phosphate (Knoll 2003).

One of the most remarkable aspects of the evolution of biomineralization in marine animals possessing calcium carbonate skeletons is evidence that the mineralogy of these skeletons may have been determined by extrinsic processes happening deep within the Earth. Phil Sandberg showed that there has been a cyclic alternation between times when inorganic calcium carbonate precipitated in the sea as calcite, and others when aragonite was the primary precipitate. This was later shown to have been controlled by the Mg/Ca ratio of seawater which in turn reflects the rate of formation of new crust at spreading ridges: during times of rapid seafloor spreading, seawater had a low Mg/Ca ratio giving a 'Calcite Sea', but when it was slow the Mg/Ca ratio was high giving an 'Aragonite Sea', as at the present-day. The existence of these two oceanic geochemical conditions seems to have played a major role in biomineralogical evolution. For example, in the Early Cambrian there was a switchover from an Aragonite Sea

to a Calcite Sea and those clades that acquired biomineralization before the change had aragonite skeletons whereas those doing so after had calcite skeletons (Porter 2010). While the mineralogy of some of these groups has remained fixed through geological time, in others it seems to have changed with subsequent switches from Calcite to Aragonite Seas and vice-versa.

Take the Bryozoa, a phylum of colonial metazoans that evolved biomineralization twice from soft-bodied ancestors, once in the Early Ordovician (480 Ma) and a second time in the Late Jurassic (155 Ma). In both biomineralizing clades, the earliest representatives have calcite skeletons, which match the Calcite Seas when they first appeared. However, the ancient clade has remained calcitic through its entire Ordovician-Recent duration, whereas an increasing number of species in the younger clade have evolved skeletons of mixed calcite-aragonite (i.e. bimineralic) or entirely aragonite mineralogy. Most of the transitions to aragonite occurred after the switch to an Aragonite Sea about 35 Ma, but some evidently happened during a Calcite Sea. While environmental factors is likely to have influenced biomineralogy, a degree of organismal control has been exerted by these animals.

Of more direct relevance to this symposium is the evolution of biomineralization in vertebrates. Fragmentary fossils of calcium phosphate known from the Late Cambrian may represent the oldest examples of vertebrates with hard skeletons but their affinities are by no means certain (Janvier 2015). Articulated skeletons of indisputable fishes are, however, found in the Ordovician. Many early fishes had extensive exoskeletons with a large headshields and scaly bodies but lacked a bony endoskeleton, which appeared subsequently in the Devonian.

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Biomineralization in Health and Disase: Biomineralization of the Arteries

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Every osteotropic agent has vasculotropic actions. This pithy summary reflects the fundamental relationship that exists in the cellular biology controlling skeletal and vascular mineralization. However, most often the relationships between vascular mineral accrual and skeletal mineral mass are reciprocal. Jean Lobstein, the 19th century surgeon-pathologist who coined the terms osteoporosis and arteriosclerosis, was amongst the first to formally note perturbations in bone and vascular mineral metabolism with advanced age. In addition to advanced age, diabetes, dyslipidemia, hypertension, tobacco use, hyperphosphatemia and chronic kidney disease all increase the risk for vascular mineral deposition - frequently in association with compromised skeletal health. Even in the absence of overt vessel occlusion, arteriosclerotic calcification has severe consequence. Arteriosclerosis impairs Windkessel physiology, the rubbery elasticity of conduit vessels required for smooth distal tissue perfusion throughout the cardiac cycle. Loss of vessel compliance not only increases the risk for stroke, cognitive impairment, and heart failure, but also increases the risk for declining renal function and lower extremity amputation.

Once considered only a passive process of dead and dying cells, arteriosclerotic calcification has clearly emerged as an actively regulated form of mineralized matrix metabolism. As in bone, osteogenic transcription programs are activated — but within the vascular smooth cell lineage (VSM) — by osteogenic morphogens of the BMP and Wnt families. This osteogenic "trans-differentiation" is in part an extension of a well-known characteristic of arterial VSM called phenotypic modulation; during phenotypic modulation, the normal healthy contractile programs of VSM

are down-regulated and collagenous matrix synthetic programs characteristic of wound fibroblasts. The paracrine morphogenetic cues driving VSM osteogenic differentiation are elaborated not only by myofibroblastic cells but also leukocytes, highlighting the tight coupling of vascular mineralization to innate immunity. Indeed, the expression of vascular osteogenic morphogens is activated by oxidized lipoproteins, advanced glycosylation end products, and inflammatory cytokines (TNF, IL1) that are components of toll like receptor innate immunity that walls off mycobacterial and fungal infections (calcified granulomas).

Conditional deletion of osteogenic transcription factors Msx1, Msx2, and Runx2 in the VSM lineage reduces arterial calcification, fibrosis, and vascular stiffness -- thus confirming the importance of osteogenic transcription factor programs to vascular mineralization in vivo. Likewise, matrix vesicles containing ectozymes (alkaline phosphatase, Phospho1, Enpp1), phospholipids (phosphatidylserine), calcium binding proteins (annexins, osteopontin, matrix Gla protein) and proteinases first characterized in skeletal mineralization are also elaborated by VSM driving vascular mineralization. Mineral nucleation occurs on elastin as well as collagen in the vasculature. However, even thought the cell biology is similar, the global endocrine regulation Inflammation most frequently drives net bone loss in the skeleton — yet promotes arteriosclerotic calcification in the vasculature. Intriguing clues as to the endocrine underpinnings arise from studies of the PTH/PTHrP receptor (PTH1R), wherein tissue-autonomous roles of the PTH1R support net bone formation yet suppress arterial reactive oxidative stress (ROS), vascular calcification and fibrosis in response to prosclerotic milieus of diabetes and dyslipidemia. Because stretch-activated VSM PTHrP is the most important physiological agonist for vascular PTH1R signaling, a better understanding of this autocrine / paracrine axis — and its perturbation with sustained hyperparathyroidism — is needed.

As in the skeleton, morphogenetic, metabolic, mechanical, endocrine and inflammatory cues control vascular mineral deposition — and these processes differ somewhat dependent upon histoanatomic venue. Severe calcific aortic stenosis, afflicting 2% of our patient population over age 60, is one such process that deserves individual consideration. Calcific nodules, stippled calcification, and woven bone replete with marrow hematopoietic elements can be seen — the latter noted in up to 15% of advanced lesions. Pre-clinical data in murine disease models has converged with human genetics to identify some key commonalities (Ddx58, IFIH1, LRP6, LDLR, Notch1) in the pathobiology of aortic valve and vascular calcification. However, recent data from human studies newly implicate a very specific role for Lp(a) -- the oxyphospholipid plasma lipoprotein -- in providing an osteogenic G-protein receptor agonist, lysophosphatic acid (LPA), to aortic valve interstitial cells to drive osteogenic mineral deposition. Of note, Lp(a) is genomically absent in vast majority of preclinical disease models deployed. However, the oxidative inflammation pathways elucidated in rodent models once again appear to be important. Small molecule activators of the soluble guanylate cyclase - nitric oxide relay that inhibit ROS signals have shown promise in phase II clinical studies in humans.

Much has yet to be learned concerning the osteogenic regulation of vascular calcification and potential pharmacological intervention. Data from the Multiethnic Study of Atherosclerosis (MESA) demonstrated that aminobisphosphonate use reduces aortic and aortic valve calcification in post-menopausal women — yet the relationship appears to be reversed in premenopausal women so treated. Sex hormone- and age-dependent changes in innate immune modulation may be important, since aminobisphosphonates alter not only relevant small G-protein signaling but also prenyl phosphate metabolites relevant to innate immunity. Nevertheless, recent cardiovascular mortality data in aminobisphosphonate users support the apparent benefit noted in MESA for postmenopausal

women. The recent differences in major adverse events favoring the aminobisphosphonate, when comparing anti-sclerostin/SOST antibody to alendronate treatment for osteoporosis, also seem to support this notion. Nevertheless, almost 15 years ago we demonstrated basal and activated SOST protein expression in vivo in arteries undergoing Wnt-dependent calcification. Moreover, others recently demonstrated that arterial SOST is epigenetically silenced in aortic aneurysmal disease in humans, and that recombinant and transgenic SOST can mitigate aneurysmal arterial remodeling in preclinical models. Of note, the current anti-sclerostin therapy for osteoporosis has a boxed advisory indicating that it may increase risk for cardiovascular death, myocardial infarction and stroke — and that a prior stroke or heart attack within the past year is a contraindication to therapy. The cardiovascular PK-PD is unknown.

Thus, while every osteotropic agent has a vasculotropic action, the net impact on cardiovascular health will differ depending upon the therapeutic strategy undertaken. Important clues to the "rules of the game" are likely to be forthcoming from a better understanding of how neuroendocrine signals globally integrate calcium, phosphate, and matrix metabolism in skeletal and vascular venues. Intriguingly, two osteotropic hormone receptors —PTH1R and LRP6 — have now been shown to support the accrual of skeletal bone mass and inhibit vascular mineralization via cell-autonomous actions in osteoblast and VSM lineages, respectively. As in bone, a foundational triumvirate — via cells of the mesenchymal, monocyte/macrophage, and endothelial lineages — regulates vascular mineralized matrix metabolism. A healthy endothelium maintains vascular integrity and stabilizes the contractile VSM phenotype — but we have almost no mechanistic insight into how the endothelium coordinates osteogenic responses to the pathogenic mechanical "loads" necessary to drive arteriosclerotic calcification. An osseocentric view of arteriosclerosis may help guide our experimental design in ways that illuminate strategies to (a) mitigate vascular mineralization while (b) maintaining skeletal health in our increasingly aged and dysmetabolic population.

Biomineralization: Definition

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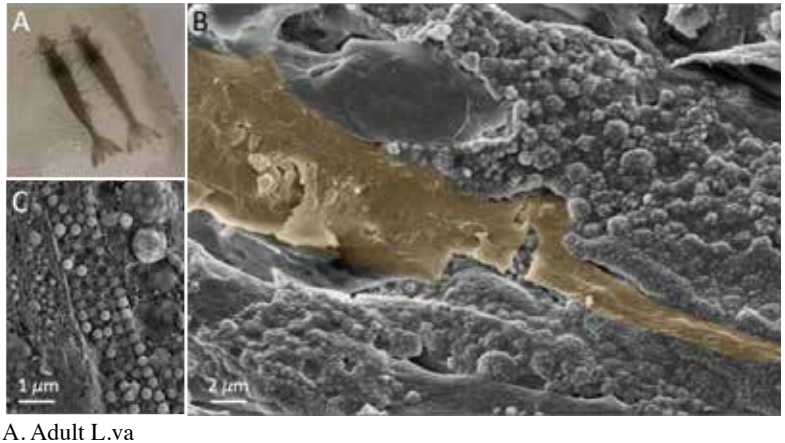
Mineral formation by organisms is widespread, and occurs in all 5 kingdoms. The processes involved in biomineralization are extremely diverse, and few generalizations can be made. Lowenstam differentiated between biologically induced mineralization and biologically controlled mineralization, with the latter almost always occurring in confined spaces (Lowenstam, 1981). Lowenstam was also the first to document in vivo that the formation of magnetite by a mollusk was preceded by the formation of a more disordered precursor mineral phase. In 1997 it was shown that sea urchin larvae form their calcitic skeletons via a highly disordered precursor phase, and since then this phenomenon has been documented in most of the major mineral forming phyla, including vertebrates. Thus in biology, mineral formation does not generally occur from saturated solutions, but rather via the prior formation of disordered phases that can be shaped into desired forms and then be induced to crystallize (Weiner and Addadi, 2011).

The term biomineralization refers to the biological formation of minerals, that are inorganic solid compounds. However the same processes could be involved in the formation of solid phases composed of ions and organic molecules (such as oxalates), and solid phases composed only of organic molecules. Guanine crystals are by far the best documented case of biogenic organic solids (Gur et al., 2017). Guanine crystals were first identified in 1861 as being responsible for the silvery color of fish scales. Guanine crystals are now known to be used by many animals to produce structural colors, as well as in vision where they function mainly as reflectors due primarily to their very high refractive index in one direction.

Guanine crystals have also been identified in a marine single-celled dinoflagellate, and their spatial association suggests that they might be involved in enhancing photosynthesis. Little is known about the formation of guanine crystals, but one study does point to the possibility that they too form via a disordered precursor phase. A few isolated cases of biogenic organic solids, such as uric acid, are known to occur. But does our present knowledge really reflect the true diversity of biogenic organic solids?

We have recently demonstrated that different crustaceans belonging to the large family of Decapoda all contain small crystals of the organic molecule isoxanthopterin. These crystals have an even higher refractive index than guanine, and can be found either as thin platelets, and intriguingly also as small hollow spheres. These spheres are effective scatterers of light, and they are thought to return photons that initially escaped detection, back into the retina. In this way they enhance vision in dimly lit environments (Palmer et al., 2018).



A. Adult L.va
nnamei with conspicuous eye-shine from the tapetum reflector. (B,C) Cryo-SEM images of the arrays of isoxanthopterin nanoparticles enveloping the rhabdom-the photosensitive unit of the retina.

There are many difficulties involved in identifying and characterizing biogenic organic crystals. They are usually very small, radiation sensitive and they are not much denser than the tissues in which they form. Organic crystals are therefore difficult to extract and characterize. We suspect that many more biologically produced organic crystals will be found. Thus the definition of the field of biomineralization should be expanded to include biogenic organic crystals and their possible precursor phases. Are we entering into the era of biological solid phase formation?

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Inhibitors of Biomineralisation

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Biomineralisation is the tightly orchestrated process by which living organisms synthesise and deposit inorganic materials to form hard tissue structures. In humans, this process contributes to normal development and the continued maintenance of tissues including healthy bone. The key biomineral components, calcium and phosphate, are subject to tissue-dependent environments, including varying mechanical and chemical stimuli. Dysregulation of the biomineralisation pathways can lead to pathological accumulation of mineral deposits, in disease conditions such as kidney stones; or deficiency of key mineral matrix structures, leading to disorders of the hard tissue such as rickets.

Mineralisation may also occur under pathological conditions in the blood vessels, where hydroxyapatite, similar to that found in bone, is deposited in a process known as vascular calcification. It is now well characterised that this calcification occurs through a regulated process, whereby vascular smooth muscle cells (vSMC) undergo trans-differentiation into bone-like osteogenic cells, which will eventually deposit a mineralised matrix. This vascular calcification process is triggered following an induction of osteogenic differentiation factors and concurrent loss of biomineralisation inhibitors. A number of reports have confirmed that the expression of various bone-associated factors including osteopontin, bone morphogenic proteins, alkaline phosphatase and matrix Gla protein, are differentially regulated in calcified vessels obtained from patients with artery disease.

Vascular disorders are highly prevalent in diabetes mellitus and cardiovascular complications are the major cause of morbidity

and mortality for individuals with diabetes. Patients with diabetes, exhibit enhanced vascular calcification when compared to age-and gender matched non-diabetic patients, indeed 90% of those with the diabetic foot disorder known as Charcot neuoroarthropathy, have evidence of vascular calcification on plain x-rays of the foot. The reason for the elevated calcification in association with diabetes is unclear and given the limited current treatment options, there is an urgent need to identify novel biomarkers and targets for therapy. This forms the focus of our group's research.

We have previously shown that the RANKL/RANK/OPG pathway is a key regulator of vascular calcification in diabetic patients. This was particularly relevant for patients with diabetic Charcot neuoroarthropathy where it was found that human vSMCs cultured in serum from these patients showed accelerated mineralisation, through the interaction between RANK and its ligand, leading to osteoblastic differentiation. This was found to be attenuated by co-incubation with OPG, the decoy receptor for RANKL (Ndip et al., 2011).

Sirtuin 1 (SIRT1), an NAD+ dependent deacetlylase enzyme, has been implicated in a number of cellular processes, including glucose homeostasis, calcium signalling and DNA damage repair. Recent investigations identified SIRT1, as having a protective role against vascular calcification in a high phosphate environment in-vitro. We sought to establish a potential role of SIRT1 in vSMC calcification in the context of diabetes. A key finding was that the serum levels of SIRT1 were reduced by 80% in patients with diabetes, compared to healthy controls (Bartoli-Leonard et al., 2019). Furthermore, immunohistochemical analysis revealed that SIRT1 staining was clearly supressed in calcified vessels obtained from diabetic patients with critical limb ischemia. We found that exposing human vSMCs to physiologically relevant hyperglycaemic conditions in vitro, increased mineralisation and decreased the expression of SIRT1. Interestingly, pharmacological activation of SIRT1 attenuated the

high glucose-induced calcification, demonstrating a protective effect.

Molecular analyses demonstrated that in the presence of osteogenic conditions and high glucose, loss of SIRT1 leads to an increase in the expression of osteogenic factors RUNX2, osteocalcin and alkaline phosphatase. This in turn, drives the osteogenic differentiation of vSMCs and the deposition of a calcified matrix. The incubation of the vascular cells in the hyperglycaemic conditions also led to the induction of a senescent phenotype, which is known to exacerbate the calcification process. Collectively, this data suggests that the suppression of SIRT1 might perpetuate the increased occurrence of vascular calcification in diabetes.

Our current research efforts aim to extend our knowledge surrounding the role of SIRT1 in osteogenic vSMC differentiation, under pathological conditions. This may occur through the activation of Wnt signalling and via the contribution of other members of the sirtuin family (reviewed in Bartoli-Leonard et al., 2018). A role for SIRT1 has previously been defined in the DNA damage response and an association has been observed between vascular mineralisation and DNA damage. We are therefore also examining whether SIRT1 activation may provide a therapeutic target for the inhibition of DNA damage and subsequent vessel calcification.

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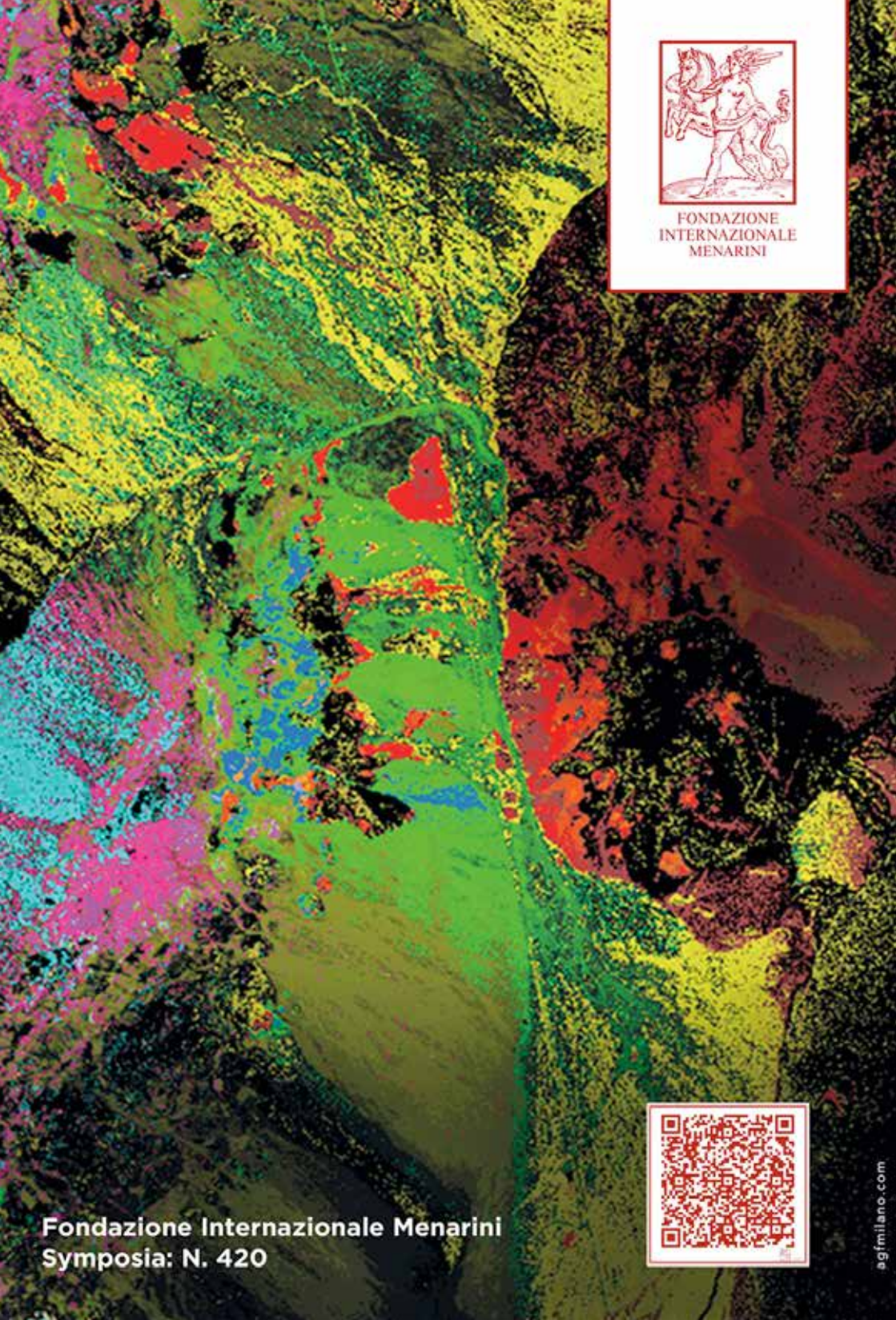

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