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REVIEW

TNAP as a therapeutic target for cardiovascular calcification: a discussion of its pleiotropic functions in the body

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Abstract

Cardiovascular calcification (CVC) is associated with increased morbidity and mortality. It develops in several diseases and locations, such as in the tunica intima in atherosclerosis plaques, in the tunica media in type 2 diabetes and chronic kidney disease, and in aortic valves. In spite of the wide occurrence of CVC and its detrimental effects on cardiovascular diseases (CVD), no treatment is yet available. Most of CVC involve mechanisms similar to those occurring during endochondral and/or intramembranous ossification. Logically, since tissue-nonspecific alkaline phosphatase (TNAP) is the key-enzyme responsible for skeletal/dental mineralization, it is a promising target to limit CVC. Tools have recently been developed to inhibit its activity and preclinical studies conducted in animal models of vascular calcification already provided promising results. Nevertheless, as its name indicates, TNAP is ubiquitous and recent data indicate that it dephosphorylates different substrates *in vivo* to participate in other important physiological functions besides mineralization. For instance, TNAP is involved in the metabolism of pyridoxal phosphate and the production of neurotransmitters. TNAP has also been described as an anti-inflammatory enzyme able to dephosphorylate adenosine nucleotides and lipopolysaccharide. A better understanding of the full spectrum of TNAP's functions is needed to better characterize the effects of TNAP inhibition in diseases associated with CVC. In this review, after a brief description of the different types of CVC, we describe the newly uncovered additional functions of TNAP and discuss the expected consequences of its systemic inhibition *in vivo*.

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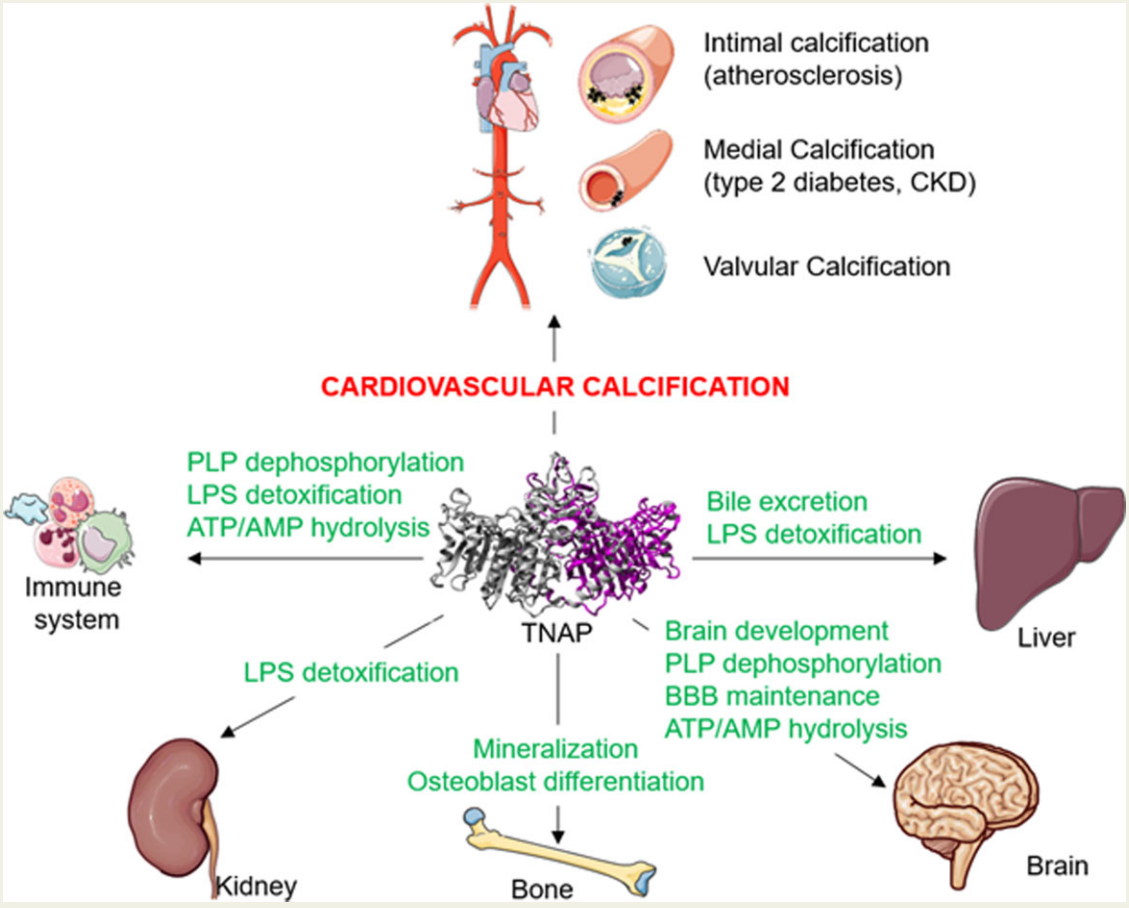
Graphical Abstract



Keywords Tissue non-specific alkaline phosphatase • Cardiovascular calcification • Therapeutic target • Inhibition • Inflammation

1. Introduction

Cardiovascular calcification (CVC) is an independent risk factor for cardiovascular morbidity and mortality. CVC is a common process across ethnicities in the general population and increases with age.[1] It is essential to distinguish between three main types of CVC. Two types of CVC with common risk factors and pathogenesis may occur in most ageing adults: intimal atherosclerotic plaque calcification and aortic valve calcification.[1,2] The third type of CVC affects the tunica media, in individuals with chronic kidney disease (CKD) or with type 2 diabetes. Media calcification indeed causes arterial stiffness, increased pulse pressure, and left ventricular hypertrophy.[3] In CKD, calcification is very likely responsible for the high cardiovascular mortality,[4] patients with end-stage renal disease have a 30 times higher risk of death compared to the general population.[5] In type 2 diabetes, media calcification first appears in the feet and develops proximally, worsening the risk of lower limb amputation.[6,7] Consequences of atherosclerotic intimal plaque calcification are less clear,[3] although it is virtually present in every adult.[1] Since the calcium score is positively associated with cardiovascular mortality risk,[8] it was commonly thought that advanced plaques with heavy calcifications are unstable. This view has been challenged, particularly by the fact that statins increase the calcium score,[9] and a consensus has emerged that strongly calcified plaques could be in fact more stable.[10] More recently, microcalcifications have been evidenced in early plaques,[11–13] where they may destabilize plaques by exerting pro-inflammatory effects and generating mechanical stress within the fibrous cap.[14–17] Finally, although aortic valve calcification share many features with intimal calcification, it undoubtedly has a harmful biomechanical and clinical impact.[2] Until recently, lack of therapeutic targets and/or of pharmacological inhibitors have prevented CVC inhibition. The identification of tissue-nonspecific alkaline phosphatase (TNAP) as a central player in physiology as well as several types of CVC, and the development of approaches to inhibit TNAP have opened new possibilities.

2. TNAP, a therapeutic target to block cardiovascular calcification

All types of CVC develop at least in part through mechanisms mimicking endochondral (through a cartilage template) and/or intramembranous

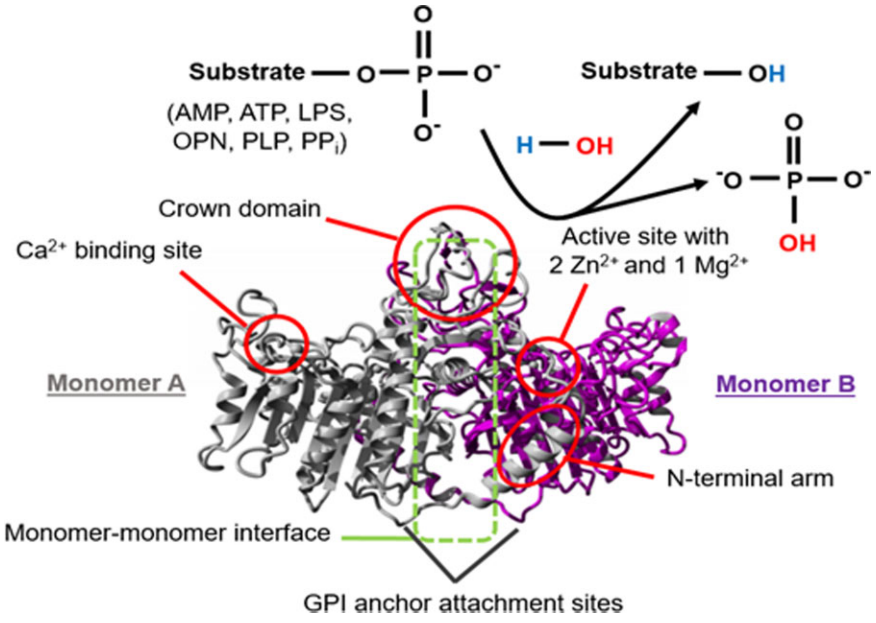


Figure 1 Structural features of TNAP. AMP, adenosine monophosphate; ATP, adenosine triphosphate; GPI, glycosyl phosphatidyl inositol; LPS, lipopolysaccharide; OPN, osteopontin; PP_i, inorganic pyrophosphate; PLP, pyridoxal phosphate; TNAP, tissue-nonspecific alkaline phosphatase. Modified from Ref.[47]

(without a cartilage template) ossification. Both ossification types have been evidenced in the tunica media of arteries in both humans and rodents with CKD[18,19] and diabetes,[20,21] in human aortic valves,[22,23] and in mouse and human atherosclerotic plaques.[10,24–26] In humans, mineralization in endochondral and intramembranous ossification relies on TNAP activity. Indeed, the most severe genetic TNAP deficiencies (hypophosphatasia, HPP) lead to the perinatal death of foetuses devoid of minerals in their whole skeleton.[27] Increasing evidence indicates that TNAP plays a role in most types of CVC. TNAP appears as a central player in valve calcification, based on *ex vivo* experimental models.[28,29] In *Apolipoprotein (Apo)E*-deficient mice, TNAP activity precedes calcification in atherosclerotic plaques.[30] In rats, nephrectomized to mimic CKD, TNAP was also detected slightly before vascular calcification (VC) initiation.[31] Importantly, local TNAP activation in arteries may be sufficient to trigger VC since its overexpression in vascular smooth muscle cells (VSMCs) or in endothelial cells is sufficient to induce massive and lethal arterial calcification in mice.[32–34] Besides its local action on CVC, TNAP may accelerate CVC from the circulation. Indeed, circulating TNAP activity is an independent predictor of mortality in the general population and in individuals with metabolic syndrome (MetS), who have increased serum TNAP levels.[35–40] Serum TNAP activity is associated with coronary artery calcium score.[41] Moreover, circulating TNAP is associated with increased risk of cardiovascular death in patients with CKD.[42–44] In the light of these results, TNAP has emerged as a promising therapeutic target to block CVC, but until recently this target lacked tools to be efficiently inhibited *in vivo*. Two approaches, based on inhibition of expression or activity, have recently been developed and several preclinical studies have been conducted with encouraging results. These advances open the way to clinical studies aiming to prevent or treat CVC. On the other hand, increasing evidence indicates that TNAP is not only the key player in mineralization but also a ubiquitous enzyme that exerts various and necessary functions in different organs. It is therefore mandatory to thoroughly analyse these functions.

3. TNAP, a ubiquitous enzyme with broad substrate specificity

3.1 TNAP is a membrane-anchored homodimeric enzyme

Humans have in their genome four different loci expressing four distinct alkaline phosphatase (AP) isoenzyme.[45] Three of these genes, *ALPI, ALPP*, and *ALPPL2*, are tissue-specific in their expression pattern restricted to the intestine, placenta, and germ cells, respectively. The fourth gene, *ALPL*, is designated as TNAP, since it is expressed in bone, liver, kidney, brain, among others. The 3D structure of mammalian TNAP has not been elucidated so far and can only be assessed based on its 57% identity and 74% homology to the human ALPP and *Escherichia coli* AP for which crystal structures are known. Both function physiologically as homodimers[46] (*Figure 1*). Structural features of APs involve the monomer–monomer interface the active site with divalent cation-binding sites, the crown domain, the N-terminal arm, a glycosylphosphatidylinositol (GPI) anchor, and N-linked glycosylation sites. The monomer–monomer interface exhibits a strong hydrophobic character demonstrating that <30% of the amino acid residues are involved in two hydrogen-bonding interactions.[48] This feature is crucial for stability and enzymatic function. The active site contains three metal-binding sites surrounding the catalytic serine residue that are essential for TNAP enzymatic activity.[49] The metal-binding site M1 and M2 are occupied by Zn^{2+} and M3 is occupied by Mg^{2+}. An additional metal-binding site—M4—is suggested for the binding of Ca^{2+} that does not alter TNAP catalytic activity.[50] The N-terminal α-helix domain together with the crown domain, which is a flexible

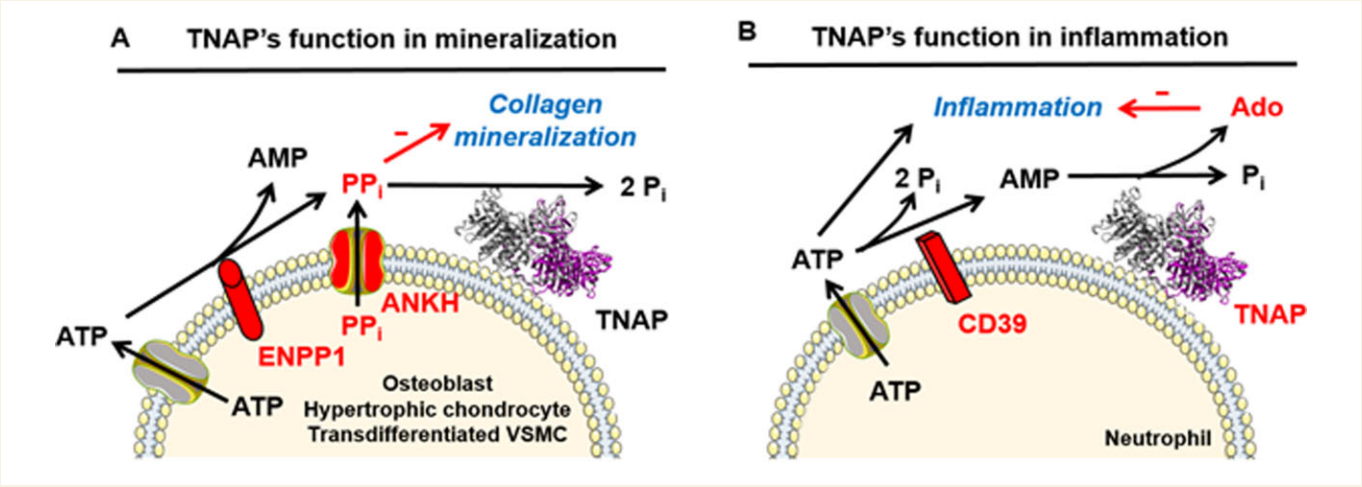


Figure 2 TNAP's involvement in PP_i dephosphorylation leading to the mineralization of collagen-rich tissues (*A*) and in AMP dephosphorylation leading to resolution of inflammation (*B*). Ado, adenosine; AMP, adenosine monophosphate; ANKH, progressive ankylosis protein homolog; ATP, adenosine triphosphate; ENPP1, ectonucleotidase pyrophosphatase phosphodiesterase 1; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; TNAP, tissue-nonspecific alkaline phosphatase; VSMC, vascular smooth muscle cell.

loop, stabilize the dimeric structure, and determine allosteric properties (*Figure 1*). In all mammalian AP homodimers, the N-terminal α-helix (residues 9–25) of one monomeric subunit encircle the contralateral subunit reaching towards its active site. These N-terminal α-helical folding is crucial for the structural stability of the second monomer in TNAP and is largely responsible for the allosteric behaviour of mammalian APs.[51] Mutation of Arg374 (R374A) and deletion of 5 and 9N-terminal amino acids cause structural and functional disruption.[52] In addition, TNAP contains five putative N-linked glycosylation sites (N123, N213, N254, N286, and N413), which are important for catalytic activity.[53,54] Another crucial structural element is the GPI anchor enabling the binding of mammalian APs to the surface of the plasma membrane. Plasma membrane-localized phospholipases can enzymatically cleave the anchor, releasing TNAP in the circulation. Both anchored and free APs are active.

3.2 TNAP has weak substrate specificity *in vitro*

APs catalyse the hydrolysis of a broad range of phosphate monoesters to form inorganic phosphate (P_i) and alcohol (or phenol) (*Figure 1*). For example, purified TNAP is able to hydrolyze adenosine triphosphate (ATP), ADP, AMP, inorganic pyrophosphate (PP_i), glucose-6-phosphate, β-glycerophosphate, or *p*-nitrophenyl-phosphate *in vitro*.[55] The reaction mechanisms have been investigated in AP from *E. coli*, which has an active site with strong homology to mammalian APs, with differences at only three positions,[56] suggesting similar reaction mechanisms.[57] The reaction generates a serine-phosphate intermediate to produce P_i and an alcohol or phenol. P_i then fills the entire volume of the active site pocket and acts as a strong competitive inhibitor of the enzyme, in a negative feedback loop.[58] The two Zn^{2+} ions play a crucial role in the reaction by coordinating the hydroxyl group of serine within the active site, preparing it for nucleophilic attack of the phosphate monoester substrate and binding the substrate/product.[59] Additionally, an arginine residue is important in the initial binding of the substrate and in the release of P_i.[56] Finally, the presence of the Mg^{2+} ion is required for deprotonation of the serine residue.[49] The presence of the two Zn^{2+} ions but not that of the Mg^{2+} ion in the nucleotide pyrophosphatase phosphodiesterase (NPP) enzymes suggests that the Mg^{2+} ion could play a fundamental role in the discrimination between the binding and hydrolysis of phosphate monoester and diester.[60,61] Indeed, in addition to their phosphomonoesterase activity, APs may have a weaker but significant phosphodiesterase activity,[60,61] including TNAP that is able to hydrolyze phosphodiesters *in vitro*.[62] Whether it is present *in vivo* remains speculative, since all *in vivo* demonstrated TNAP substrates are monoesters.

3.3 TNAP has a small but growing number of pathophysiological substrates

The mineralizing function of TNAP relies on inorganic pyrophosphate (PP_i) hydrolysis rather than on P_i generation. In 1962, Fleisch and Bisaz[63] proposed that PP_i in the plasma reaches tissues to prevent collagen mineralization, which is triggered by the local hydrolysis of PP_i through TNAP. This hypothesis was confirmed by Russell *et al.* who measured increased PP_i excretion in patients with HPP.[64] In the 2000s, the group of JL Millan crossed TNAP-deficient mice (*Alpl*-deficient mice) with mice deficient in genes involved in the generation of extracellular PP_i. One of these genes is *Ank*, the ortholog of the human *ANKH* gene, encoding an exporter of PP_i in the extracellular compartment, whose mutations result in craniometaphyseal dysplasia.[65] The other one is *Enpp1* (ectonucleotidase pyrophosphatase phosphodiesterase 1) encoding an enzyme that generates PP_i from extracellular ATP, whose deficiency in humans is associated with generalized arterial calcification of infancy.[66] Crossing *Alpl*-deficient mice with *Ank* mice or *Enpp1*-deficient mice not only reduced the ectopic calcification phenotype of PP_i-deficient mice but also prevented the mineralization defects of *Alpl*-deficient mice.[67,68] Finally, Murshed *et al.*[69] demonstrated that TNAP induces mineralization only in tissues containing a fibrillar collagen, which serves as a template for crystal deposition, explaining why only bones and teeth are physiologically mineralized (*Figure 2A*). However, TNAP is not involved in crystal nucleation but in crystal growth. Bones from HPP patients and TNAP-deficient mice still contain small crystals in matrix vesicles [the so-called extracellular vesicles (EVs) released by mineralizing bone cells[70]] that

have failed to growth and multiply within collagen fibrils.[47] This paradigm is strengthened by the fact that PP_i ions may not act by inhibiting crystal nucleation, but by binding to the hydration shell of apatite crystals, and hindering P_i binding.[71] The molecular mechanisms involved in crystal nucleation remain obscure. Intravesicular dephosphorylation of phosphocholine by phospho1 and calcium ion import by annexins have been proposed as mechanisms to increase ion concentration and induce crystal nucleation.[70] Alternatively, poly-ADP ribose released in EVs by senescent bone cells and VSMCs has been proposed to participate to crystal nucleation in bone and the vasculature.[72,73] Recent evidence also suggest that phosphatidylserine-mediated nucleation could be the predominant mechanism to produce the very first mineral nuclei during MV-mediated bone/cartilage mineralization.[74]

Another *in vivo* substrate of TNAP is pyridoxal phosphate (PLP), the major circulating form of vitamin B_6. PLP dephosphorylation in the blood is necessary to allow pyridoxal uptake by cells, where it is phosphorylated again to participate as a cofactor in many reactions.[47] In particular, PLP is required for the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the central nervous system, explaining in part why severe HPP is associated with epileptic seizures.[47] Moreover, *Alpl*-deficient mice have increased levels of cystathionine in their brain, a molecule of the transsulfuration pathway that is controlled by PLP-dependent enzymes.[75] Since the transsulfuration pathway is responsible for the synthesis of important anti-oxidant molecules, such as glutathione and hydrogen sulphide, TNAP may indirectly participate in anti-oxidant capacity.

Increasing evidence suggests that adenosine nucleotides are *in vivo* TNAP substrates. In addition to its intracellular energetic role, ATP is released by all cells, during necrosis, apoptosis, or in response to inflammatory or mechanical stresses.[76] Extracellular ATP binds to P2 purinergic receptors in neighbouring cells and activates multiple intracellular pathways. These effects are restrained by CD39 that dephosphorylates ATP into AMP removing two P_i, and CD73 that removes the third P_i to generate adenosine.[76] To date, convincing data indicate that TNAP participates at least in AMP dephosphorylation (*Figure 2B*). *Alpl*-deficient mice have less adenosine in their brain than control mice,[75] suggesting that other tissues also exhibit lack of adenosine. In the blood, TNAP might be a significant contributor to AMP hydrolysis.[77] In addition, human neutrophils, which are the most abundant leucocytes in the blood, have very low expression of CD73 and high levels of TNAP,[77,78] which is responsible for AMP dephosphorylation[77] (*Figure 2B*). A contribution of TNAP to the dephosphorylation of high AMP levels has also been reported in bronchial epithelial cells.[79,80] Given the well-known anti-inflammatory role of adenosine, these data identify TNAP as a possible anti-inflammatory ectonucleotidase.[78] The contribution of TNAP to AMP hydrolysis increases with increasing AMP concentrations, suggesting that TNAP is particularly involved in intense pro-inflammatory responses. TNAP's function in AMP dephosphorylation may significantly interfere with CVC. Patients with arterial calcification due to deficiency of CD73 (ACDC) present with extensive medial calcification.[81] The group of St Hilaire demonstrated that decreased adenosine production in absence of CD73 stimulates the expression of TNAP to compensate for deficient AMP dephosphorylation, leading to parallel PP_i hydrolysis and calcification.[82] Importantly, stimulated TNAP expression due to CD73 deficiency relies on the transcription factor FOXO1 in cells from ACDC patients, and calcified femoropopliteal arteries form non-ACDC patients exhibit increased FOXO1 levels as compared from non-calcified arteries.[83] Considered together with the fact that the pro-inflammatory cytokines TNF-α and IL-1β stimulate TNAP expression and calcification in human mesenchymal stem cells (MSCs) while they decrease RUNX2 levels and osteoblast differentiation,[84] these results indicate that dysregulated expression of TNAP in pro-inflammatory conditions might be sufficient to trigger CVC. Finally, TNAP dephosphorylates ATP in culture of neurons,[85] hypertrophic chondrocytes,[78] and MSCs,[86] but the *in vivo* relevance of these findings and whether they are associated with TNAP involvement in inflammation or calcification remains uncertain and deserve further investigation.

Lipopolysaccharide (LPS) is a well-known pro-inflammatory compound synthesized by Gram-negative bacteria. The pro-inflammatory effects of LPS relies on the phosphate groups in the lipid A region.[87] IAP dephosphorylates LPS in the gut and prevents the development MetS resulting from repeated LPS absorption accompanying high fat diets.[88] LPS is indeed absorbed during fatty meals,[89] and contributes to postprandial inflammation and metabolism.[90] IAP would thus limit postprandial endotoxemia and keep postprandial inflammation in physiological limits.[88] It is likely that in the blood TNAP participates in the dephosphorylation of LPS that has escaped from IAP in the gut. Increasing evidence indicates that human TNAP has the ability to dephosphorylate LPS *in vitro* and *in vivo*.[91–96] Whether LPS dephosphorylation by TNAP also participates in the control of postprandial inflammation and metabolism is yet only speculative but deserves investigation.

Finally, TNAP may dephosphorylate proteins *in vivo*. To our knowledge, the first protein that has been suggested to be a TNAP substrate is osteopontin (OPN). OPN is a highly phosphorylated glycoprotein expressed in multiple cell types and may exert multiple functions. OPN can act as an inflammatory cytokine,[97] an inducible inhibitor of ectopic calcification,[98] and a promoter of regression of ectopic calcification[99] and bone resorption.[100] Importantly, phosphorylated but not unphosphorylated OPN has been proposed to inhibit ectopic calcification.[101] *Alpl*-deficient mice have increased levels of OPN, and crossing *Alpl*-deficient mice with *Opn*-deficient mice partly corrects their mineralization defects.[102] TNAP likely dephosphorylates OPN on residues from two distinct regions to stimulate mineralization.[103] These effects of TNAP on mineralization mediated by OPN dephosphorylation seem to counter-regulate those relying on PP_i hydrolysis since the extracellular P_i/PP_i ratio and OPN appear to regulate the expression of the same genes involved in the control of mineralization.[102,103] Among other proteins that have been proposed to be TNAP substrates, Tau protein will be discussed in chapter 3.4.

4. Physiological TNAP's functions and potential consequences of its inhibition

4.1 Inhibition of TNAP expression and/or activity to prevent CVC

Until recently, the most potent available TNAP inhibitor was levamisole (or tetramisole, the racemic mixture of levamisole and its enantiomer dexamisole). However, this inhibitor has TNAP-independent effects,[104] for instance, on voltage-dependent sodium channels and/or noradrenaline uptake.[104] The first potent and selective TNAP inhibitor is arylsulfonamide 2,5-dimethoxy-N-(quinolin-3-yl) benzenesulfonamide, also known as MLS-0038949.[105] MLS-0038949 specifically inhibits TNAP with no effect on IAP. It is however unknown whether MLS-0038949 differently inhibits liver and bone TNAP, which have the same amino acid sequence but differ by their glycosylation pattern.[53] MLS-0038949

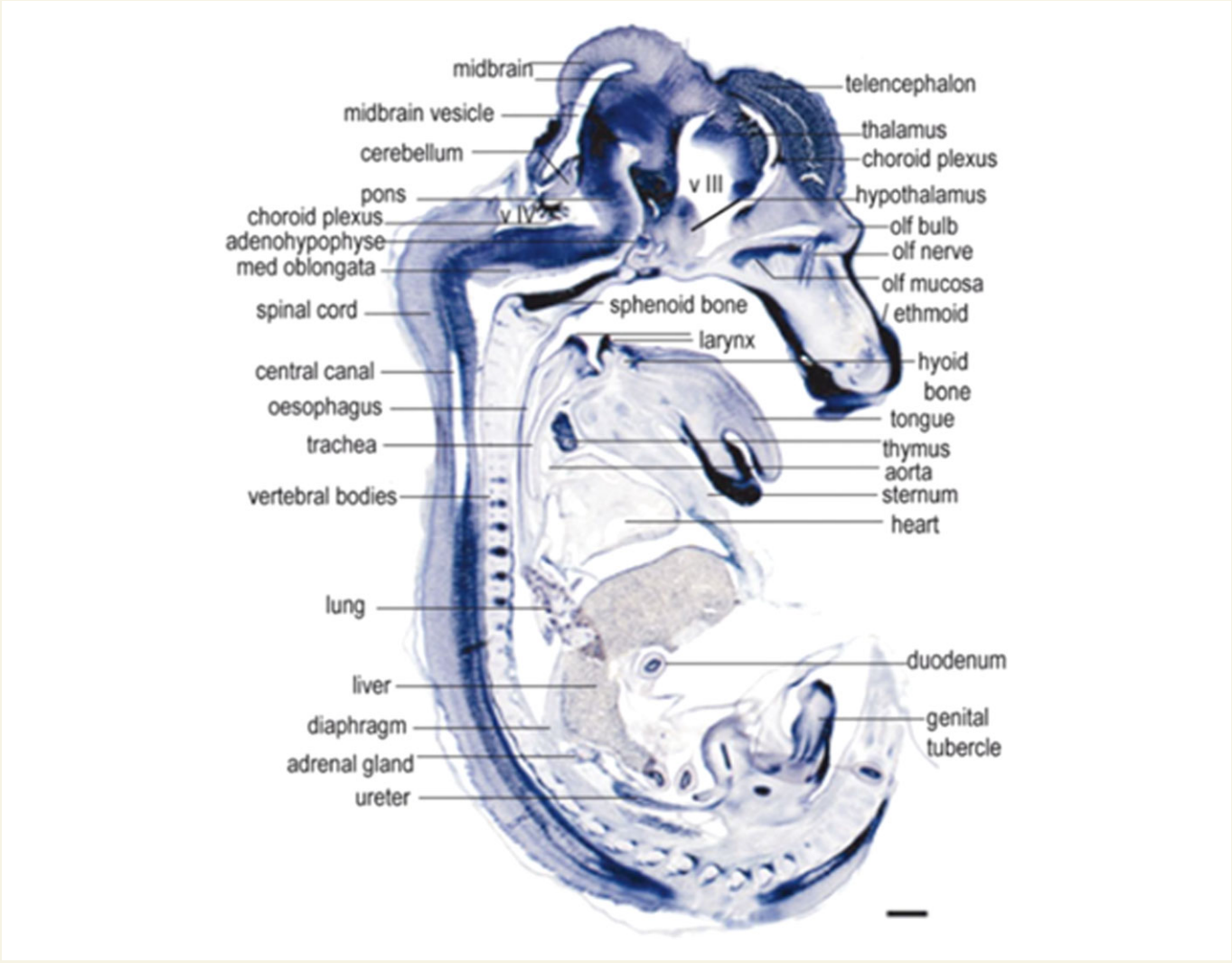


Figure 3 TNAP activity detected by histochemistry in E15 mouse embryo (bar 500 µm). Taken with permission from Ref.[120]

reduced calcification in culture of VSMCs.[106,107] However, MLS-0038949 has only modest pharmacokinetics properties, and had to be modified for translational approaches. In 2018, the group of JL Millan developed 5-((5-chloro-2-methoxyphenyl)sulfonamido)nicotinamide, also known as SBI-425, as a potent and highly selective TNAP inhibitor adapted for *in vivo* experiments. This inhibitor was first validated in mice overexpressing TNAP under the control of the *Tagln* promoter active in VSMCs.[32] These mice develop extensive arterial calcification, left ventricular hypertrophy and fibrosis, higher systolic blood pressure, and early and sudden mortality, consistent with pressure overload-induced hypertrophy and progression to heart failure. Mice that orally or intravenously received a daily single dose of 10 mg/kg of SBI-425 showed a complete inhibition of plasma TNAP activity, and reduced arterial calcification and prolonged lifespan in TNAP-overexpressing mice.[32] SBI-425 also prevented VC in a mouse model of *pseudoxanthoma elasticum*, a genetic disease associated with impaired PP_i generation,[108] and in mice with CKD induced by adenine and phosphorus-rich diet.[109] Treatment with SBI-425 at 10 or 30 mg/kg/day did not affect kidney function, but fully prevented early death.[109] This was associated with the prevention of aorta calcification but not of heart calcification, likely explained by the low penetration of SBI-425 in tissues.[110] Nevertheless, these results indicate that TNAP inhibition in general, and SBI-425 in particular, may represent a very promising strategy to treat patients with CKD. On the other hand, patients with CKD also have atherosclerotic intimal plaque calcification that has a very different impact on the vasculature than the specific medial calcification developed by these patients.[3] To date, one published article reported the effects of SBI-425 on atherosclerotic plaque calcification in a mouse model of familial hypercholesterolemia, but these mice were crossed with mice overexpressing TNAP in endothelial cells,[34] making results difficult to interpret. Oral supplementation of SBI-425 30 mg/kg/day in the wicked high cholesterol (WHC) mouse model induced by a point mutation in the low-density lipoprotein receptor, decreased coronary calcium accumulation, and left ventricular hypertrophy.[34] These results indicate that SBI-425 may inhibit intimal atherosclerosis plaque calcification in addition to inhibiting medial calcification associated with CKD. More studies are needed to have a clearer vision of the effects of SBI-425 on cardiovascular mortality.

Besides SBI-425, potential novel inhibitors were tested chemically and putative binding modes were suggested by molecular docking simulations. The isonicotinohydrazone derivative (E)-N'-(4-hydroxy-3-

methoxybenzylidene)isonicotinohydrazide was identified as the most potent TNAP inhibitor of the tested derivatives.[111] Further, derivatives of 4-quinolones, hybrid compounds from chalcone and 1,2-benzothiazine pharmacophores and trinary benzocoumarin-thiazoles-azomethine derivatives were suggested as selective inhibitors of AP isoenzymes.[112–114] Whether these compounds are suitable for *in vitro* and/or *in vivo* experiments remains to be determined.

Another approach to diminish TNAP activity is to reduce its protein levels. Recently, the epigenetic regulator apabetalone, an orally available bromodomain and extraterminal (BET) protein inhibitor which is in clinical development for cardiovascular disease treatment, was identified as a TNAP inhibitor acting on expression level. Apabetalone diminished the induction of *ALPL* mRNA, TNAP protein and enzyme activity in primary human hepatocytes and VSMCs via the transcriptional regulator BRD4 that caused reduction of VC.[115] Additionally, apabetalone prevented induction of inflammatory cytokines *in vitro* and reduced multiple mediators of lipid metabolism and chronic vascular inflammation in the plasma of CVD patients.[116] Potential beneficial cardiovascular effects of apabetalone are under investigation. While apabetalone treatment reduced cardiovascular risk and improved kidney function in CKD patients, patients with recent acute coronary syndrome, type 2 diabetes, and low high-density lipoprotein cholesterol levels, did not demonstrate beneficial cardiovascular effects.[117–119] In CKD and CVD patients, apabetalone also reduced circulating TNAP levels.[119] However, whether the potential favourable cardiovascular effects of apabetalone are causally linked to TNAP reduction remains to be demonstrated.

Therefore, tools now exist to inhibit TNAP and block CVC. A tight evaluation of TNAP inhibitors is however needed *in vivo*, since as its name indicates, TNAP is ubiquitous, as perfectly illustrated by its pattern of activity during development (*Figure 3*).[120] During vertebrate development and growth, TNAP is active in bone, liver, kidney, nervous, and immune systems, where it exerts known or unknown effects. Its activity changes during ageing, being progressively weaker in bone, but higher in other tissues depending on the presence or absence of different age-related diseases. The next subchapters review these known TNAP functions.

4.2 TNAP as an anti-inflammatory enzyme

TNAP is present in the blood as a soluble (anchorless) isoform, originating primarily from bone and liver. Bone is the main source of blood TNAP during growth, when the skeleton is being built, whereas liver progressively becomes the main source of blood TNAP in ageing adults when bone formation slows down.[121] Distinction between bone and liver TNAPs in the blood is possible but technically challenging. Bone TNAP levels can be quantified by ELISA, although cross-reactivity as high as 18% has been reported with liver TNAP.[122] Unfortunately, there is to date no available ELISA that specifically measures liver TNAP in the blood, which can only be assessed through total TNAP activity. Nevertheless, since increased bone TNAP levels are relatively uncommon in adults, increased TNAP activity in the blood often reflects liver disorders, in particular, during impaired bile flow or cholestasis in primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) but also non-alcoholic fatty liver disease (NAFLD),[123] and more generally MetS.[35–40] Circulating TNAP may generate adenosine from AMP to exert anti-inflammatory effects,[77] and/or dephosphorylate LPS to detoxify it, in a context of sepsis and/or to regulate postprandial endotoxaemia.[92] TNAP is also present in the blood anchored at the membrane of neutrophils,[124] the most abundant leukocytes in human blood, where it exerts anti-inflammatory functions.[77,78,125] Neutrophil TNAP may support soluble TNAP to hydrolyze PLP, AMP, and LPS, but its presence at the membrane more likely participates in the control of the autocrine effects of adenosine and its nucleotides, in particular on neutrophil survival,[126] migration,[127] or secretion of IL-1β.[78] Finally, TNAP is also present in the blood attached to the membrane of endothelial cells in arterioles and capillaries.[34,128,129] Its specific function in endothelial cells is unknown. It might also be related to nucleotide and/or LPS dephosphorylation, or to transport from blood to the endothelium.

If indeed TNAP in the blood exerts important anti-inflammatory effects, its inhibition may have detrimental effects in several age-related diseases. Ageing is associated with a low-grade systemic inflammation, characterized by increased levels of C-reactive protein (CRP), and of cytokines, such as TNF-α and IL-1β.[130–132] Exacerbation of this systemic age-associated inflammation may therefore adversely impact health during ageing. Moreover, TNAP inhibition may not only impact systemic inflammation but also tissue inflammation in several diseases, since neutrophils are often the first cells to be recruited in inflamed tissues, e.g. in atherosclerotic plaques, where neutrophils are the first cells to enter and stimulate inflammation.[133,134] In plaques, TNAP inhibition may therefore have more complex consequences than only blocking calcification.

4.3 TNAP'S crucial role in skeletal/dental mineralization

The most severe form of HPP leads to the perinatal death of foetuses completely devoid of bone mineral,[135] highlighting the requirement for TNAP to mineralize the skeleton and teeth. Bone formation proceeds either by endochondral ossification, in particular, in long bones, or by intramembranous ossification in flat bones. In the former process, mesenchymal cells differentiate under the control of the transcription factor SOX9 into proliferative chondrocytes, which then maturate into mineralizing hypertrophic cells expressing the transcription factor RUNX2.[136] During this process, TNAP is expressed in proliferative chondrocytes, but its expression is strongly stimulated by RUNX2 in hypertrophic chondrocytes, where TNAP hydrolyzes extracellular PP_i allowing growth plate mineralization.[137–140] During endochondral ossification, osteoblasts form bone on this calcified cartilage plate, whereas during endomembranous ossification, they produce bone without the need for a cartilage template. In both cases, osteoblasts express TNAP under the control of RUNX2 and anchor it to their cell membrane to hydrolyze PP_i.[140] In the teeth, TNAP is expressed in odontoblasts and ameloblasts, the cells responsible for dentin and enamel mineralization, respectively.[140,141] This explains why HPP in humans and lack of TNAP in mice result not only in bone hypomineralization but also in decreased dentin and enamel mineralization,[141] and lack of acellular cementum, a tissue highly sensitive to extracellular PP_i concentration.[142,143] In addition to its pro-mineralizing role relying on PP_i hydrolysis in bone and tooth cells, TNAP may exert functions in MSCs. TNAP has indeed been identified as a marker of human MSCs,[144] where its stimulation may lead to ATP hydrolysis leading to the promotion of osteoblastogenesis at the expense of adipogenesis.[86] In this context, it is logical to anticipate that TNAP inhibition will slow down bone formation and exacerbate age-related osteoporosis. These side effects of TNAP inhibition are a concern for patients with CKD, who are prone to develop mineral bone disorder (MBD). They are also relevant for post-menopausal women, who may be at increased risk of developing osteoporosis with a severity inversely

associated to the calcium score,[145] and have a high risk of cardiovascular mortality.[146] The first reports of TNAP inhibition using SBI-425, in a mouse model of *pseudoxanthoma elasticum*[108] and in a mouse model of CKD-MBD[109] did not report significant effects on bone architecture. These reports in mice with daily administration of SBI-425 for several weeks were reassuring and paved the way to subsequent studies. On the other hand, one article reported that in addition to preventing the warfarin-induced VC in rats, administration of SBI-425 for 7 weeks decreased bone formation rate and mineral apposition rate, and increased osteoid maturation time, effects that did not yet impact bone architecture as determined by μCT.[147] Collectively, these studies indicate that TNAP inhibition with SBI-425 for several weeks has no harmful effects on bone architecture, but that in case of long treatments with high doses, bone homoeostasis has to be carefully controlled. Moreover, if indeed TNAP is an anti-inflammatory enzyme acting by dephosphorylating adenosine nucleotides and LPS, its inhibition on the long term may adversely impact bone homoeostasis through enhanced inflammaging. It is indeed well-known that inflammaging drives the decline in bone mass associated with ageing.[148] TNF-α in particular strongly inhibits the expression of RUNX2 in osteoblasts[149] and induces its degradation by the proteasome, resulting in impaired osteoblastogenesis.[150] Inflammaging-associated reduction of bone TNAP expression may render bones more susceptible to inflammation in a vicious cycle. *Alpl*$^{+/-}$ mice have increased levels of IL-1β and IL-6 in their bones, which is probably due to TNAP's nucleotidase activity.[78] As mentioned above, SBI-425 does not seem to have dramatic effects on bone homoeostasis but most of the experiments were not realized in aged animals with osteoporosis, which deserve further consideration.

4.4 Emerging functions of TNAP in the central nervous system

TNAP is strongly active in the brain during development (*Figure 3*), where it is expressed by endothelial and neuronal cells. In endothelial cells, TNAP is active in both the luminal and abluminal sides of the cell membrane, particularly in the arterial part of the microvasculature, but not the venous system.[151,152] In human brain vessels, TNAP activity can be detected from gestational ages,[153,154] while in mouse brain vessels TNAP is active several days after birth.[151,155] This subcellular distribution suggests a role in active transport across capillary endothelial cells, and in blood brain barrier (BBB) permeability.[156,157] This role likely includes PLP dephosphorylation in the blood and pyridoxal transport across the BBB. Lack of TNAP in both *Alpl*-deficient mice and newborns with HPP results in higher PLP levels in blood, reduced GABA synthesis, and epileptic seizures. In addition to endothelial TNAP, neuronal TNAP is likely crucial for normal brain development and functions. During development, neuronal TNAP activity is observed in both grey matter and white matter.[158] Interestingly, the association of TNAP with neurogenesis in embryonic rodent brain is maintained in adulthood, with elevated TNAP activity in neurogenic niches.[159] Multiple neuroanatomical abnormalities have been observed by MRI in HPP infants, such as hypodensity of the white matter, dilated ventricles, multicystic encephalopathy, parenchymal lesions,[160–164] while delay in myelination and synaptogenesis, and abnormalities in the spinal nerve roots were reported in *Alpl*-deficient mice.[139,165] Several mechanisms have been proposed to explain TNAP contribution in brain development: dephosphorylation or interaction with extracellular matrix proteins, such as laminin and collagen,[166–168] a role in signal transduction through interaction with PrPc, a plasma membrane GPI anchored protein in lipid rafts highly expressed in the central nervous system,[166] regulation of PLP-dependent enzymes, impacting synthesis of GABA, serotonin and dopamine,[75,169,170] and a ectonucleotidase function involved in axon formation and growth,[85] neurotransmission,[171,172] and contributing to the epileptic phenotype of TNAP-deficient pups.[173]

SBI-425 administration in mice does not trigger epileptic seizures, suggesting that the remaining TNAP activity is sufficient to dephosphorylate PLP in the blood and allow GABA synthesis in the brain. It is however possible that TNAP inhibition may trigger seizures in some patients. Indeed, epileptic seizures have been reported in children treated for paediatric nephrotic syndrome with levamisole.[104] In this case, pyridoxine supplementation could prevent these seizures as it does in the infantile and perinatal forms of hypophosphatasia.[160,174,175] In addition, SBI-425 does not cross the BBB in healthy mice,[176] indicating that it should not alter TNAP functions in brain cells in animals and patients with a normal BBB. However, SBI-425 may cross the BBB whose integrity is compromised by systemic and local inflammation,[176] which might be the case in ageing adults with MetS and neurodegenerative disorders, such as Alzheimer's disease (AD). Analyses of post-mortem AD brains showed an increase in TNAP protein, activity, or expression in hippocampus and temporal gyrus, which are cerebral regions targeted by tau pathology.[177,178] Patients with AD also seem to have increased serum levels of TNAP, which are associated with cognitive dysfunction.[177,179–181] Activation of TNAP in AD may have both beneficial and harmful effects. First, TNAP may exert protective effects on neuroinflammation by its ability to dephosphorylate ATP into adenosine.[75,85,182,183] Second, TNAP may also protect from AD development helping to maintain a functional BBB.[176] On the other hand, a scenario based on *in vitro* approaches, proposed that TNAP participates to AD progression by dephosphorylating extracellular hyperphosphorylated Tau protein.[178] Unphosphorylated Tau interacting with muscarinic receptors induces an increase in intracellular calcium that affects calcium homoeostasis triggering neuronal cell death[184]; it promotes phosphorylation of intracellular Tau which has neurotoxic effects, and it increases TNAP expression.[178,185] Thus, a positive feedback loop would result in a link between increases in TNAP and Tau levels, increase in neuronal loss and decline in brain functions. In this complex context, exploring the effects of TNAP inhibition in models of AD and neuroinflammation deserve consideration.

Finally, TNAP inhibition may participate in brain damages induced by ischaemic stroke. Ischaemic strokes account for 85% of strokes and are due to large vessel atherosclerosis in 15–20% of cases.[186] Serum TNAP activity is increased in patients with acute ischaemic stroke and is associated with a poor functional outcome, stroke recurrence, and mortality.[187–193] TNAP may not only be a biomarker but also an active player in post-ischaemic reperfusion injuries. TNAP may play a protective role in post-ischaemic BBB leakage.[176,194] Moreover, TNAP may also reduce neuroinflammation following stroke. Stroke leads to cell necrosis, which triggers the release of ATP and a subsequent inflammation that is resolved by the dephosphorylation of ATP into adenosine.[195] The reported involvement of TNAP in ATP hydrolysis and/or adenosine generation in neurons[85,182,183] and in neutrophils,[77,78] which are recruited by ATP release,[196] and play multiple pro-inflammatory roles during stroke,[197–199] may suggest that TNAP inhibition might worsen post-ischaemic neuroinflammation. Therefore, the effects of TNAP inhibition on brain damages and functional outcome after stroke deserves investigation.

4.5 TNAP'S functions in liver and kidneys

TNAP's former name was liver/bone/kidney (L/B/K) AP. The expression of TNAP in liver and kidney is known for a long time, but its function in these tissues remains elusive. In the liver, TNAP is localized at the canalicular membrane of hepatocytes and the apical area of the cytoplasm of bile duct epithelial cells.[140,200,201] Cholangitis is associated with increased release of liver TNAP into the blood,[123] suggesting that TNAP participates in bile excretion. TNAP may dephosphorylate ATP at the cholangiocyte surface to participate in the regulation of bile pH in response to the secretion of bile salts.[202] Additionally, liver TNAP may participate in LPS detoxification and excretion in the bile.[91–94,203] Whether this regulation takes place under pathological conditions during bacterial infection, or physiologically to regulate postprandial endotoxemia is worth exploring. Finally, it is possible that liver expresses TNAP mainly to release it in the blood in adults to control systemic inflammation when bone formation is reduced and bone TNAP release in the blood is decreased. To our knowledge, no effect of TNAP inhibition on the liver has been reported to date, but most studies were conducted in mice which, in contrast to humans and rats, have very weak expression of liver TNAP.[201]

In the kidney, TNAP is expressed at the brush borders of proximal renal tubules.[140,204] This renal expression of TNAP may appear surprising since prevention of mineralization in urine is supposed to rely on the presence of PP_i, the mineralization inhibitor inactivated by TNAP.[205] However, the main production site of PP_i may be the distal nephron where TNAP is absent,[205] which may explain why TNAP does not induce urinary tract calcification. Instead, in the kidney brush border, TNAP induced by LPS may be able to dephosphorylate and detoxify it.[206,207] Whether this function occurs in physiological and/or in pathological situations remains obscure, but it is worth exploring.

5. Conclusion

An increasing number of articles now indicate that TNAP is responsible, or at least involved, in CVC, and tools to inhibit its expression and activity are now available. Several preclinical studies showed that TNAP inhibition strongly reduces VC.[108,109,147] Since TNAP is ubiquitous and likely has several important functions in addition to its well-established role in the control of the Pi/PPi ratio during physiological and ectopic mineralization, there is now a compelling need to perform studies to better understand these additional functions, identifying its associated substrates, and elucidating these new metabolic pathways.

Conflict of interest: J.L.M. is a co-inventor on a patent covering SBI-425 (PCT WO 2013126608).

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