



Endogenous Calcification Inhibitors in the Prevention of Vascular Calcification: A Consensus Statement From the COST Action EuroSoftCalcNet

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The physicochemical deposition of calcium-phosphate in the arterial wall is prevented by calcification inhibitors. Studies in cohorts of patients with rare genetic diseases have shed light on the consequences of loss-of-function mutations for different calcification inhibitors, and genetic targeting of these pathways in mice have generated a clearer picture on the mechanisms involved. For example, generalized arterial calcification of infancy (GACI) is caused by mutations in the enzyme ecto-nucleotide pyrophosphatase/phosphodiesterase-1 (eNPP1), preventing the hydrolysis of ATP into pyrophosphate (PP_i). The importance of PP_i for inhibiting arterial calcification has been reinforced by the protective effects of PP_i in various mouse models displaying ectopic calcifications. Besides PP_i, Matrix Gla Protein (MGP) has been shown to be another potent calcification inhibitor as Keutel patients carrying a mutation in the encoding gene or *Mgp*-deficient mice develop spontaneous calcification of the arterial media. Whereas PP_i and MGP represent locally produced calcification inhibitors, also systemic factors contribute to protection against arterial calcification. One such example is Fetuin-A, which is mainly produced in the liver and which forms calciprotein particles (CPPs), inhibiting growth of calcium-phosphate crystals in the blood and thereby preventing their

soft tissue deposition. Other calcification inhibitors with potential importance for arterial calcification include osteoprotegerin, osteopontin, and klotho. The aim of the present review is to outline the latest insights into how different calcification inhibitors prevent arterial calcification both under physiological conditions and in the case of disturbed calcium-phosphate balance, and to provide a consensus statement on their potential therapeutic role for arterial calcification.

Keywords: arterial calcification, pyrophosphate, gla proteins, klotho, osteoprotegerin, osteopontin, fetuin

Vascular calcification (VC) is a common occurrence in patients affected with chronic diseases including diabetes, chronic kidney disease (CKD), or atherosclerosis. VC is also a hallmark of rare genetic diseases including pseudoxanthoma elasticum (PXE), generalized arterial calcification of infancy (GACI), Keutel syndrome, and progeria (1). Although the pathogenesis and clinical significance of VC are dependent on the etiology, the endpoint is invariably the formation of hydroxyapatite (HA) deposits in the arterial wall. Over the last two decades, studies have identified a number of calcification inhibitors in the healthy vessel wall that act to protect the vascular smooth muscle cells (VSMCs) from calcification. These factors act by either directly interfering with molecular pathways and/or sequestering hydroxyapatite components impairing their assembly and deposition. Their actions also depend on the stage of crystal formation and environmental context. Tremendous efforts have been put into the understanding of the mechanisms involved in the activity of these endogenous inhibitors that represent attractive factors with therapeutic relevance to VC treatment.

INORGANIC PYROPHOSPHATE

Inorganic pyrophosphate (PP_i), which consists of two inorganic phosphate molecules joined by a hydrolyzable ester, was first recognized as a key endogenous inhibitor of biomineralization in the 1960s (2). The major source of PP_i is extracellular ATP, which is released from cells through a highly regulated process (3). Subsequently, ATP can be rapidly hydrolyzed by ecto-nucleotide pyrophosphatase/phosphodiesterases (eNPPs) to produce PP_i. Additionally, the membrane protein ANK (progressive ankylosis or ANKH) regulates PP_i levels through the transport of intracellular PP_i to the extracellular environment (4). Furthermore, a crucial source of systemic PP_i is provided through ATP-binding cassette subfamily C member 6 (ABCC6)-mediated ATP release from hepatocytes (5).

The formation of calcium phosphates and homogeneous precipitation is not thermodynamically favored in blood and solutions, but it still can take place through the nucleating activity of matrix proteins such as collagen or elastin (6, 7). Nucleation of amorphous calcium phosphate is prevented by PP_i, which also inhibits the crystallization toward hydroxyapatite and crystal growth by binding to the hydroxyapatite surface (2, 8).

Reduced circulating PP_i concentration is commonly present during vascular calcification, as observed in hemodialysis patients (9). PP_i is hydrolyzed by local phosphatases, such as

tissue-non-specific alkaline phosphatase (TNAP). Consequently, when the expression of TNAP is selectively increased, ectopic calcification is observed (10). During CKD, aortic calcification is accompanied by TNAP overexpression (11), an event that precedes the first observed calcium nanodeposits and hyperphosphatemia in a rat CKD model (12). In those, calcium deposition is followed by an unexpected local increase in ANKH expression and late increase in ENPP1 expression. These expression changes are followed by reduced plasma PP_i concentrations as a later event (12). Therefore, the local concentration of PP_i may be a relevant factor for the initial deposition of calcium in soft tissue, whereas reduced circulating PP_i levels may play a role during ESRD and hemodialysis.

A number of animal models have further contributed to our understanding of the role of reduced PP_i levels in VC. Mice lacking ABCC6 (*Abcc6*^{-/-}) display a 40% reduction in plasma PP_i levels (13) and present arterial calcification and an enhanced myogenic response (14). *Enpp1*-knockout mice also show depressed levels of circulating PP_i, with concomitant increased calcification in articular cartilage, peri-spinal ligament and aorta (15). A comparable phenotype can be found in the so-called tiptoe-walking (*ttw/ttw*) mouse (16), a naturally occurring mutant with a non-sense mutation in *Enpp1*, and the *asj/asj* mouse, which carries a V246D missense mutation (17). Furthermore, a naturally occurring truncation mutation of the C-terminal cytosolic domain of ANK appears to attenuate PP_i channeling in *ank/ank* mutant mice, which display VC (18). Intriguingly, intraperitoneal administration of PP_i in adenine-induced uremic calcification reduced calcium content by 70% (19), and a recent study has shown that orally administered PP_i also inhibits arterial calcification in *ttw/ttw* and *Abcc6*^{-/-} mice (20), reinforcing the central role of PP_i in the protection against VC.

GLA PROTEINS

Matrix Gla protein (MGP) and Gla-rich protein (GRP), also known as Upper zone of growth plate and Cartilage Matrix Associated protein (UCMA) because of its original discovery in cartilage chondrocytes, are small secreted matrix proteins. They are members of the vitamin K-dependent (VKD) protein family containing, in their mature forms, several γ -carboxylated glutamate (Gla) residues. These VKD post-translational modifications (5 in human MGP, 15 in human GRP), enable MGP and GRP to bind calcium and calcified matrices (21, 22), which can modulate their function (23, 24).

Under normal physiological conditions, both MGP and GRP are synthesized by a variety of cell types including VSMCs and chondrocytes, where they function locally (21, 22). In agreement with this finding is the observation that both carboxylated and uncarboxylated MGP are localized at different levels in mineralized elastic fibers (25–27). Reverse genetics has clearly shown that MGP is a potent physiological inhibitor of calcification (28) since *Mgp*-deficient mice exhibit lethal early spontaneous medial calcification of their arterial trunk. Mutations in the human *MGP* gene cause Keutel syndrome, a rare autosomal recessive disease characterized by abnormal cartilage calcification, short stature, multiple peripheral pulmonary stenoses, brachytelephalangia, and inner ear deafness (29–31). However, in contrast to the mouse, humans rarely develop arterial calcifications (32). This has been suggested to be due to compensatory up-regulation of osteopontin (OPN, see below) in the vessel wall, which may have a protective effect in Keutel syndrome patients (33).

Interestingly, beside mutations, post-translational modifications (i.e., γ -carboxylation and/or phosphorylation for MGP) can further influence the clinical phenotype in patients. For MGP, its dephosphorylated and uncarboxylated form (dp-ucMGP) is a surrogate marker in CKD patients (34) and is associated with increased incidence of cardiovascular diseases (35, 36).

Several studies have also implicated GRP in vascular and soft tissue calcification, osteoarthritis, inflammation and carcinoma (37). Similar to MGP, GRP inhibits phosphate-induced VSMC calcification via SMAD-dependent BMP signaling (38). However, in contrast to *Mgp*-deficient mice, GRP deletion does not induce a clear phenotype (39), which contradicts a putative essential role as a physiological calcification inhibitor *in vivo*.

FETUIN-A

Fetuin-A, also known as alpha2-Heremans-Schmid glycoprotein, is a liver-derived protein, which was initially isolated from fetal calf serum (40) and later also found in human serum (41, 42). Fetuin-A is the strongest circulating proteinaceous calcification inhibitor, being able to bind ~ 100 Ca^{2+} ions per molecule, i.e., ~ 50 x the calcium-binding capacity of an albumin molecule (43). The Fetuin-A cystatin 1-domain contains a functional site, which is able to bind clusters of amorphous calcium phosphate ($\text{Ca}_9(\text{PO}_4)_6$).

When pure Fetuin-A or Fetuin-A-containing serum is exposed to high calcium and phosphate concentrations, mineral-laden Fetuin-A molecules coalesce to form so-called primary calciprotein particles (CPP) (44, 45). These particles contain amorphous calcium phosphate and have a diameter of 50–100 nm. In analogy to lipoprotein particles, which solubilize fatty acids, CPP keep calcium phosphate in solution and prevent it from precipitating (46). Over time, however, primary CPP undergo spontaneous transformation toward secondary CPP, which are larger (>100 nm), of elongate shape and contain crystalline calcium phosphate (HA) (47). CPP can be regarded as the nano-morphological correlate of a *humoral mineral buffering*

system in blood. Interestingly, both primary and secondary CPP have been found in blood samples from patients with CKD (48, 49). Recent work suggests that circulating CPP may predominantly represent primary CPP or even earlier forms (“low molecular weight CPP”) (50).

Consistent with the important calcification-inhibiting properties of Fetuin-A, mice deficient in *fetuin-A* develop heavy and diffuse soft tissue calcifications throughout the whole body (51). In contrast, upon induction of vascular injury, calcifications are primarily found in the intimal plaques, indicating an interaction between systemic and local calcification facilitators (51).

Fetuin-A is a negative acute phase protein, and, accordingly, its blood concentrations are commonly lower in the presence of inflammation (52). Furthermore, circulating Fetuin-A concentrations have been found to be associated with SNPs in the genetic region coding for the fetuin-A protein (53). Low fetuin-A concentrations have also been found in CKD patients and these low levels been associated with poor long-term cardiovascular outcome (54). Recent data indicate that fetuin-A should not be considered as an isolated factor only. In contrast, it should rather be seen in the functional context of the formation of mineral-fetuin-complexes/CPP and thus the performance of the humoral mineral buffering system (55, 56). Specifically, a newly developed blood test measures the transformation (T_{50} -) time point from primary to secondary CPP *in vitro*, and thus the calcification homeostasis in blood beyond single factors. This provides more insight and functional information about the net effect of the humoral factors, which inhibit or promote calcification (57–61). These recent findings have the potential to vastly widen our view and to open new and exciting possibilities for research and clinical care alike.

KLOTHO

Klotho is a single pass transmembrane protein that acts as a co-receptor for fibroblast growth factor-23 (FGF23) (62). Signaling through the Klotho and FGF receptor heterodimer decreases both phosphate reabsorption, via down-regulation of the renal proximal tubule type-II sodium phosphate co-transporters as well as 1,25(OH) $_2$ vitamin D synthesis. Thus, Klotho plays a major role in calcium-phosphate equilibrium. Additionally, a soluble form of Klotho, produced by alternative splicing and cleavage by secretases, can be found in the circulation. This soluble form acts as an endocrine factor exerting its functions by its glycosidase activity (62). Soluble Klotho has been implicated in Wnt signaling inhibition (63) as well as maintenance of endothelial integrity (64).

Genetic deletion of *Klotho* in mice is characterized by a reduced lifespan, osteoporosis, arteriosclerosis, hyperphosphatemia, and ectopic calcification (65), hallmarks of CKD. Indeed, downregulation of Klotho is observed in CKD patients as well as in animal models of CKD (66–68). Interestingly, targeted deletion of *Klotho* in the murine kidney mimics the phenotype of the full body knockout mice (69). Taken together, these observations hence point to the kidney

as the main producer and effector of Klotho in VC. However, transgenic overexpression of Klotho prevents CKD-induced medial calcification despite only modest serum phosphate reduction (67), suggesting that Klotho can also prevent medial calcification through alternative mechanisms other than reducing phosphate. Moreover, as mentioned previously, Klotho can act as an endocrine factor. This is further supported by the stable delivery of soluble Klotho to *Klotho*-deficient mice, which prevents VC despite a modest decrease in serum phosphate and an increase in serum calcium (70). In support of direct effects of Klotho in the vascular wall, treatment of rat VSMCs with recombinant soluble Klotho reduces both phosphate-induced calcification and sodium-dependent phosphate uptake (67). However, it is still debated if Klotho is endogenously produced by VSMCs (71). Therefore, whether these effects on VC are the consequence of circulating or locally produced Klotho remains unknown.

Two mutations in the α KLOTHO gene have been described in humans, which resemble the observed phenotype in mice. First, a homozygous missense mutation leading to an attenuated production of Klotho translated in hyperphosphatemia, hypercalcemia, and both vascular and ectopic calcification in the brain and the Achilles tendon (72). Second, a balanced chromosomal translocation in the proximity of the α KLOTHO gene resulted conversely in increased soluble Klotho levels, leading to hypophosphatemic rickets and skeletal abnormalities (73). In CKD, serum Klotho levels decrease alongside disease progression (74, 75). Moreover, in a small group of patients, urinary Klotho was decreased in stage 1 CKD patients, and the decrease correlated with the severity of the decline of the estimated glomerular filtration rate (67). However, in a prospective observational study of stage 2–4 CKD patients circulating Klotho levels did not predict atherosclerotic or acute heart failure events or death after 2.6 years of follow-up (76). It is worth noting that none of these studies explored the relationship between Klotho and VC. Nonetheless, decreased levels of circulating serum Klotho have been associated with increased arterial stiffness (77). In summary, serum and urinary Klotho could hence serve as predictors of CKD progression but not mortality, whereas their role as biomarkers for VC remains to be established.

OSTEOPONTIN

Osteopontin (OPN) is a member of the SIBLING (small integrin-binding ligand, N-linked glycoprotein) protein family of bone and teeth mineralization regulators (78). It is a multifunctional protein with a clear role in opsonization and chemotaxis via integrin signaling in non-mineralized tissues and is highly expressed by a variety of cell types including macrophages where it acts as a cytokine (79). Besides these roles, OPN was also one of the earliest regulators of mineralization identified in the vessel wall, although its mechanisms of action in regulating VC still remain incompletely resolved.

Independent studies identified OPN as a protein highly expressed in synthetic VSMCs in culture and subsequent studies

in vivo identified OPN invariably at sites of mineralization in both atherosclerotic plaques and the vessel media (80–82). OPN. When expressed at sites of calcification, it forms a bridging protein that links the cellular extracellular matrix with mineral. It may also play a role in the dissolution of calcification by inducing macrophages to express carbonic anhydrase, which acts to acidify the local environment (83). Knockout mouse studies have shown that OPN is not an endogenous inhibitor of VC, as *Opn*-deficient mice do not develop spontaneous calcification, which is consistent with its low expression in contractile VSMCs (80, 82, 84). However, when *Opn*-deficient mice are crossed with *Mgp*-deficient mice or are subjected to a high phosphate diet, then calcification is exacerbated, suggesting that OPN functions as an inducible inhibitor of calcification (84, 85).

OSTEOPROTEGERIN

Osteoprotegerin (OPG) is a protein endogenously expressed by contractile VSMCs. Its role in calcification was first identified when the *Opg*-knockout mouse was found to develop not only osteoporosis, but also VC and this was one of the first pathways linking these two age-associated pathologies (86). OPG acts as a neutralizing decoy receptor for RANKL and TRAIL and it has a major function in regulating osteoclast differentiation via this pathway (87). Mice lacking OPG develop osteoporosis because of increased osteoclast activity—however, the role of OPG in regulating VC has been more problematic to solve. Mouse OPG knockout studies showed that in the vessel wall the RANKL system is activated in the absence of OPG and this is associated with the presence of multinucleate osteoclast-like cells (88). *In vitro* studies have further elaborated the roles of OPG showing it can affect a number of cell types and processes including blocking osteoblastic change in VSMCs via direct and paracrine secretion from endothelial cells and this occurs via multiple signaling pathways (89, 90). OPG also appears to play an important role in the context of diabetes by regulating inflammatory responses (91, 92). Therefore, its actions in protecting the vessel wall from calcification may be context-dependent and clearly further work is required to delineate its multifunctional roles. Interestingly, epidemiological studies have shown that circulating levels of OPG are increased in patients with VC (93, 94). However, the significance of this biomarker remains unclear. It is not known whether its elevation reflects increased OPG to combat calcification, while the cellular origin of the circulating OPG has not been identified.

ENDOGENOUS VASCULAR CALCIFICATION INHIBITOR AS THERAPEUTIC AGENTS

The use and/or stimulation of the endogenous calcification inhibitors described herein constitute a tempting therapeutic strategy. Yet, limited data are available on successful attempts for the reversal of already established calcification.

The delicate balance of pro-calcifying P_i and the major anti-calcifying molecule PP_i (the P_i/PP_i ratio) is regulated

by numerous factors, opening up for intervention at several distinct levels. The dietary uptake of P_i can be hindered by phosphate binders (e.g., sevelamer and aluminum salts) or novel therapies (e.g., tenapanor), which inhibit P_i absorption from the gastrointestinal (GI) tract leading to a decreased P_i/PP_i ratio. These molecules are used in hyperphosphatemia in patients suffering from CKD (95, 96).

Besides decreasing P_i , the P_i/PP_i ratio could potentially be reduced through elevating blood PP_i levels. This can be experimentally achieved via the intraperitoneal or oral administration of PP_i in rodent models, the latter being effective in humans as well (20, 97, 98). Although oral delivery has clinical potential as it halts crystal growth in the PXE or progeria mouse models (97, 98) and prevents calcification even as a gestational treatment in the GACI mouse model (20), it might have several limitations. First, only ~0.1% of dietary PP_i is absorbed (20) as presumably the vast majority of PP_i is degraded in the GI tract by the microbiome. Second, dietary PP_i and P_i intake are variable particularly as PP_i is a frequently used food additive (E450). Additionally, considering the short half-life of PP_i in plasma, several daily doses of PP_i might be necessary, although repetitive administration of PP_i might lead to GI and other side effects (19, 20). Therefore, maintaining sufficient P_i/PP_i plasma levels might be difficult to obtain via oral administration. However, analogs of PP_i , the bisphosphonates, are already in clinical use for the treatment of osteoporosis, despite the rare but severe adverse effects (e.g., jaw necrosis). Moreover, bisphosphonates have been shown to reduce ectopic calcification in patients with GACI (99, 100) or PXE (101), and in animal models of PXE and CKD (17, 97, 102).

Besides direct administration of PP_i or uncleavable derivatives, alternative strategies could target endogenous enzymes involved in the maintenance of PP_i concentration. The serum PP_i level can thus be increased by the recombinant soluble Enpp1 enzyme, as shown in laboratory conditions (103). Finally, a novel promising target is TNAP, which cleaves PP_i into two P_i ions increasing thereby the P_i/PP_i ratio and the propensity for calcification (104, 105). SBI-425 is a recently developed specific TNAP inhibitor (106), with sufficient oral bioavailability and efficacy in mouse models (103–105, 107).

CONSENSUS STATEMENTS

Endogenous calcification inhibitors represent a crucial defense mechanism against VC. Although the function of the endogenous

VC inhibitors has been extensively studied, there are still some important clues lacking to fully elucidate their role in the development of VC. To attain this knowledge, the EuroSoftCalcNet COST Action consortium here emphasizes the following:

1. The deep phenotyping of genetic alterations in calcification inhibitor pathways in both humans and mice represents a powerful tool to better define their clinical and therapeutic relevance and to increase our understanding of the alteration of the pro- and anti-calcifying balance during different stages of VC and the influence of local and systemic inhibitors on the cellular response of VSMCs and/or the physical-chemical properties of mineral deposit.
2. The calcification inhibitors need to be studied from an integrated point of view, including detailed analysis of the molecular pathways and the interactions involved, the relation to altered phosphate (P_i/PP_i) balance and the association with different calcification phenotypes. Altogether, this would help identify an ideal biomarker measure that should reflect calcification homeostasis beyond single factors.
3. The central role of the P_i/PP_i ratio in the regulation of VC makes PP_i an interesting candidate as an effective and low-cost treatment against VC.
4. The exploration of the therapeutic potential of PP_i and other calcification inhibitors should focus on bioavailability and tolerability as well as efforts to avoid bone loss as a consequence of stimulating these pathways within a long-term treatment perspective.

AUTHOR CONTRIBUTIONS

All authors listed significantly participated in the content and writing of the article. MB and HK contributed to the conception of the article, and to the writing, reviewing, and editing of the manuscript.

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REFERENCES

1. Rashdan NA, Rutsch F, Kempf H, Varadi A, Leftheriotis G, MacRae VE. New perspectives on rare connective tissue calcifying diseases. *Curr Opin Pharmacol.* (2016) 28:14–23. doi: 10.1016/j.coph.2016.02.002
2. Fleisch H, Bisaz S. Mechanism of calcification: inhibitory role of pyrophosphate. *Nature* (1962) 195:911. doi: 10.1038/195911a0
3. Lomashvili KA, Narisawa S, Millan JL, O'Neill WC. Vascular calcification is dependent on plasma levels of pyrophosphate. *Kidney Int.* (2014) 85:1351–6. doi: 10.1038/ki.2013.521
4. Ho AM, Johnson MD, Kingsley DM. Role of the mouse ank gene in control of tissue calcification and arthritis. *Science* (2000) 289:265–70. doi: 10.1126/science.289.5477.265
5. Jansen RS, Duijst S, Mahakena S, Sommer D, Szeri F, Varadi A, et al. ABCG6-mediated ATP secretion by the liver is the main source of the mineralization inhibitor inorganic pyrophosphate in the systemic circulation—brief report. *Arterioscler Thromb Vasc Biol.* (2014) 34:1985–9. doi: 10.1161/ATVBAHA.114.304017

6. Hortells L, Sosa C, Millan A, Sorribas V. Critical parameters of the *in vitro* method of vascular smooth muscle cell calcification. *PLoS ONE* (2015) 10:e0141751. doi: 10.1371/journal.pone.0141751
7. O'Neill WC. The fallacy of the calcium-phosphorus product. *Kidney Int.* (2007) 72:792–6. doi: 10.1038/sj.ki.5002412
8. Fleisch H, Russell RG, Straumann F. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. *Nature* (1966) 212:901–3. doi: 10.1038/212901a0
9. Lomashvili KA, Khawandi W, O'Neill WC. Reduced plasma pyrophosphate levels in hemodialysis patients. *J Am Soc Nephrol.* (2005) 16:2495–500. doi: 10.1681/ASN.2004080694
10. Sheen CR, Kuss P, Narisawa S, Yadav MC, Nigro J, Wang W, et al. Pathophysiological role of vascular smooth muscle alkaline phosphatase in medial artery calcification. *J Bone Miner Res.* (2015) 30:824–36. doi: 10.1002/jbmr.2420
11. Lomashvili KA, Garg P, Narisawa S, Millan JL, O'Neill WC. Upregulation of alkaline phosphatase and pyrophosphate hydrolysis: potential mechanism for uremic vascular calcification. *Kidney Int.* (2008) 73:1024–30. doi: 10.1038/ki.2008.26
12. Hortells L, Sosa C, Guillen N, Lucea S, Millan A, Sorribas V. Identifying early pathogenic events during vascular calcification in uremic rats. *Kidney Int.* (2017) 92:1384–94. doi: 10.1016/j.kint.2017.06.019
13. Jansen RS, Kucukosmanoglu A, de Haas M, Saptho S, Otero JA, Hegman IE, et al. ABC6 prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release. *Proc Natl Acad Sci USA.* (2013) 110:20206–11. doi: 10.1073/pnas.1319582110
14. Kauffenstein G, Pizard A, Le Corre Y, Vessieres E, Grimaud L, Toutain B, et al. Disseminated arterial calcification and enhanced myogenic response are associated with *abcc6* deficiency in a mouse model of pseudoxanthoma elasticum. *Arterioscler Thromb Vasc Biol.* (2014) 34:1045–56. doi: 10.1161/ATVBAHA.113.302943
15. Mackenzie NC, Zhu D, Milne EM, van't Hof R, Martin A, Darryl Quarles L, et al. Altered bone development and an increase in FGF-23 expression in *Enpp1*(^{-/-}) mice. *PLoS ONE* (2012) 7:e32177. doi: 10.1371/journal.pone.0032177
16. Okawa A, Nakamura I, Goto S, Moriya H, Nakamura Y, Ikegawa S. Mutation in *Npps* in a mouse model of ossification of the posterior longitudinal ligament of the spine. *Nat Genet.* (1998) 19:271–3. doi: 10.1038/956
17. Li Q, Sundberg JP, Levine MA, Terry SF, Uitto J. The effects of bisphosphonates on ectopic soft tissue mineralization caused by mutations in the *ABCC6* gene. *Cell Cycle* (2015) 14:1082–9. doi: 10.1080/15384101.2015.1007809
18. Harmey D, Hesse L, Narisawa S, Johnson KA, Terkeltaub R, Millan JL. Concerted regulation of inorganic pyrophosphate and osteopontin by *akp2*, *enpp1*, and *ank*: an integrated model of the pathogenesis of mineralization disorders. *Am J Pathol.* (2004) 164:1199–209. doi: 10.1016/S0002-9440(10)63208-7
19. O'Neill WC, Lomashvili KA, Malluche HH, Faugere MC, Riser BL. Treatment with pyrophosphate inhibits uremic vascular calcification. *Kidney Int.* (2011) 79:512–7. doi: 10.1038/ki.2010.461
20. Dedinszki D, Szeri F, Kozak E, Pomozi V, Tokesi N, Mezei TR, et al. Oral administration of pyrophosphate inhibits connective tissue calcification. *EMBO Mol Med.* (2017) 9:1463–70. doi: 10.15252/emmm.201707532
21. Cancela ML, Conceicao N, Laize V. Gla-rich protein, a new player in tissue calcification? *Adv Nutr.* (2012) 3:174–81. doi: 10.3945/an.111.001685
22. Cancela ML, Laize V, Conceicao N. Matrix Gla protein and osteocalcin: from gene duplication to neofunctionalization. *Arch Biochem Biophys.* (2014) 561:56–63. doi: 10.1016/j.abb.2014.07.020
23. Cranenburg EC, Koos R, Schurgers LJ, Magdeleyns EJ, Schoonbrood TH, Landewe RB, et al. Characterization and potential diagnostic value of circulating matrix Gla protein (MGP) species. *Thromb Haemost.* (2010) 104:811–22. doi: 10.1160/TH09-11-0786
24. Cranenburg EC, Vermeer C, Koos R, Boumans ML, Hackeng TM, Bouwman FG, et al. The circulating inactive form of matrix Gla Protein (ucMGP) as a biomarker for cardiovascular calcification. *J Vasc Res.* (2008) 45:427–36. doi: 10.1159/000124863
25. Boraldi F, Garcia-Fernandez M, Paolinelli-Devincenzi C, Annovi G, Schurgers L, Vermeer C, et al. Ectopic calcification in beta-thalassemia patients is associated with increased oxidative stress and lower MGP carboxylation. *Biochim Biophys Acta* (2013) 1832:2077–84. doi: 10.1016/j.bbdis.2013.07.017
26. Gheduzzi D, Boraldi F, Annovi G, DeVincenzi CP, Schurgers LJ, Vermeer C, et al. Matrix Gla protein is involved in elastic fiber calcification in the dermis of pseudoxanthoma elasticum patients. *Lab Invest.* (2007) 87:998–1008. doi: 10.1038/labinvest.3700667
27. Vanakker OM, Martin L, Schurgers LJ, Quagliano D, Costrop L, Vermeer C, et al. Low serum vitamin K in PXE results in defective carboxylation of mineralization inhibitors similar to the GGCC mutations in the PXE-like syndrome. *Lab Invest.* (2010) 90:895–905. doi: 10.1038/labinvest.2010.68
28. Luo G, Ducey P, McKee MD, Pinero GJ, Loyer E, Behringer RR, et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* (1997) 386:78–81. doi: 10.1038/386078a0
29. Khosroshahi HE, Sahin SC, Akyuz Y, Ede H. Long term follow-up of four patients with Keutel syndrome. *Am J Med Genet A* (2014) 164A:2849–56. doi: 10.1002/ajmg.a.36699
30. Marulanda J, Eimar H, McKee MD, Berkvens M, Nelea V, Roman H, et al. Matrix Gla protein deficiency impairs nasal septum growth, causing midface hypoplasia. *J Biol Chem.* (2017) 292:11400–12. doi: 10.1074/jbc.M116.769802
31. Munroe PB, Olgunturk RO, Fryns JP, Van Maldergem L, Ziereisen F, Yuksel B, et al. Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome. *Nat Genet.* (1999) 21:142–4. doi: 10.1038/5102
32. Bjorklund G, Svanberg E, Dadar M, Card DJ, Chirumbolo S, Harrington DJ, et al. The role of matrix Gla protein (MGP) in vascular calcification. *Curr Med Chem.* (2018) doi: 10.2174/0929867325666180716104159. [Epub ahead of print].
33. Kaartinen MT, Murshed M, Karsenty G, McKee MD. Osteopontin upregulation and polymerization by transglutaminase 2 in calcified arteries of Matrix Gla protein-deficient mice. *J Histochem Cytochem.* (2007) 55:375–86. doi: 10.1369/jhc.6A7087.2006
34. Schurgers LJ, Barreto DV, Barreto FC, Liabeuf S, Renard C, Magdeleyns EJ, et al. The circulating inactive form of matrix gla protein is a surrogate marker for vascular calcification in chronic kidney disease: a preliminary report. *Clin J Am Soc Nephrol.* (2010) 5:568–75. doi: 10.2215/CJN.070.81009
35. van den Heuvel EG, van Schoor NM, Lips P, Magdeleyns EJ, Deeg DJ, Vermeer C, et al. Circulating uncarboxylated matrix Gla protein, a marker of vitamin K status, as a risk factor of cardiovascular disease. *Maturitas* (2014) 77:137–41. doi: 10.1016/j.maturitas.2013.10.008
36. Ueland T, Gullestad L, Dahl CP, Aukrust P, Aakhus S, Solberg OG, et al. Undercarboxylated matrix Gla protein is associated with indices of heart failure and mortality in symptomatic aortic stenosis. *J Intern Med.* (2010) 268:483–92. doi: 10.1111/j.1365-2796.2010.02264.x
37. Bordoloi J, Dihingia A, Kalita J, Manna P. Implication of a novel vitamin K dependent protein, GRP/Ucma in the pathophysiological conditions associated with vascular and soft tissue calcification, osteoarthritis, inflammation, and carcinoma. *Int J Biol Macromol.* (2018) 113:309–16. doi: 10.1016/j.ijbiomac.2018.02.150
38. Willems BA, Furmanik M, Caron MMJ, Chatrou MLL, Kusters DHM, Welting TJM, et al. Ucma/GRP inhibits phosphate-induced vascular smooth muscle cell calcification via SMAD-dependent BMP signalling. *Sci Rep.* (2018) 8:4961. doi: 10.1038/s41598-018-23353-y
39. Eitzinger N, Surmann-Schmitt C, Bosl M, Schett G, Engelke K, Hess A, et al. Ucma is not necessary for normal development of the mouse skeleton. *Bone* (2012) 50:670–80. doi: 10.1016/j.bone.2011.11.017
40. Pedersen KO. Fetuin, a new globulin isolated from serum. *Nature* (1944) 154:575. doi: 10.1038/154575a0
41. Heremans JF. *Les Globulines Sérique du Système Gamma*. Brussels: Arscia (1960).
42. Schmid K, Burgi W. Preparation and properties of the human plasma Ba-alpha2-glycoproteins. *Biochim Biophys Acta* (1961) 47:440–53. doi: 10.1016/0006-3002(61)90539-X

43. Heiss A, DuChesne A, Denecke B, Grotzinger J, Yamamoto K, Renne T, et al. Structural basis of calcification inhibition by alpha 2-HS glycoprotein/fetuin-A. Formation of colloidal calciprotein particles. *J Biol Chem.* (2003) 278:13333–41. doi: 10.1074/jbc.M210868200
44. Heiss A, Eckert T, Aretz A, Richtering W, van Dorp W, Schafer C, et al. Hierarchical role of fetuin-A and acidic serum proteins in the formation and stabilization of calcium phosphate particles. *J Biol Chem.* (2008) 283:14815–25. doi: 10.1074/jbc.M709938200
45. Heiss A, Jahnen-Dechent W, Endo H, Schwahn D. Structural dynamics of a colloidal protein-mineral complex bestowing on calcium phosphate a high solubility in biological fluids. *Biointerphases* (2007) 2:16–20. doi: 10.1116/1.2714924
46. Schinke T, Amendt C, Trindl A, Poschke O, Muller-Esterl W, Jahnen-Dechent W. The serum protein alpha2-HS glycoprotein/fetuin inhibits apatite formation *in vitro* and in mineralizing calvaria cells. A possible role in mineralization and calcium homeostasis. *J Biol Chem.* (1996) 271:20789–96.
47. Wald J, Wiese S, Eckert T, Jahnen-Dechent W, Richtering W, Heiss A. Formation and stability kinetics of calcium phosphate–fetuin-A colloidal particles probed by time-resolved dynamic light scattering. *Soft Matter* (2011) 7:2869–74. doi: 10.1039/c0sm01191f
48. Holt SG, Smith ER. Fetuin-A-containing calciprotein particles in mineral trafficking and vascular disease. *Nephrol Dial Transplant.* (2016) 31:1583–7. doi: 10.1093/ndt/gfw048
49. Smith ER, Hewitson TD, Cai MMX, Aghagolzadeh P, Bachtler M, Pasch A, et al. A novel fluorescent probe-based flow cytometric assay for mineral-containing nanoparticles in serum. *Sci Rep.* (2017) 7:5686. doi: 10.1038/s41598-017-05474-y
50. Miura Y, Iwazu Y, Shiizaki K, Akimoto T, Kotani K, Kurabayashi M, et al. Identification and quantification of plasma calciprotein particles with distinct physical properties in patients with chronic kidney disease. *Sci Rep.* (2018) 8:1256. doi: 10.1038/s41598-018-19677-4
51. Schafer C, Heiss A, Schwarz A, Westenfeld R, Ketteler M, Floege J, et al. The serum protein alpha 2-Heremans-Schmid glycoprotein/fetuin-A is a systemically acting inhibitor of ectopic calcification. *J Clin Invest.* (2003) 112:357–66. doi: 10.1172/JCI17202
52. Gangneux C, Daveau M, Hiron M, Derambure C, Papaconstantinou J, Salier JP. The inflammation-induced down-regulation of plasma Fetuin-A (alpha2HS-Glycoprotein) in liver results from the loss of interaction between long C/EBP isoforms at two neighbouring binding sites. *Nucleic Acids Res.* (2003) 31:5957–70. doi: 10.1093/nar/gkg788
53. Jensen MK, Jensen RA, Mukamal KJ, Guo X, Yao J, Sun Q, et al. Detection of genetic loci associated with plasma fetuin-A: a meta-analysis of genome-wide association studies from the CHARGE Consortium. *Hum Mol Genet.* (2017) 26:2156–63. doi: 10.1093/hmg/ddx091
54. Ketteler M, Bongartz P, Westenfeld R, Wildberger JE, Mahnken AH, Bohm R, et al. Association of low fetuin-A (AHS) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study. *Lancet* (2003) 361:827–33. doi: 10.1016/S0140-6736(03)12710-9
55. Hamano T, Matsui I, Mikami S, Tomida K, Fujii N, Imai E, et al. Fetuin-mineral complex reflects extraosseous calcification stress in CKD. *J Am Soc Nephrol.* (2010) 21:1998–2007. doi: 10.1681/ASN.2009090944
56. Pasch A. Novel assessments of systemic calcification propensity. *Curr Opin Nephrol Hypertens.* (2016) 25:278–84. doi: 10.1097/MNH.0000000000000237
57. Dahle DO, Asberg A, Hartmann A, Holdaas H, Bachtler M, Jenssen TG, et al. Serum calcification propensity is a strong and independent determinant of cardiac and all-cause mortality in kidney transplant recipients. *Am J Transplant.* (2016) 16:204–12. doi: 10.1111/ajt.13443
58. Keyzer CA, de Borst MH, van den Berg E, Jahnen-Dechent W, Arampatzis S, Farese S, et al. Calcification propensity and survival among renal transplant recipients. *J Am Soc Nephrol.* (2016) 27:239–48. doi: 10.1681/ASN.2014070670
59. Pasch A, Block GA, Bachtler M, Smith ER, Jahnen-Dechent W, Arampatzis S, et al. Blood calcification propensity, cardiovascular events, and survival in patients receiving hemodialysis in the evolve trial. *Clin J Am Soc Nephrol.* (2017) 12:315–22. doi: 10.2215/CJN.04720416
60. Pasch A, Farese S, Graber S, Wald J, Richtering W, Floege J, et al. Nanoparticle-based test measures overall propensity for calcification in serum. *J Am Soc Nephrol.* (2012) 23:1744–52. doi: 10.1681/ASN.2012030240
61. Smith ER, Ford ML, Tomlinson LA, Bodenham E, McMahon LP, Farese S, et al. Serum calcification propensity predicts all-cause mortality in predialysis CKD. *J Am Soc Nephrol.* (2014) 25:339–48. doi: 10.1681/ASN.2013060635
62. Mencke R, Hillebrands JL, consortium N. The role of the anti-ageing protein Klotho in vascular physiology and pathophysiology. *Ageing Res Rev.* (2017) 35:124–46. doi: 10.1016/j.arr.2016.09.001
63. Satoh M, Nagasu H, Morita Y, Yamaguchi TP, Kanwar YS, Kashihara N. Klotho protects against mouse renal fibrosis by inhibiting Wnt signaling. *Am J Physiol Renal Physiol.* (2012) 303:F1641–51. doi: 10.1152/ajprenal.00460.2012
64. Kusaba T, Okigaki M, Matui A, Murakami M, Ishikawa K, Kimura T, et al. Klotho is associated with VEGF receptor-2 and the transient receptor potential canonical-1 Ca²⁺ channel to maintain endothelial integrity. *Proc Natl Acad Sci USA.* (2010) 107:19308–13. doi: 10.1073/pnas.1008544107
65. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, et al. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* (1997) 390:45–51. doi: 10.1038/36285
66. Aizawa H, Saito Y, Nakamura T, Inoue M, Imanari T, Ohyama Y, et al. Downregulation of the Klotho gene in the kidney under sustained circulatory stress in rats. *Biochem Biophys Res Commun.* (1998) 249:865–71. doi: 10.1006/bbrc.1998.9246
67. Hu MC, Shi M, Zhang J, Quinones H, Griffith C, Kuro-o M, et al. Klotho deficiency causes vascular calcification in chronic kidney disease. *J Am Soc Nephrol.* (2011) 22:124–36. doi: 10.1681/ASN.2009121311
68. Koh N, Fujimori T, Nishiguchi S, Tamori A, Shiomi S, Nakatani T, et al. Severely reduced production of klotho in human chronic renal failure kidney. *Biochem Biophys Res Commun.* (2001) 280:1015–20. doi: 10.1006/bbrc.2000.4226
69. Lindberg K, Amin R, Moe OW, Hu MC, Erben RG, Ostman Wernerson A, et al. The kidney is the principal organ mediating klotho effects. *J Am Soc Nephrol.* (2014) 25:2169–75. doi: 10.1681/ASN.2013111209
70. Hum JM, O'Bryan LM, Tatiparthi AK, Cass TA, Clinkenbeard EL, Cramer MS, et al. Chronic hyperphosphatemia and vascular calcification are reduced by stable delivery of soluble klotho. *J Am Soc Nephrol.* (2017) 28:1162–74. doi: 10.1681/ASN.2015111266
71. Yamada S, Giachelli CM. Vascular calcification in CKD-MBD: roles for phosphate, FGF23, and klotho. *Bone* (2017) 100:87–93. doi: 10.1016/j.bone.2016.11.012
72. Ichikawa S, Imel EA, Kreiter ML, Yu X, Mackenzie DS, Sorenson AH, et al. A homozygous missense mutation in human KLOTHO causes severe tumoral calcinosis. *J Clin Invest.* (2007) 117:2684–91. doi: 10.1172/JCI31330
73. Brownstein CA, Adler F, Nelson-Williams C, Iijima J, Li P, Imura A, et al. A translocation causing increased alpha-klotho level results in hypophosphatemic rickets and hyperparathyroidism. *Proc Natl Acad Sci USA.* (2008) 105:3455–60. doi: 10.1073/pnas.0712361105
74. Kim HR, Nam BY, Kim DW, Kang MW, Han JH, Lee MJ, et al. Circulating alpha-klotho levels in CKD and relationship to progression. *Am J Kidney Dis.* (2013) 61:899–909. doi: 10.1053/j.ajkd.2013.01.024
75. Pavik I, Jaeger P, Ebner L, Wagner CA, Petzold K, Spichtig D, et al. Secreted Klotho and FGF23 in chronic kidney disease Stage 1 to 5: a sequence suggested from a cross-sectional study. *Nephrol Dial Transplant.* (2013) 28:352–9. doi: 10.1093/ndt/gfs460
76. Seiler S, Rogacev KS, Roth HJ, Shafein P, Emrich I, Neuhaus S, et al. Associations of FGF-23 and sKlotho with cardiovascular outcomes among patients with CKD stages 2–4. *Clin J Am Soc Nephrol.* (2014) 9:1049–58. doi: 10.2215/CJN.07870713
77. Kitagawa M, Sugiyama H, Morinaga H, Inoue T, Takiue K, Ogawa A, et al. A decreased level of serum soluble Klotho is an independent biomarker associated with arterial stiffness in patients with chronic kidney disease. *PLoS ONE* (2013) 8:e56695. doi: 10.1371/journal.pone.0056695
78. Staines KA, MacRae VE, Farquharson C. The importance of the SIBLING family of proteins on skeletal mineralisation and bone remodelling. *J Endocrinol.* (2012) 214:241–55. doi: 10.1530/JOE-12-0143

79. Scatena M, Liaw L, Giachelli CM. Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. *Arterioscler Thromb Vasc Biol.* (2007) 27:2302–9. doi: 10.1161/ATVBAHA.107.144824
80. Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. *J Clin Invest.* (1993) 92:1686–96. doi: 10.1172/JCI116755
81. Shanahan CM, Cary NR, Metcalfe JC, Weissberg PL. High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. *J Clin Invest.* (1994) 93:2393–402. doi: 10.1172/JCI117246
82. Shanahan CM, Weissberg PL, Metcalfe JC. Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. *Circ Res.* (1993) 73:193–204. doi: 10.1161/01.RES.73.1.193
83. Steitz SA, Speer MY, McKee MD, Liaw L, Almeida M, Yang H, et al. Osteopontin inhibits mineral deposition and promotes regression of ectopic calcification. *Am J Pathol.* (2002) 161:2035–46. doi: 10.1016/S0002-9440(10)64482-3
84. Speer MY, McKee MD, Gulberg RE, Liaw L, Yang HY, Tung E, et al. Inactivation of the osteopontin gene enhances vascular calcification of matrix Gla protein-deficient mice: evidence for osteopontin as an inducible inhibitor of vascular calcification *in vivo*. *J Exp Med.* (2002) 196:1047–55. doi: 10.1084/jem.20020911
85. Paloiian NJ, Leaf EM, Giachelli CM. Osteopontin protects against high phosphate-induced nephrocalcinosis and vascular calcification. *Kidney Int.* (2016) 89:1027–36. doi: 10.1016/j.kint.2015.12.046
86. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, et al. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev.* (1998) 12:1260–8. doi: 10.1101/gad.12.9.1260
87. Collin-Osdoby P. Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. *Circ Res.* (2004) 95:1046–57. doi: 10.1161/01.RES.0000149165.99974.12
88. Min H, Morony S, Sarosi I, Dunstan CR, Capparelli C, Scully S, et al. Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis. *J Exp Med.* (2000) 192:463–74. doi: 10.1084/jem.192.4.463
89. Callegari A, Coons ML, Ricks JL, Rosenfeld ME, Scatena M. Increased calcification in osteoprotegerin-deficient smooth muscle cells: dependence on receptor activator of NF- κ B ligand and interleukin 6. *J Vasc Res.* (2014) 51:118–31. doi: 10.1159/000358920
90. Di Bartolo BA, Kavurma MM. Regulation and function of Rankl in arterial calcification. *Curr Pharm Des.* (2014) 20:5853–61. doi: 10.2174/1381612820666140212205455
91. Ndip A, Wilkinson FL, Jude EB, Boulton AJ, Alexander MY. RANKL-OPG and RAGE modulation in vascular calcification and diabetes: novel targets for therapy. *Diabetologia* (2014) 57:2251–60. doi: 10.1007/s00125-014-3348-z
92. Ndip A, Williams A, Jude EB, Serracino-Inglott F, Richardson S, Smyth JV, et al. The RANKL/RANK/OPG signaling pathway mediates medial arterial calcification in diabetic Charcot neuroarthropathy. *Diabetes* (2011) 60:2187–96. doi: 10.2337/db10-1220
93. Siasos G, Oikonomou E, Maniatis K, Georgiopoulos G, Kokkou E, Tsigkou V, et al. Prognostic significance of arterial stiffness and osteoprotegerin in patients with stable coronary artery disease. *Eur J Clin Invest.* (2018) 48:e12890. doi: 10.1111/eci.12890
94. Venuraju SM, Yerramasu A, Corder R, Lahiri A. Osteoprotegerin as a predictor of coronary artery disease and cardiovascular mortality and morbidity. *J Am Coll Cardiol.* (2010) 55:2049–61. doi: 10.1016/j.jacc.2010.03.013
95. King AJ, Siegel M, He Y, Nie B, Wang J, Koo-McCoy S, et al. Inhibition of sodium/hydrogen exchanger 3 in the gastrointestinal tract by tenapanor reduces paracellular phosphate permeability. *Sci Transl Med.* (2018) 10:eam6474. doi: 10.1126/scitranslmed.aam6474
96. Vo TM, Disthabanchong S. Are there ways to attenuate arterial calcification and improve cardiovascular outcomes in chronic kidney disease? *World J Cardiol.* (2014) 6:216–26. doi: 10.4330/wjc.v6.i5.216
97. Pomozi V, Brampton C, van de Wetering K, Zoll J, Calio B, Pham K, et al. Pyrophosphate supplementation prevents chronic and acute calcification in ABCC6-deficient mice. *Am J Pathol.* (2017) 187:1258–72. doi: 10.1016/j.ajpath.2017.02.009
98. Villa-Bellocosta R, Rivera-Torres J, Osorio FG, Acin-Perez R, Enriquez JA, Lopez-Otin C, et al. Defective extracellular pyrophosphate metabolism promotes vascular calcification in a mouse model of Hutchinson-Gilford progeria syndrome that is ameliorated on pyrophosphate treatment. *Circulation* (2013) 127:2442–51. doi: 10.1161/CIRCULATIONAHA.112.000571
99. Rutsch F, Boyer P, Nitschke Y, Ruf N, Lorenz-Depierreux B, Wittkamp T, et al. Hypophosphatemia, hyperphosphaturia, and bisphosphonate treatment are associated with survival beyond infancy in generalized arterial calcification of infancy. *Circ Cardiovasc Genet.* (2008) 1:133–40. doi: 10.1161/CIRCGENETICS.108.797704
100. Yapicioglu-Yildizdas H, Ozbarlas N, Erdem S, Yilmaz MB, Ozlu F, Buyukurt S, et al. Two newborn babies with generalized arterial calcification of infancy, two new mutations. *Turk J Pediatr.* (2016) 58:419–23. doi: 10.24953/turkjped.2016.04.013
101. Kranenburg G, de Jong PA, Bartstra JW, Lagerweij SJ, Lam MG, Ossewaarde-van Norel J, et al. Etidronate for prevention of ectopic mineralization in patients with pseudoxanthoma elasticum. *J Am Coll Cardiol.* (2018) 71:1117–26. doi: 10.1016/j.jacc.2017.12.062
102. Lomashvili KA, Monier-Faugere MC, Wang X, Malluche HH, O'Neill WC. Effect of bisphosphonates on vascular calcification and bone metabolism in experimental renal failure. *Kidney Int.* (2009) 75:617–25. doi: 10.1038/ki.2008.646
103. Albright RA, Stabach P, Cao W, Kavanagh D, Mullen I, Braddock AA, et al. ENPP1-Fc prevents mortality and vascular calcifications in rodent model of generalized arterial calcification of infancy. *Nat Commun.* (2015) 6:10006. doi: 10.1038/ncomms10006
104. Varadi A, Fulop K, Aranyi T, Szeri F. Tissue-nonspecific alkaline phosphatase: a promising target for pseudoxanthoma elasticum therapy. *Ann Transl Med.* (2017) 5:489. doi: 10.21037/atm.2017.10.01
105. Ziegler SG, Ferreira CR, MacFarlane EG, Riddle RC, Tomlinson RE, Chew EY, et al. Ectopic calcification in pseudoxanthoma elasticum responds to inhibition of tissue-nonspecific alkaline phosphatase. *Sci Transl Med.* (2017) 9:eal1669. doi: 10.1126/scitranslmed.aal1669
106. Pinkerton AB, Sergienko E, Bravo Y, Dahl R, Ma CT, Sun Q, et al. Discovery of 5-((5-chloro-2-methoxyphenyl)sulfonamido)nicotinamide (SBI-425), a potent and orally bioavailable tissue-nonspecific alkaline phosphatase (TNAP) inhibitor. *Bioorg Med Chem Lett.* (2018) 28:31–4. doi: 10.1016/j.bmcl.2017.11.024
107. Li Q, Huang J, Pinkerton AB, Millan JL, van Zelst BD, Levine MA, et al. Inhibition of tissue-nonspecific alkaline phosphatase attenuates ectopic mineralization in the Abcc6(-/-) mouse model of PXE but not in the Enpp1 mutant mouse models of GACI. *J Invest Dermatol.* (2018). doi: 10.1016/j.jid.2018.07.030. [Epub ahead of print].

Conflict of Interest Statement: AP is an inventor of the calcification propensity (T50) test, and an employee and stockholder in Calciscon AG (Nidau, Switzerland) which commercializes this blood test.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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