Review

Age-Associated Calcification: Insights from Murine Models

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Abstract: Calcification refers to the deposition of calcium-containing crystals either intracellularly or within the extracellular matrix. Physiologic calcification is a normal process occurring during bone and tooth development and growth. In contrast, pathologic calcification occurs in soft tissues that typically do not undergo mineralization, such as blood vessels, cartilage, tendons, and skin. Pathologic calcification is significantly associated with tissue impairment and the development of secondary diseases, such as atherosclerosis, osteoarthritis, tendinopathy, and skin ulcers. Aging, a natural process linked to numerous pathologic conditions, is one of the most recognized risk factors for pathologic calcification. In this manuscript, we review the current state of knowledge regarding the role of aging in calcification across different tissues. We focus on the mechanisms activated during normal aging, including cellular senescence, decreased pyrophosphate levels, increased secretion of extracellular vesicles, elevated oxidative stress, and higher levels of pro-mineralizing cytokines, all of which can contribute to pathological calcification. Finally, we discuss the available animal models used to study the impact of aging on calcification.

Keywords: aging; pathologic calcification; murine models

1. Introduction

Aging is an increasing tendency worldwide, and it has been estimated that about 128 million people will be older than 80 years in 2050 [1]. Aging is a complex naturally occurring process, resulting from cellular and molecular processes, often leading to the onset of age-related conditions, including pathological calcification [2]. The latter is the process by which calcium-containing crystals are deposited in extra-osseous structures of the cardiovascular system (vessels, heart valves), in the musculoskeletal system (cartilage, tendons), in the eye, and in the brain [1]. These crystals include calcium pyrophosphate dihydrate (CPP) and basic calcium phosphate (BCP) crystals. BCP crystals encompass carbonated-substituted hydroxyapatite (HA), tricalcium phosphate (TCP), octacalcium phosphate (OCP), and whitlockite [3]. Crystal formation is carried out by calcification-competent cells and their cell membrane-derived matrix vesicles (MVs) (Figure 1). A complex machinery of enzymes and transporters is involved: cell-associated ankylosis protein (ANK) extrudes ATP in the extracellular space [4]. Ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) converts ATP into pyrophosphate (PPi). ATP can also be released through various membrane transporters, such as pannexins, connexins, and others, or via exocytosis. The principal supplier of ATP likely varies among tissues and remains unknown [5]. Excessive PPi reacting with Ca2+ leads to the formation of CPP crystals. In contrast, when PPi is hydrolyzed by tissue-nonspecific alkaline phosphatase (Tnap) in P1, the latter can be transported into MVs by the Na/P1 cotransporters (P1T1/2). If Ca2+ is concomitantly concentrated in MVs through the Annexin V channel (ANX V), the nucleation and growth of BCP crystals occur. CPP and BCP are therefore the two main families of calcium-containing crystals found in pathologic calcification and the P1/P1...
equilibrium is the main determinant of their respective formation [6–8]. While a mixture of BCP and CPP crystals have been found in cartilage [9,10], in other pathologically calcifying tissues, BCP crystals are prominent [11,12].

**Figure 1.** Calcium-containing crystal formation in calcification-competent cells. The extracellular concentration of PP\(_i\) and P\(_i\) is tightly regulated by different enzymes and transporters. This leads to either CPP or BCP crystal formation. The triggers of pathologic calcification include alteration of PP\(_i\), P\(_i\), and Ca\(^{2+}\) homeostasis, impaired mitochondrial homeostasis, blockage of DNA reparation, a jammed cell cycle, compromised autophagy and proteostasis, deregulation of the circadian rhythm, and an increase in pro-mineralization cytokines.

2. Tissues Affected by Pathologic Calcification in Aging and Their Associated Diseases

2.1. Cardiovascular Calcification

Cardiovascular calcification is the process of calcium-containing crystal deposition in cardiovascular tissues such as aortic valves, the myocardium, and large and small arteries [13]. The incidence of cardiovascular calcification increases with age, affecting approximately 12% of individuals older than 80 [14]. Moreover, it appears that cardiovascular calcification represents a 4-fold higher risk factor for mortality [15] and a 3.5-fold higher risk for any cardiovascular event [16], such as heart disease, stroke and atherosclerotic plaque rupture [17–20].

Calcific aortic valve disease (CAVD) is the most common disorder affecting the heart valves. It can range from mild stiffening and fibrosis of the valve leaflets without obstruction of the blood flow (aortic sclerosis) to severe leaflet calcification that reduces their movement and blood flow (aortic stenosis) [20]. Currently, there is no curative treatment to stop the progression of CAVD [20]. Age appears to be an important risk factor for CAVD, as its prevalence peaks in the age group of 65–69 years for men and 70–74 years for women [21]. Epidemiological studies show that 3% of adults older than 75 have some sign of CAVD and up to 25% of adults over 65 old have at least aortic sclerosis [22–24]. Age-related functional impairment of the aortic valve might be caused by disorganized collagen fibers, inflammatory cell recruitment, and active formation of crystals, ultimately leading to valvular stiffness and narrowing.

Myocardial calcification affects the myocardium, the cardiac muscle tissue that forms the middle layer of the heart walls. It is composed of cells called cardiomyocytes and is responsible for the contractile function of the heart and for maintaining adequate tissue and organ perfusion throughout the entire body [25]. Three forms of myocardial calcification
are recognized: dystrophic, metastatic, and idiopathic [26]. Dystrophic calcifications are focal and linear, and they often result from a local myocardial injury such as myocardial infarction. Metastatic calcifications are more diffuse and globular, and they are an important sign of a systemic pathological status such as hypercalcemia and/or abnormalities in calcium homeostasis. Idiopathic calcifications are dystrophic or metastatic calcifications with occult and unidentifiable pathological process. Aging is mainly associated with dystrophic rather than metastatic myocardial calcification due to the increased incidence of myocardial infarction in elderly people. Indeed, an older age has been associated with a greater proportion of heart failure and coronary disease, and a higher mortality rate, after myocardial infarction [27].

Vascular calcification (VC) is the deposition of calcium-containing crystals in the intimal or medial layers of the arteries. Intimal calcification is linked with the development of atherosclerotic plaques [28,29], while medial calcification does not normally cause luminal narrowing [30,31]. VC is associated with several age-related diseases, such as atherosclerosis, type II diabetes, chronic kidney disease, and chronic inflammatory disease [1,32,33]. Large-scale studies, including the Multi-Ethnic Study of Atherosclerosis [14,34], the St. Francis Heart Study [35], and the study by Tota-Maharaj et al. in 1015 asymptomatic South Korean subjects [36] have demonstrated that coronary atherosclerotic calcification increases with age. Other studies also demonstrated the presence of increased calcification with aging in the carotid region [37,38].

Age-induced alterations in the ECM structure and composition of the artery wall include increased collagen deposition and cross-linking, accumulation of advanced glycation end products, and elastin fiber breakage. This leads to progressive fibrosis, one of the suspected underlying mechanisms predisposing people to atherosclerosis during aging [39,40]. Finally, osteoblastic transdifferentiation of vascular smooth muscle cells seems to be a crucial mechanism leading to vascular calcification and atherosclerosis during aging [41].

2.2. Musculoskeletal Calcification

Aging is associated with the calcification and/or ossification of musculoskeletal soft tissues, including tendons [42], ligaments [43], and cartilage [44,45]. This phenomenon, which is accompanied by increased pain, impaired mobility, and disability, often leads to the development of very common musculoskeletal disorders in the elderly, such as calcific tendinopathy (CT), ossification of the posterior longitudinal ligament (OPLL) and osteoarthritis (OA).

CT in aged subjects affects the rotator cuff tendons, Achilles tendons, and biceps tendons, with a prevalence varying from 42% for the rotator cuff tendons to 5% for other tendons [46]. In adult progeria patients diagnosed with Werner syndrome, tendon calcification is one of the most common phenotypes [47]. CT occurs because tenocytes transdifferentiate into hypertrophic chondrocytes, which deposit calcium-containing crystals in the extracellular matrix [48]. Chondrocytes might further undergo osteoblastogenesis and tendon tissue is finally replaced by bone tissue [43].

Like tendons, ligaments calcify because of an endochondral ossification process, although an intramembranous ossification process can also occur [43]. OPLL is a disease characterized by the thickening of the posterior longitudinal ligament and appearance of osteoblasts and chondrocytes in ligament tissue, with a prevalence of around 3% in aged individuals [49]. A prospective multicenter study [50] identified age as the primary risk factor for neurological impairment in patients with cervical OPLL. Indeed, the compressive effects of the ossified tissue would lead to the development of cervical myelopathy. Talbot et al. performed computed tomography scans of the cervical spines of horses and found a significant association between the degree of calcification of the longitudinal ligament and the age [51].

The articular cartilage is another tissue affected by calcification, a cause of inflammation and cartilage degeneration. Age is one of the main risk factors for the development of
cartilage calcification [52], which is more prevalent in individuals aged 60 years and above. Two different types of calcium-containing crystals have been found in cartilage: BCP and CPP crystals [9,10]. BCP crystals, rather than CPP, appear prevalent in OA and are present in 100% of cartilage samples at the time of joint replacement [9]. The consensus on the role of crystals in OA remains controversial. While some studies suggest they are not etiologically significant [53,54], recent clinical and experimental data support their contribution to OA development and progression. Radiographically detected mineralization has been linked to an increased risk of knee pain over two years in individuals both under 60 years old [55] and over 60 years old [56]. Additionally, cartilage calcification has been associated with increased cartilage and meniscus degeneration over a four-year period [57]. In knee specimens from 56 individuals, the relative calcium deposition had a significant positive correlation with age and with the severity of OA, at least until a Kellgren–Lawrence score of 3 [52]. In another study, quantitative PET/CT analysis of the costal cartilage revealed increased calcification with age in both males and females at similar rates [58]. Currently, it is believed that the dedifferentiation of resting chondrocytes into hypertrophic calcifying chondrocytes could explain this phenomenon. In addition, chondrocyte apoptosis could contribute to cartilage calcification [44]. Finally, aging can affect the cartilage matrix composition [59]. ECM changes that occur during osteoarthritis, such as increased collagen I and reduced proteoglycans, may promote cartilage calcification [60].

2.3. Skin Calcification

The elastin fibers of the skin are more calcified and degraded in aged skin [61]. Ca^{2+} binds to elastin, elastin becomes positively charged and attracts counterions such as phosphate and carbonate, and this initiates the crystallization process [62].

In patients diagnosed with Hutchinson–Gilford progeria syndrome (HGPS), skin calcification was noted mainly in the distal digits but also in the heel, trunk, upper and lower leg, chest, and abdomen [63]. In patients diagnosed with Werner syndrome (WS), an autosomal recessively inherited progeroid disorder caused by homologous mutations in a RecQ family DNA helicase, skin is often present. It can be asymptomatic but often results in severe pain and may promote skin ulcer formation [64–67].

2.4. Eye Calcification

Ocular calcification can be found in several ocular structures, especially the cornea and the retina, but also Bruch’s membrane and the optic nerve [68]. These calcifications are associated with either trauma or idiopathic causes. In aged people, the most common form of ocular calcification is found in the retinal pigment epithelium (RPE), which leads to age-related macular degeneration (AMD) [68]. This disease affects millions of people worldwide and is a leading cause of visual impairment in the elderly population. Three different calcification structures are present in AMD: spherules, plaques, and nodules. Spherules have been characterized as whitlockite, the plaques as amorphous apatite, and the nodules as apatite. How RPE calcific deposits contribute to AMD pathogenesis is not fully understood; however, it is suggested that calcification in the RPE blocks the metabolic exchange between photoreceptor cells and the choroidal vasculature, ultimately leading to vision loss. Moreover, the size and frequency of spherules may affect the severity of AMD [69].

Cataract is a very well-known ocular aging phenotype, with an incidence as high as 45% in the people between 75 and 85 years of age [70]. Senile cataract is defined as cataracta ossea when calcifications are found, with a bone-like structure [71]. This condition seems to be caused by the transdifferentiation of lens epithelial cells into calcification-competent osteo-/chondrogenic cells [72].

Finally, calcified senile sclera plaques (CSSPs) are commonly seen in elderly people, with a prevalence of 8.2%. They are usually found anterior to the insertion of the horizontal recti muscles because of stress and strain on the sclera by the action of muscles. It occurs
in aged subjects due to dehydration and scleral ischemia due to atherosclerosis. They can lead to scleromalacia and perforation [73].

2.5. Brain Calcification

Intracranial calcifications are often incidental findings on brain tomography, and their prevalence ranges from 1% in young individuals up to 38% in elderly subjects [74–76]. In these patients, calcification can be considered a normal phenomenon and mainly localizes in the basal ganglia, pineal gland, choroid plexus, and habenula [77]. Intracranial calcifications are mainly asymptomatic, but in some cases, they are associated with migraine, parkinsonism, psychosis or dementia. The most common symptomatic condition where calcifications form in the basal ganglia is called Fahr’s disease. Pathological calcifications can be distinguished from age-related physiological calcifications by their features. Physiological calcifications are often small, symmetrical and confined to the globus pallidus, while pathological ones are bigger and extend in the putamen and the dentate nucleus [78].

Different mechanisms accounting for brain calcification have been suggested in the literature and previously reviewed [79]. Progressive basal ganglia calcification can compress the vessel lumen, leading to impaired blood flow, tissue injury and calcification deposition [79]. Iron may also play a critical role in brain calcification. Increased iron levels in the basal ganglia may induce reactive oxygen species (ROS) in this tissue and accelerate neuronal degeneration. Indeed, oxidative stress is linked to other degenerative processes enhancing cellular death, such as mitochondrial dysfunction, excitotoxicity, DNA damage and inflammation [79]. It remains unknown whether oxidative stress is the cause or the consequence of these events. Finally, elevated intracellular calcium levels have been implicated in the pathogenesis of brain calcification as well. Overload of calcium intracellularly promotes the conversion of xanthine dehydrogenase to xanthine oxidase, which produces superoxide anion and cell damage [80]. Calcium may also participate in excitotoxicity by activating proteases and destroying the cytoskeleton [81].

3. Animal Models of Pathologic Calcification during Aging

Aged mice have been extensively used in the context of aging research as they recapitulate many of the typifying molecular and functional alterations of age-associated human diseases in different organs [42,82–85]. It is no surprise therefore that aged mice have been also exploited to investigate pathological calcification of the cardiovascular system, tendons, cartilage, and joints [86–89]. However, despite their relatively short lifespan, studying age-related disorders in mice presents challenges, such as the high maintenance costs and time investment. In recent years, genetic manipulation of rodents has provided the unprecedented opportunity to investigate aging in appositely generated mouse models, allowing researchers to overcome time constrains and reduce costs. Accelerated aging can be achieved in mice by the alteration of specific genes involved in different pathways mediating the life- and health-span, i.e., calcium and phosphate homeostasis, mitochondrial homeostasis, DNA repair and stability, cell cycle, proteostasis, and circadian rhythm. Importantly, many of the models in these categories present pathologic calcification because of their rapid aging and therefore can be useful models for studying this age-related condition. These models are discussed below and summarized in Table 1.

3.1. Alteration of Pyrophosphate, Phosphate, and Calcium Homeostasis

Inorganic pyrophosphate (PPI) is one of the most potent calcification inhibitors in humans. It binds to hydroxyapatite crystals, thereby inhibiting further growth of the calcifications [90–92]. Lmna<sup>G609G</sup> mice express a mutated form of Lmna, which mimics the human mutation characterizing HGPS patients [93]. These mice display a shortened lifespan, reduced body weight, and bone and cardiovascular abnormalities, including extensive calcification of the vascular smooth muscle and aortic media [93,94]. Vascular calcification is due to a four-fold lower plasma pyrophosphate concentration than in wild-
type mice [94]. Indeed, daily injection of exogenous pyrophosphate prevents vascular calcification in Lmna\textsuperscript{G609G} rats and mice [94–97].

Phosphate and calcium are the principal ions involved in the deposition of mineral in tissues; hence, perturbation of their metabolism is associated with ectopic mineral deposition and related pathologies. KLOTHO is a transmembrane protein that plays a role in many crucial cell processes, such as calcium homeostasis, Wnt signaling, nitric oxide production and control of vascular tone, suppression of oxidative stress, and insulin/IGF-1 signaling, modulating the de facto health- and lifespan. Consistent with its role in cellular and organism homeostasis, transgenic mice lacking functional Klotho already display at 8 weeks of age a severe aging phenotype, including infertility, arteriosclerosis, skin atrophy, osteoporosis and emphysema, which ultimately leads to premature death [98,99]. KLOTHO also possesses the ability to act as an obligatory co-receptor for FGF23, a bone-derived hormone that induces a negative phosphate balance [100,101]. Interestingly, Fgf23 \textsuperscript{KO} mice present a progeroid phenotype strikingly similar to the one observed in Klotho-deficient mice, which is in line with the physiological role of Klotho in activating the FGF23 pathway [100,101]. Importantly, one of the most prominent features of both Klotho and Fgf23 KO animals is the pronounced ectopic calcification in multiple organs and tissues, including the kidney, skeletal muscle, cardiac muscle and arterial walls, brain and choroid plexuses, lung, bronchial mucosa and alveolar cells, skin, urinary bladder, stomach, and testes [99,102,103]. Consequently, they are among the most well-established models used to investigate age-dependent calcification and its relationship with calcium and phosphate homeostasis.

Consistent with the role of calcium and phosphate metabolism in aging and age-associated calcification, the mutant mouse strain D2,Ahsg\textsuperscript{−/−}, combining Fetuin-A deficiency with the calcification-prone DBA/2 genetic background, also presents severe calcification of soft tissues with concomitant organ dysfunction, growth retardation and reduced lifespan [104,105]. Fetuin-A is a liver-synthetized plasma protein acting as a mineral scavenger; indeed, Fetuin-A is capable of binding clusters of calcium and phosphate ions, therefore preventing ectopic mineralization [106,107]. Despite the fact that Fetuin-A deficiency was shown to protect against certain age-related metabolic alterations, including insulin insensitivity [108], its absence in DBA/2 mice promotes premature aging and pathological calcification of the majority of the organs, although most prominently in the kidney, skin, testis, adipose tissue and blood vessels [104,105].

As mentioned above, Enpp1 is a transmembrane protein that generates P\textsubscript{i} [109,110]. Cartilage-specific Enpp1 conditional knockout mice exhibited various aging-related phenotypes, including osteoporosis and ectopic calcium deposition in tissues, such as the kidney and spinal ligament, under phosphate overload conditions. Thus, the authors concluded that Enpp1 activity in cartilage is a regulator of systemic phosphate metabolism, ectopic calcification, and aging [111].

3.2. Impaired Mitochondrial Homeostasis

Accumulating evidence has described an age-dependent impairment of mitochondrial function across species from yeast to humans [112,113]. Mitochondria are essential components of eukaryotic cells, providing them with 90% of the chemical energy (in the form of ATP) they need to survive through oxidative phosphorylation (OXPHOS); moreover, they also contribute to cellular metabolism, calcium homeostasis and apoptosis. The unique feature of mitochondria is the ability to self-synthesize through their own genome and translation machinery, including proteins involved in their activity and function. A crucial player in this process is the nuclear encoded mitochondrial DNA polymerase, whose catalytic activity is mediated by POLG. Knock-in mice that express a proof-reading-deficient version of PolgA, present high levels of point mutations, as well as increased amounts of deleted mtDNA, and therefore are commonly referred to as “mtDNA mutator mice”. This increase in somatic mtDNA mutations is associated with a reduced lifespan and premature onset of aging-related phenotypes, including reduced body size, decreased fertility, hearing
loss, kyphosis, sarcopenia, alopecia, reduced bone density, anemia, heart hypertrophy, and loss of intestinal crypts [114,115]. Importantly, these animals also present elevated numbers of hypertrophic chondrocytes in the articular cartilage [116], which is in line with the role of mitochondrial dysfunction in the onset of pathological calcification [117].

ROS are produced by mitochondria during OXPHOS. Elevated ROS levels trigger oxidative stress and oxidative stress-induced signaling, two common features of aging, cardiovascular, and metabolic diseases, representing a major driving factor for calcification and stiffness [118–121]. In vitro studies have further demonstrated a direct effect of H2O2 on promoting the osteogenic differentiation and calcification of VSMC [122] and calcifying bovine vascular cells [120]. In cartilage, ROS induce chondrocyte hypertrophy, a condition ultimately leading to pathologic calcification [123]. Senescence-accelerated mouse/prone (SAMP) mice exhibit increased ROS generated by mitochondria or other cellular sites, triggering amyloidosis, osteoporosis, osteoarthritis, and other age-associated conditions [124]. However, none of the studies have explored the occurrence of pathologic calcification in these mice.

### 3.3. Blockage of DNA Reparation

In line with the observation of premature aging in mtDNA mutator mice, the alteration of nuclear DNA integrity can also play a crucial role in the development of age-associated pathologies. DNA damage occurs frequently in cells, which are therefore equipped with specific mechanisms to correct such errors. One such mechanism is performed by the chromatin-associated protein SIRT6, which acts as a DNA damage sensor and mediates the base excision- and double-strand break-repair of damaged DNA by non-homologous end joining and by homologous recombination. SIRT6 is part of the sirtuin family of deacylases, which coordinates not only DNA repair but also mitochondrial function, metabolism, and aging. Indeed, Sirt6 expression is decreased during aging and, accordingly, Sirt6-knockout mice have a marked progeroid phenotype, characterized by a reduced lifespan, altered glucose homeostasis, impaired vision, and development of lordokyphosis and osteopenia [125,126]. Increasing evidence also suggests a higher level of calcification of the aortic valve in SIRT6-deficient mice, which is in line with the protective role of other sirtuins, e.g., SIRT1. This is mediated by a modulation of endothelial nitric oxide production, inhibition of endothelial and vascular smooth muscle cells apoptosis, and attenuation of the transition of vascular smooth muscle cells toward an osteoblastic phenotype [127,128]. Very recently, SIRT7 have been shown to protect against vascular calcification via modulation of ROS and senescence of vascular smooth muscle cells [129].

### 3.4. Jammed Cell Cycle

Cell cycle arrest is the first step in a cascade of molecular events leading to cellular senescence, a prominent feature of aging and age-associated diseases. Cyclin-dependent kinase 7 (Cdk7) is the catalytic subunit of the Cdk-activating kinase (CAK) complex and has been implicated in the control of cell cycle progression. While ablation of Cdk7 in tissues with low cellular turnover has no detrimental effects, its removal from actively dividing tissues leads to progressive depletion of stem cell pools and concomitant onset of aging features, including body weight loss, alopecia, pervasive hair-graying and kyphosis [130]. Moreover, lack of Cdk7 also mediates severe kidney medullary calcification and consequent damage [130].

During their physiological cycle, cells undergo mitotic division, which leads to the generation of cell progeny genetically identical to the original cell. To ensure correct division and an error-free replication, different proteins participate to this delicate process. This group of proteins includes BUB3 and RAE1, which ensures the fidelity of the chromosome segregation, avoiding chromosome instability and carcinogenesis. Bub3 and Rae1 show extensive sequence homology, indicative of functional similarity. While mice haploinsufficient for either of these genes do not display any significant alteration in their phenotype (in line with the notion that Bub3 and Rae1 could have overlapping roles), Bub3/Rae1-haploinsufficient mice age much
faster. These animals have a significantly reduced lifespan, early development of kyphosis and severe cataracts characterized by profound calcification of the inner eye [131]. This is also in accordance with the role of DNA integrity in maintaining homeostasis and preventing the onset of age-related pathologies, as previously described (see the “Altered mitochondrial homeostasis” and “Blockage of DNA reparation” sections).

In the frame of the correct cell cycle and DNA integrity, a crucial role is played by the nuclear architecture, particularly by the nuclear envelope. In fact, this membrane, placed at the interface between the nucleus and cytoplasm, not only mediates molecules trafficking into and out of the nucleus but also provides anchoring sites for chromatin domains and regulates mitosis. Mutations in the gene encoding nuclear Lamin A, a key protein of the nuclear envelope, have indeed been associated with impaired cell division and several human diseases, including muscular dystrophy, dilated cardiomyopathy, heart–hand syndrome, restrictive dermopathy, mandibulocranial dysplasia, lipodystrophy, Charcot–Marie–Tooth disease, and, interestingly, HGPS [132,133]. HGPS, characterized by premature aging, can be partially recapitulated in mice by alterations to the Lmna gene. One example are the Lmna

3.5. Compromised Autophagy and Proteostasis

One of the most common factors mediating age-associated collapse of cellular and tissue homeostasis is impaired autophagy, which is associated with the intracellular accumulation of damaged organelles and molecules. An abundant body of literature has outlined the critical role of dysfunctional autophagy in the onset of age-related disorders such as neurodegenerative diseases, muscular degeneration, cardiac dysfunction, and cancer. A key model that enables the characterization of the link between altered autophagy and aging is the glycogen synthase kinase-3 (Gsk3α) global KO mouse strain. These animals display an accelerated aging phenotype in different tissues, including striated muscles, gut, liver, and joints because of impaired autophagy [134]. Indeed, GSK3α acts as a negative regulator of different metabolic pathways, including amino acid and nutrient sensing, whose master regulator is the mTOR kinase; as a result, in Gsk3α-KO mice, mTOR is constitutively activated, preventing proper autophagic flux. Importantly, one of the most prominent features of Gsk3α-KO mice is profound calcification of the knee joint, which extends into the subchondral bone and into the meniscal area [134].

3.6. Deregulation of the Circadian Rhythm

All living organisms have evolved to adapt to the Earth’s 24 h rotation pattern using a series of endogenous molecular oscillators that compose the circadian clock. This molecular clock, which is present in all cells, is composed of the transcriptional activator proteins CLOCK and BMAL1, which drive rhythmic gene expression. Inhibition of either of these two master regulators of the circadian rhythm leads to a decrease in lifespan and the development of age-related pathologies. Indeed, although Bmal1-KO mice do not display anomalies in early development, by 30 weeks of age, they present evident sarcopenia and osteoporosis, reduced hair growth, various grades of cataract and severe joint ankylosis due to the flowing ossification of ligaments and tendons and almost complete immobilization of weight-bearing and non-weight bearing joints. Importantly, pathological calcification of the joints is observed also in animals in which Bmal1 is selectively ablated in the skeletal muscle during adulthood, suggesting a close relation between joints’ calcium homeostasis and the circadian rhythm [135,136].

3.7. Increase of Pro-Mineralization Cytokines

A persistent pro-inflammatory state is a prevalent characteristic of aging. Termed “inflammaging,” this chronic low-grade inflammation, occurring without evident infection, represents a substantial risk factor for morbidity and mortality among the elderly [137]. Inflammatory
cytokines such as IL-6, IL-1β, and TNF-α are the most commonly involved [137]. All of them have been shown to induce pathologic calcification in different tissues [136,138–140].

Mice with a gain of function mutation in one of the factors involved in the NF-kB signaling pathway represent aging models with an inflammation phenotype. These mice have a shortened lifespan, kyphosis, osteoporosis, and many other aging-related conditions [141].

A mouse model of inducible IL-6 expression (IL-6^{TET-ON/+} mice) developed increased aging features such as the frailty index, a decrease in grip strength, and disrupted muscle mitochondrial homeostasis following administration of doxycycline in food [142]. In 80-week-old mice, TNF-α was shown to be elevated, together with a loss of skeletal muscle mass and grip strength, known signs of sarcopenia in the elderly [143].

While none of these animal models have been examined for the presence of ectopic calcification thus far, we hypothesize that they may exhibit calcification in multiple anatomical sites.

<table>
<thead>
<tr>
<th>Model</th>
<th>Target Gene</th>
<th>Altered Physiological Processes</th>
<th>Effect on Health-Span and Lifespan</th>
<th>Calcification Area</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klotho^{−/−}</td>
<td>Klotho</td>
<td>Calcium/pyrophosphate/phosphate homeostasis, Wnt signaling, nitric oxide production, oxidative stress response, insulin/IGF-1 signaling, FGFR3 signaling.</td>
<td>Reduced lifespan, reduced growth, hypogonadism and infertility, osteopenia, emphysema, decreased cognitive function.</td>
<td>Heart, vascular system, kidney, trachea, lung, stomach, intestine, brain, gonads.</td>
<td>[99,102]</td>
</tr>
<tr>
<td>Fgf23^{−/−}</td>
<td>Fgf23</td>
<td>Calcium/pyrophosphate/phosphate homeostasis, Klotho signaling.</td>
<td>Reduced lifespan, reduced growth, kyphosis, muscle wasting, hypogonadism and infertility, osteopenia, emphysema, uncoordinated movement, T cell dysregulation, atrophy of intestinal villi, skin, thymus, and spleen.</td>
<td>Heart, vascular system, kidney, lung, skeletal muscle, skin, urinary bladder, testes.</td>
<td>[103]</td>
</tr>
<tr>
<td>D2Ahsγ^{−/−}</td>
<td>Fetuin-A</td>
<td>Calcium/pyrophosphate/phosphate homeostasis, removal of clusters of calcium and phosphate ions.</td>
<td>Reduced lifespan, reduced growth, reduced breeding performance, heart failure.</td>
<td>Heart, vascular system, kidney, lung, pancreas, skin, gonads, spleen, adipose tissue.</td>
<td>[104,105]</td>
</tr>
<tr>
<td>Enpp1^{−/−}, conditional in cartilage</td>
<td>Enpp1</td>
<td>Calcium/pyrophosphate/phosphate homeostasis, diminished pyrophosphate</td>
<td>Reduced lifespan, severely impaired movement</td>
<td>Cartilage, tendons, ligaments, kidney</td>
<td>[111]</td>
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<tr>
<td>mtDNA mutator mice</td>
<td>PolgA</td>
<td>Mitochondrial homeostasis, mitochondrial DNA integrity.</td>
<td>Reduced body size, decreased fertility, hearing loss, kyphosis, muscle wasting, reduced hair density and alopecia, osteoporosis, anemia, heart hypertrophy, loss of intestinal crypts.</td>
<td>Joints.</td>
<td>[116]</td>
</tr>
<tr>
<td>Sirt6^{−/−}</td>
<td>Sirt6</td>
<td>DNA damage responses and repair, genomic stability, chromatin compaction, transcriptional repression.</td>
<td>Reduced lifespan, hypoglycemia, reduced IGF-1, kyphosis, lymphopenia, reduced subcutaneous fat.</td>
<td>Aortic valve.</td>
<td>[125]</td>
</tr>
<tr>
<td>Cdk7^{−/−}</td>
<td>Cdk7</td>
<td>Cell cycle progression, stem cell pool.</td>
<td>Reduced lifespan, reduced growth, weight loss, alopecia, hair-graying, kyphosis, osteoporosis, nephropathy, reduced subcutaneous fat, intestine atrophy.</td>
<td>Kidney.</td>
<td>[130]</td>
</tr>
<tr>
<td>Bub3^{−/−} / Rae1^{−/−}</td>
<td>Bub3 / Rae1</td>
<td>Chromosome segregation and integrity.</td>
<td>Reduced lifespan, weight loss, muscle wasting, kyphosis, cataract, alopecia, reduced subcutaneous fat.</td>
<td>Eye.</td>
<td>[131]</td>
</tr>
<tr>
<td>Lmna^{G97R/G97R}</td>
<td>Lmna</td>
<td>Cell division, nuclear integrity, pyrophosphate levels</td>
<td>Reduced lifespan, reduced body weight, reduced growth, infertility, kyphosis, osteoporosis, reduced grip strength, reduced subcutaneous fat, cardiovascular alterations, reduced IGF-1, atrophy of lymphoid organs, thymus and spleen.</td>
<td>Cardiovascular system.</td>
<td>[93,94]</td>
</tr>
<tr>
<td>Gsk3α^{−/−}</td>
<td>Gsk3α</td>
<td>Autophagy, mTOR signaling.</td>
<td>Reduced lifespan, sarcopenia, cardiac hypertrophy and contractile dysfunction, impaired diastolic relaxation, cardiac fibrosis, tubular protein aggregation, liver senescence, intestinal senescence, joint ankylosis.</td>
<td>Knee joint.</td>
<td>[134]</td>
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<tr>
<td>Bmal1^{−/−}</td>
<td>Bmal1</td>
<td>Circadian rhythm, oxidative stress response.</td>
<td>Reduced lifespan, growth retardation, reduced body weight, male infertility, sarcopenia, osteoporosis, reduced hair growth, cataract, joint ankylosis, reduced subcutaneous fat, atrophy of spleen, kidney, testis, heart, and lung.</td>
<td>Joints, tendon.</td>
<td>[135,144,145]</td>
</tr>
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Table 1. Animal Models of Pathologic Calcification during Aging.
4. Conclusions

Ectopic calcification represents a significant pathological process associated with aging, contributing to various age-related diseases and conditions. Understanding the mechanisms underlying ectopic calcification is crucial for developing effective therapeutic strategies. Animal models have been indispensable in unraveling these mechanisms, providing insights into the genetic, molecular, and environmental factors that drive calcification in tissues where it should not occur. These models have allowed researchers to identify some of the pathways involved in ectopic calcification, offering potential targets for intervention. It is imperative to continue refining these animal models and other technologies to enhance our comprehension of ectopic calcification and to translate the findings into clinical applications. These efforts hold promise for mitigating the burden of ectopic calcification in the aging population, improving health outcomes and quality of life.

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