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Review

New insights into NPP1 function: Lessons from clinical and animal studies

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Abstract

The recent elucidation of rare human genetic disorders resulting from mutations in ectonucleotide pyrophosphatase/phosphodiesterase (ENPP1), also known as plasma cell membrane glycoprotein 1 (PC-1), has highlighted the vital importance of this molecule in human health and disease. Generalised arterial calcification in infants (GACI), a frequently lethal disease, has been reported in recessive inactivating mutations in ENPP1. Recent findings have also linked hypophosphataemia to a lack of NPP1 function. A number of human genetic studies have indicated that NPP1 is a vital regulator that influences a wide range of tissues through various signalling pathways and when disrupted can lead to significant pathology.

The function of Enpp1 has been widely studied in rodent models, where both the mutant tip toe walking (ttw/ttw) mouse and genetically engineered Enpp1−/− mice show significant alterations in skeletal and soft tissue mineralisation, calcium/phosphate balance and glucose homeostasis. These models therefore provide important tools with which to study the potential mechanisms underpinning the human diseases associated with altered NPP1.

This review will focus on the recent advances in our current knowledge of the actions of NPP1 in relation to bone disease, cardiovascular pathologies and diabetes. A fuller understanding of the mechanisms through which NPP1 exerts its pathological effects may stimulate the development of novel therapeutic strategies for patients at risk from the devastating clinical outcomes associated with disrupted NPP1 function.

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Introduction

Rare human genetic disorders resulting from loss-of-function mutations in the ectonucleotide pyrophosphatase/phosphodiesterase (ENPP1) gene, also known as plasma cell membrane glycoprotein 1 (PC-1), have highlighted the importance of this molecule in human health and disease. Generalised arterial calcification in infants (GACI) and severe hypophosphataemia have been reported in recessive inactivating mutations in the ENPP1 gene [1–4]. Together with the association between polymorphisms in ENPP1 and ALPL, the gene encoding tissue non-specific alkaline phosphatase (TNAP), and reduced bone size and mineral density in the Caucasian population [5] these findings indicate that the ENPP1 gene is required for normal inhibition of ectopic
mineralisation while also being essential for mineralisation in the bone. Furthermore, levels of ENPP1 expression have been reported to be elevated in humans showing high levels of insulin resistance [6-8] suggesting an important role in glucose homeostasis and insulin signalling. These human studies indicate that the NPP1 protein is a vital regulator that influences a wide range of tissues through various signalling pathways and when disrupted can lead to significant pathology.

The function of NPP1 has been widely studied in rodent models, where both the mutant tiptoe walking (ttw/ttw) mouse [9-14], and the transgenically engineered Enpp1−/− mice [15,16], show changes in skeletal and soft tissue mineralisation, calcium/phosphate and glucose homeostasis, mimicking the diseases seen in human subjects. Furthermore, by acting remotely on the balance of circulating minerals and glucose, NPP1 has a wider reaching impact on both skeletal and soft tissue structure and metabolism. This review will focus on the recent advances in current understanding of the role of the NPP1 protein in these pathways and outline the importance of this research in bone diseases, cardiovascular diseases and diabetes.

Genetics and function of NPP1

The nucleoside pyrophosphatase/phosphodiesterases (NPPs) are an important group of enzymes with an extensive functional range that are distributed widely and are highly conserved between species. In humans the NPP family consists of 5 proteins of which NPP1 and NPP3 show similar structure and function and the genes encoding for these two proteins have been mapped to human chromosome 6q22-23 [17,18]. Despite the close sequence homology of the NPP genes between species it has been reported that the 5' flanking region is far less conserved, leading to different regulation and gene expression patterns in different species [19].

The NPP1 protein is a membrane spanning homodimer and, when cleaved, the extracellular domain can function as a secreted circulating protein. In a very revealing review Bollen and colleagues have discussed the biochemistry of the NPP family and have summarised the localisation of ENPP1 gene expression [19]. ENPP1 is expressed in a wide range of tissues including cartilage, heart, kidney, parathyroid and skeletal muscle, and it is highly expressed in vascular smooth muscle cells (VSMCs), osteoblasts and chondrocytes [20-22].

NPPs have wide substrate specificity, and the hydrolysis of pyrophosphate bonds (for example, in ATP) and phosphodiester bonds (for example, in oligonucleotides) to produce nucleoside 5'-monophosphates makes NPPs extremely important in extracellular nucleotide metabolism and extracellular signalling. NPP1 (EC3.1.4.1) is a 104 kDa type II transmembrane protein consisting of a small intracellular region (between 10 and 80 residues) and a larger extracellular domain (830 residues) which contains the catalytic site [23]. Phosphodiesterases are classified as enzymes that hydrolyse diesters of phosphoric acid into phosphomonesters, and can be classified into two main groups — those that act on lipids or on nucleotides. Pyrophosphatases are acid anhydride hydrolases that catalyse the breakdown of diphosphate bonds and are biologically important in the cleavage of ATP. NPP1 hydrolyses ATP to generate either inorganic pyrophosphate (PPi) plus AMP or inorganic phosphate (P_i) plus ADP in a two stage process via either AMP or a phosphate bound intermediate, respectively (Fig. 1) [19,24]. It has also been reported that NPPs can convert AMP into adenosine and P_i [25,26] although conflicting reports suggest that AMP competitively inhibits NPP activity [27]. All of the products of these hydrolysis reactions are essential in cellular signalling and function, the effects of which vary between tissues.

Basic mechanisms of bone formation and the role of NPP1 in skeletal mineralisation

In order to understand the functions of NPP1 it is important to appreciate the physiological process of mineralisation in bone. This relies on the deposition of hydroxyapatite (HA) onto a collagenous matrix, and is a highly regulated process that requires the correct concentration of calcium (Ca^{2+}) and P_i to precipitate as HA crystals. Mineralisation is thought to be a two stage process, the first of which occurs within matrix vesicles (MVs) [28] where the conditions are optimal for the initial precipitation of HA. The second stage consists of the propagation of HA formation onto the extracellular matrix.
levels of extracellular PPi, with phenotypic features including signifi-
cance alterations in bone mineralisation in long bones and calvariae, and pathologic, severe peri-spinal soft tissue and arterial calcification [16,30,32].

The calvariae of *Enpp1* /−/− mice are hyper-calcified in vivo, and calvarial osteoblasts derived from *Enpp1* /−/− mice show reduced extracellular PPi, levels, and a concomitant increase in calcification in vitro [30]. These abnormalities can be rescued by transfection with NPP1 but not with NPP3. A significant reduction in the mineralisation inhibitor OPN has also been observed in *Enpp1* /−/− osteoblasts, indicating that NPP1 not only has a direct effect on PPi, concentration, but also has an indirect effect on the process of calcification by regulating the expression of other cellular regulators [32].

*Enpp1* /−/− mice also show significant defects in long-bone mineralisation [16,40]. *Enpp1* /−/− mice have reduced trabecular bone mass (Fig. 2) and cortical thickness of both the tibia and femur, characterised by disruption of the structural and mechanical properties, the severity of which increases with age [40]. This is likely to be a direct effect of lack of NPP1 activity, but the reduced body weight observed in *Enpp1* /−/− mice will reduce the loading on the bones and thus may also have an effect on their structure.

Previous evaluation of the mineralisation of bones from 10-day-old *Enpp1* /−/− and [*Enpp1* /−/−; *Akp2* /−/−] double knockout mice has indicated that the effects of *Enpp1* ablation on an *Akp2* null background is site-specific [16]. Thus, in contrast to the normalisation of the degree of mineralisation seen in the joints, calvariae, vertebrae and soft tissues as a consequence of ablating both NPP1 and TNAP function, the long bones of these double knockout mice appeared to remain hypomineralised. Furthermore, calcified nodule formation and mineral deposition are inhibited to a higher extent in osteoblasts isolated from *Enpp1* /−/− bone marrow than calvarial osteoblasts isolated from the same animal, further indicating that loss of NPP1 activity affects skeletal sites in a site-specific manner [16]. The hypomineralisation observed in the long bones of *Enpp1* /−/− mice may be related to relatively low levels of endogenous PPi when compared to the calvaria [16]. Thus, in long bones, the complete deletion of NPP1 activity would further reduce extracellular PPi, to abnormally low levels. This would result in insufficient PPi, substrate for TNAP to generate PPi, for normal mineral formation.

It has been widely reported that PPi, functions to regulate both osteoblast and chondrocyte differentiation. However, it has recently been shown that NPP1 regulates osteoblastic gene expression and cellular differentiation in calvarial osteoblasts independent of PPi, and PPi [41]. Nam and colleagues have provided evidence that NPP1 is an inducer of osteoblast differentiation, demonstrating that IGF-2 signalling induces *Enpp1* expression in pre-osteoblasts but not in differentiatated osteoblasts. Furthermore, MC3T3E1(C4) cells that overexpressed *Enpp1* showed enhanced osteoblastic gene expression. Conversely, defective osteoblast differentiation was observed in both calvariae extracted from *Enpp1* /−/− mice and MC3T3E1(C4) cells treated with *Enpp1* targeting short hairpin RNAs. Therefore inhibition of osteoblast differentiation due to lack of NPP1 activity may also contribute to the reduced mineralisation in the long bones observed in *Enpp1* /−/− mice.

Wild-type mice show reduced bone resorption with advancing age which is consistent with the attainment of the adult skeleton. Interestingly *Enpp1* /−/− mice maintain similar levels of osteoclast activity at 6 and 22 weeks of age, indicating an increase in functional activity of osteoclasts [40], the cells that mediate bone resorption. Furthermore, treatment of *ttw/ttw* mice with calcitonin, a known inhibitor of osteoclast function and putative suppressor of osteoblastic bone formation, has been shown to reverse the osteopenic phenotype [14]. This study indicates that accelerated periosteal bone formation in *ttw/ttw* mice is suppressed by calcitonin but does not assess the role of osteoclasts in the correction of the osteopenic phenotype.

The role of NPP1 in soft tissue calcification

Ectopic calcification occurs throughout the body causing clinical complications particularly when seen in the aorta, cardiac valves and in the myocardium, where mineralisation is a serious risk factor in cardiovascular disease. It is also observed in tendons, cartilages and ligaments where severe osteoarthritides and ankylosis can occur. Mutations in the *ENPP1* gene have been associated with several rare human diseases, demonstrating the importance of NPP1 in maintaining normal tissue function [1,3,4,42]. There is a complex interaction between a wide range of molecular and genetic factors that inhibit calcification of the soft tissue and a breakdown in these pathways can lead to severe pathology. These genetic factors have been recently reviewed [43] therefore this review will focus on the roles of NPP1 in ectopic calcification in human disease, and the relevant rodent models used to study these pathological conditions.

The study of diseases such as GACI and pseudoxanthoma elasticum (PXE), which show overlapping clinical pathology in a wide range of
Generalised arterial calcification in infancy and pseudoxanthoma elasticum: disease models of ectopic tissue calcification

Generalised arterial calcification of infancy (GACI) is a rare autosomal recessive disease characterised by calcification of large and medium-sized arteries and arterial stenosis caused by intimal proliferation (Fig. 4). Most affected children die within the first 6 months of life from the sequelae of end-organ damage including myocardial infarction [44]. In a subset of patients, peri-articular calcification of the greater joints also occurs. The finding of low systemic levels of inorganic pyrophosphate in one affected proband [45] due to defective activity of the PP-generating enzyme NPP1 [2] prompted the search for mutations in the NPP1 encoding gene, and indeed, most of the patients known so far with the classical GACI phenotype were found to carry bi-allelic mutations in ENPP1 [46]. The understanding of the disease as caused by the deficiency of an inhibitor of hydroxyapatite crystal deposition, namely inorganic pyrophosphate, has paved the way for the use of bisphosphonates, i.e., synthetic analogues of PP, to effectively treat GACI patients [46,47]. The retrospective observational analysis of 55 subjects affected by generalised arterial calcification of infancy by Rutsch and colleagues showed that treatment with bisphosphonates was associated with a regression of the calcifications and an increased survival rate [46]. However, spontaneous regression of ectopic calcifications also occurs in GACI patients [48,49]. Most recently, mutations in ENPP1 were also detected in a subset of patients with generalised arterial calcification and pseudoxanthoma elasticum: up to date, a total of four patients have been described, who presented typical signs of GACI in infancy and who later developed typical signs of PXE, including angioid streaks of the retina and pseudoxanthomatosus skin lesions [42,50]. Pseudoxanthoma elasticum, a rare disease associated with soft tissue calcification at different sites including the eye, the kidneys, the arterial wall and the skin had been previously demonstrated to be caused by loss of function mutations in ABCC6 encoding MRP6, a transport protein of hitherto unknown function [51,52]. Interestingly, ABCC6 mutations have also been found to be associated with the GACI phenotype [42]. The finding of genocopy and phenocopy in GACI and PXE points to a close relationship between these two diseases and suggests common downstream mediators of ectopic tissue calcification in MRPI and NPP1 deficiency [43].

Mouse models elucidating the role of NPP1 in tissue calcification

It has been widely described that mouse models with disrupted or genetically ablated Enpp1 expression show high levels of ectopic calcification and subsequent cardiovascular pathology and hyperostosis of the joints [9,11,13–16,30,32,40]. Given that NPP1 is the primary producer of PP, an important inhibitor of HA crystallisation and chondrocyte differentiation [53], it is unsurprising that widespread soft tissue calcification is observed when NPP1 function is disrupted.

In the mutant mouse model, designated the ttw/ttw mouse, a phenotype including postnatal development of progressive ankylosing intervertebral and peripheral joint hyperostosis; increased vertebral cortical bone formation; spontaneous articular cartilage and arterial calcification is observed [9,11–14]. This mouse model provides a useful model for ossification of posterior lateral ligament (OPLL), a human condition characterised by pathological cartilage calcification in the spine and disrupted phosphate metabolism, associated with single nucleotide polymorphisms in the Enpp1 gene [54–56].

A number of studies have demonstrated that Enpp1−/− mice develop extensive arterial calcification (Fig. 3) [57]. The regulation of the phenotypic transition of VSMCs during aortic calcification is likely to involve reduced NPP1 activity and subsequent PP levels, with Enpp1−/− VSMCs showing an up-regulation of molecules associated with chondrogenic, osteoblastic and osteocytic phenotypes [57]. Recent research has also demonstrated that NPP1 activity modulates arterial calcification through the mediation of receptor for advanced glycation of end-products (RAGE) signalling [58]. Membrane bound RAGE promotes nuclear factor-kappaB (NF-kB) and oxidative stress signalling, causing an up-regulation of aortic matrix remodelling. This signalling pathway has been implicated in patients suffering from aortic aneurysms and calcific aortic valve stenosis (CAVS) [59,60]. The production of sRAGE – a soluble endogenous suppressor of RAGE signalling – has been shown to be reduced in Enpp1−/− aortic ring cultures. Additionally, treatment of cultures with sRAGE inhibits...
calcification and chondrogenic trans-differentiation [58]. Furthermore, the \textit{Rage}−/−/\textit{Enpp1}−/− double knockout mouse shows reduced arterial calcification when compared to the \textit{Enpp1}−/− mouse. It is, however, important to note that this double knockout mouse did not show a rescue of skeletal phenotype seen in \textit{Enpp1}−/− mice, suggesting that the changes in RAGE signalling mediated by loss of NPP1 activity may be specific to vascular smooth muscle cells.

The generation of \textit{ppb} by NPP1 also upregulates OPN expression, which can further inhibit mineralisation [61–64]. The complex interplay between OPN and NPP1 during ectopic calcification is confounded by the pro-atherogenic activity of OPN [65,66], and the recent finding that NPP1 promotes atherosclerotic plaque formation through OPN [20]. Furthermore, recent studies by Cote and colleagues have demonstrated that over-expression of \textit{Enpp1} can also induce mineralisation in human valve interstitial cells [67]. The authors show not only that \textit{Enpp1} expression is increased in human stenotic valve samples, but also that when over-expressed in vitro, NPP1 acts to increase apoptosis and mineralisation through a mechanism involving disrupted signalling of the P2Y2 and PI3-kinase/Akt pathways. These data indicate that expression of \textit{Enpp1} must be maintained within a physiological range, and when altered, either by a reduction or increase in \textit{Enpp1} expression, ectopic mineralisation may occur. Thus the precise role that NPP1 plays in modulating vascular calcification has yet to be fully defined, and requires further investigation.

**Calcium phosphate homeostasis**

The recent demonstration of elevated expression and circulating levels of fibroblast growth factor 23 (FGF-23) in \textit{Enpp1}−/− mice [40] is consistent with human genetic studies that have shown that mutations in \textit{ENPP1} can cause hypophosphataemic rickets resulting from increased levels of FGF-23 [4]. These findings add to a growing number of single gene mutations whose activation impairs bone mineralisation and leads to changes in FGF-23 gene transcription [68]. As well as in \textit{ENPP1}, mutations in other regulators of phosphate homeostasis, including phosphate regulating endopeptidase homolog, X-linked (\textit{PHEX}) and dentin matrix protein-1 (\textit{DMP1}), cause hypophosphatemic disorders and stimulate expression of FGF-23 [69,70]. This indicates that levels of bone metabolism and systemic phosphate homeostasis are tightly coordinated.

FGF-23 is a phosphaturic hormone that controls phosphate homeostasis, calcium homeostasis and bone mineralisation. FGF-23 binds to FGF receptors (mainly \textit{FGFR1}) and the co-receptor \textit{KLOTHO} in the kidney and promotes excretion of P, which leads to reduced serum P, [71,72] and stimulation of Cyp24 and inhibition of Cyp27b1 in the kidney to reduce circulating 1,25(OH)2D levels. Thus, the decreases in circulating calcium and phosphate levels reported in \textit{Enpp1}−/− mice are consistent with increased FGF-23 [40]. The mechanism whereby \textit{Fgf-23} gene transcription in bone is stimulated by NPP1 inactivation has yet to be defined, however, recent studies have indicated that alterations in matrix mineralisation induced by other single gene mutations in osteoblasts lead to stimulation of FGF-23 expression via FGF receptor activation [73]. It is not clear whether the increase in FGF-23 observed in \textit{Enpp1}−/− bone is intrinsic and due to pathways similar to \textit{Phex} and \textit{Dmp1} mutations [69,70] or as a result of distinct signalling pathways. The increases in serum FGF-23 levels reported in \textit{Enpp1}−/− mice [40] may regulate the \textit{Enpp1}−/− bone phenotype through the bone–kidney axis or through local effects on bone cells. There is also controversial evidence that indicates that FGF-23 may directly affect skeletal mineralisation, independent of phosphate homeostasis [74], which further confounds the relationship between NPP1 and FGF-23 in \textit{Enpp1}−/− mice. Further research is required in order to fully elucidate the mechanisms through which NPP1 and FGF-23 are acting to modulate bone mineralisation.

Furthermore, the role of the FGF-23/KLOTHO axis in mediating vascular calcification is a subject of increasing interest. Although the interaction between NPP1 and FGF-23 has not been investigated during vascular calcification it is interesting to note that there is an association between FGF-23 levels and calcium accumulation in the aorta and coronary arteries of patients with chronic kidney disease (CKD) [75–77]. Indeed, elevated FGF-23 levels in patients with CKD have also been associated with the presence of widespread atherosclerosis [78] and left ventricular hypertrophy [79,80]. High levels of ectopic calcification and disrupted bone structure have been described in Fgf-23−/− mice [81,82], similar to the phenotype described in \textit{Enpp1}−/− mice. FGF-23 over-expressing mice also show a disrupted bone phenotype, with no ectopic calcification [83–85]. Recent evidence suggests that FGF-23 plays a protective role in vascular smooth muscle cells [86] but the precise actions of FGF-23, and its possible relationship with NPP1, during vascular calcification remain unclear and require further investigation.

**Insulin signalling and glucose homeostasis**

The link between NPP1 and insulin signalling was first described in a seminal study by Maddux and colleagues nearly two decades ago. NPP1 activity was shown to be increased in dermal fibroblast cultures from patients with non-insulin-dependent type 2 diabetes and severe insulin resistance [6]. Defective insulin-stimulated autophosphorylation of the insulin receptor (IR) was also observed in these cells, leading to the hypothesis that NPP1 acts as an inhibitor of the IR [87]. Subsequently, NPP1 has been shown to directly interact with the receptor α-subunit of the IR, blocking the insulin signalling pathway [88]. Additional studies in humans have also revealed that increased NPP1 expression in muscle correlates with increased body mass index and decreased insulin stimulation of muscle glucose transport [7,89], indicating a possible link between levels of NPP1 in muscle and insulin resistance.

Studies in animal models have shown that NPP1 regulates insulin signalling in both in vitro and in vivo settings. Transgenic mice with liver specific over-expression of human \textit{ENPP1} show insulin resistance and glucose intolerance, although the animals are not overtly diabetic [90]. However, transgenic mice with human \textit{ENPP1} over-expressed in both liver and muscle have fed and fasting hyperglycaemia with hyperinsulinaemia, suggesting that NPP1 may play a role in the insulin resistance and hyperglycaemia of type 2 diabetes. These findings have been further supported by murine studies demonstrating that in the presence of a high-fat diet, \textit{Enpp1} over-expression in adipocytes induces fatty liver, hyperlipidaemia, and dysglycaemia, thus recapitulating key manifestations of the metabolic syndrome [91].

The majority of animal studies to date have focused on the effects on insulin signalling induced by over-expression of \textit{Enpp1}. However a study by Zhou and colleagues [92] investigated the biological effect of NPP1 suppression. This research demonstrated that knockdown of \textit{Enpp1} with siRNA significantly increases insulin-stimulated Akt phosphorylation in HuH7 human hepatoma cells. In vivo studies utilising the \textit{db/db} mouse model of diabetes revealed that \textit{db/db} mice treated with \textit{Enpp1}-1 short hairpin RNA adenovirus showed reduced hepatic \textit{Enpp1} mRNA levels and decreased fed and fasting plasma glucose, with a concomitant improved oral glucose tolerance. Taken together, these results demonstrate that suppression of \textit{Enpp1} expression improves insulin sensitivity, supporting the proposition that NPP1 inhibition is a potential therapeutic approach for the treatment of type 2 diabetes.

Multiple linkage studies have associated the chromosome locus mapping \textit{ENPP1}, to insulin resistance [90,93,94], hyperglycaemia [95], childhood and adult obesity and increased risk of type 2 diabetes [8]. Furthermore, specific polymorphisms have been identified, of which Lys121Gln (K121Q) [96] is the most widely investigated. Over-expression of the \textit{NPP1} Gln121 variant in vitro has been shown to have increased IR inhibition activity in cell lines representing the
liver (HepG2) and skeletal muscle (L6) when compared to the over-expression of the Lys121 variant [93]. This study showed that the Gln121 has a higher affinity to the IR, leading to a stronger inhibition of autophosphorylation. In the pancreatic β-cell line INS1E, over-expression of the Gln121 variant induced a significant increase in apoptosis, and almost abolished glucose induced insulin secretion, however the mechanism by which NPP1 mediates this reduction was not investigated. It is of particular interest that the over-expression of ENPP1 alone, regardless of the variant, induced an 80% reduction in insulin secretion in INS1E cells, and a 20% and 50% decrease in IR autophosphorylation in HepG2 and L6 cells respectively [93].

Despite the existing evidence from in vitro studies on the increased susceptibility to insulin resistance of the Gln121 variant, there are now an increasing number of population association studies that show conflicting data about the linkage of this variant to insulin resistance, type 2 diabetes and obesity, which was extensively reviewed by Goldfine et al. [97]. Two recent studies have shown no association of Gln121 with type 2 diabetes in the Iranian and northern Chinese populations while previous studies on a Finnish population showed a strong linkage to early onset type 2 diabetes [98]. However, the largest Lys121Gln meta-analysis in type 2 diabetes to date, conducted on European populations, showed a modest increase of the Gln allele to risk of type 2 diabetes [99]. It is therefore likely that ethnic origin and environmental factors influence the development of type 2 diabetes, therefore confounding the role of NPP1 as a risk factor.

A fuller appreciation of the role of NPP1 in regulating insulin signalling and glucose homeostasis in newly defined metabolic tissues such as bone, as well as in established endocrine organs such as the pancreas and liver, is essential for the advancement of new potential strategies for the prevention and control of diabetes.

Conclusions

NPP1 is known to play vital roles in calcium/phosphate regulation, and repression of soft tissue mineralisation, as well as maintaining skeletal structure and function. A greater understanding of the actions of NPP1 in novel pathways such as insulin signalling in bone, in concurrence with the full elucidation of the mechanisms underpinning and connecting the known effects of NPP1, may stimulate the development of novel therapeutic treatments for patients with bone diseases, cardiovascular pathologies and diabetes.

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