New perspectives on rare connective tissue calcifying diseases
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Connective tissue calcifying diseases (CTCs) are characterized by abnormal calcium deposition in connective tissues. CTCs are caused by multiple factors including chronic diseases (Type II diabetes mellitus, chronic kidney disease), the use of pharmaceuticals (e.g. warfarin, glucocorticoids) and inherited rare genetic diseases such as pseudoxanthoma elasticum (PXE), generalized arterial calcification in infancy (GACI) and Keutel syndrome (KTLS). This review explores our current knowledge of these rare inherited CTCs, and highlights the most promising avenues for pharmaceutical intervention. Advancing our understanding of rare inherited forms of CTC is not only essential for the development of therapeutic strategies for patients suffering from these diseases, but also fundamental to delineating the mechanisms underpinning acquired chronic forms of CTC.

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Basic mechanisms of bone mineralization
In order to understand more fully the mechanisms underpinning connective tissue calcification, it is important to appreciate the physiological process of bone mineralization, which occurs through the deposition of hydroxyapatite (HA) onto a collagenous extracellular matrix (ECM). HA crystal formation is regulated by matrix vesicles (MVs), which maintain calcium (Ca2+) and inorganic phosphate (P) concentrations at levels optimal for HA nucleation. As the HA crystals grow they disrupt the MV and deposit onto the ECM where they continue to grow [3]. The transport of Ca2+ into MVs is primarily controlled by annexin channels, whereas P is transported into the MV by the type III sodium-dependent P, co-transporter-1 (PiT-1) [4]. Intracellular to extracellular channelling of pyrophosphate (PPi) is mediated by ANK [5].

Whilst P acts to promote HA crystal formation, PP, generated by ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), has a dual role as an inhibitor of HA
generation and as a precursor to Pi [6]. The ratio of Pi to PPi is controlled by a complex interaction between the regulatory phosphatases tissue non-specific alkaline phosphatase (TNAP) and phosphoethanolamine/phosphocholine phosphatase (PHOSPHO1). TNAP hydrolyses PPi in the ECM to release Pi and PHOSPHO1 hydrolyses phosphocholine and phosphoethanolamine to produce Pi inside the MVs. Together these phosphatases control the P/Pi balance during the mineralization process [7]. Further feedback signalling allows modulation of mineralization; inorganic pyrophosphatase stimulates mineralization without reducing PPi levels [8]. Both exogenous Pi and Pi upregulate the bone sialoprotein osteopontin (OPN), which in turn inhibits mineralization through restricting HA crystal formation and growth [9]. Intriguingly, a clear dissociation in the hierarchical roles of Pi and OPN has recently been highlighted [10*].

Overview of mechanisms of connective tissue calcification

The control and regulation of connective tissue calcification is a multifactorial process which shares many similarities with that of the physiological matrix mineralization during skeletal development described previously. A wealth of knowledge emanates from research into vascular calcification, a strong and independent predictor of morbidity and mortality in cardiovascular disease [2]. Indeed normal vascular smooth muscle cell (VSMC) populations contain cells that undergo phenotypic transition to osteocytic, osteoblastic and chondrocytic cells in a calcified environment [11]. In VSMCs, MVs have been shown to nucleate hydroxyapatite crystals that contain calcium and inorganic phosphate [12**] forming the first nidus for calcification. This nucleation occurs via a tightly controlled balance of inhibitors and inducers comparable to that seen in bone, with PHOSPHO1, sphingomyelinase 3 (SMPD3), TNAP, annexins, ANK and ENPP1 playing key regulatory roles [2,12**]. Furthermore, MVs derived from VSMCs have been shown to contain negative regulators of hydroxyapatite crystal nucleation and growth, such as fetuin-A and matrix gla protein (MGP) [13*]. In cooperation with local mediators such as Pi [6], these molecules protect the arteries from mineral deposition and growth. In the absence of these inhibitors, or following the stimulation of apoptotic processes [14*], together with the osteogenic activity of VSMCs, vascular calcification readily proceeds.

A key role for pyrophosphate (Pi) in rare CTCs

It has recently been established that connective tissue calcification is contingent on circulating Pi levels rather than local PPi production [15]. As previously highlighted, Pi not only acts as a potent inhibitor of connective tissue calcification, but also contributes directly to the calcification process. Intriguingly, one of the identified sources of systemic Pi is through ATP binding cassette sub-family C member 6 (ABCC6)-mediated ATP release from hepatocytes [16]. Still within the vasculature of the liver, released ATP is rapidly converted to Pi and AMP by ENPP1, which are in turn distributed throughout the body via the circulation. In connective tissues the metabolite AMP is further hydrolysed into Pi and adenosine by ecto-5’-nucleotidase (CD73). Adenosine in turn inhibits TNAP transcription, thus decreasing Pi production and more importantly maintaining Pi levels [17]. Different perturbations in this mechanism can contribute to several rare CTCs (Table 1) which whilst varying in degree of severity and phenotype, show notable overlap.

Table 1

<table>
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<th>The cause and phenotype of significant rare inherited CTCs</th>
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<td>CTC disease</td>
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Disease models of connective tissue calcification

Pseudoxanthoma elasticum (PXE)

A defect in the ABCC6 gene is responsible for pseudoxanthoma elasticum (PXE) (#OMIM 264800), an inherited autosomal recessive multisystem disorder affecting connective tissues in humans. The ABCC6 gene carried by chromosome 16p13.11 encodes a trans-membrane ATP-binding cassette transporter, subfamily C, member 6 [15], which is primarily expressed in the hepatocyte and to a lesser extent in the proximal tubule cells in the kidney. The phenotypic expression of the disease is characterized by the fragmentation and mineralization of elastic fibres in the skin, retina and arterial wall [16–18]. The symptoms are represented by unesthetic skin folds, central blindness and adverse cardiovascular events (Figure 1).

To date, the role of ABCC6 and the nature of its substrate(s) in the PXE disease remain unknown and are presently subject to extensive research efforts worldwide. After several inconclusive hypotheses, recent seminal studies disclosed that ABCC6 participates in the release of intracellular nucleotides (such as ATP) by a yet unknown pathway in the liver [19**]. This results in a decrease in the extracellular ATP available for degradation by hepatic ecto-nucleotidase enzymes (such as ENPP1), which ultimately leads to less circulating PPi, a powerful tissue and circulating anti-calcifying factor (as highlighted above) [20]. This important study places the liver, and to a lesser extent the kidney, as the metabolic control centre of PXE-associated calcification, with almost 40% of the overall systemic PPi production generated from the liver [21**], which cannot be compensated for by the local release of PPi, through alternative ectonucleotidic pathways (i.e. ENPP1 or TNAP).

Additional confounding factors of genetic [22] and/or metabolic origin [23–25] highlight the complex direct and remote interactions in PXE. Further clinical manifestations of PXE, such as elevated thrombotic susceptibility, increased myogenic tone and vascular malformations [26,27] may be a result of altered purinergic signalling, which unequivocally mediates complex autocrine and paracrine cellular signalling pathways within various tissues [28–31].

PXE represents a prototypic metabolic disease that shares some of the features of calcifying vascular diseases of acquired metabolic origin such as type II diabetes mellitus and CKD [2]. The discovery of the role of ABCC6 raises new challenging questions on the central role of the hepato-renal axis in the connective-tissue calcifying process. From a clinical point of view, the current absence of an efficient targeted therapy requires the tight control and management of the typical cardiovascular risk factors associated with PXE, in addition to the limitation of pro-calcifying conditions. Promising new perspectives for etiologic treatments are however in progress, and may shortly yield exciting new treatment options for patients with PXE.

Generalized arterial calcification in infancy (GACI)

Mutations in ENPP1 are known to cause a rare human disease phenotype, namely generalized arterial calcification in infancy (GACI, MIM# 208000), formerly known as idiopathic infantile arterial calcification (IAC) [32]. In GACI, calcification of the media of large and medium-sized arteries (Figure 2a) is associated with intimal proliferation (Figure 2b) leading to arterial stenosis. Depending on the severity and the local distribution of the calcific stenoses, affected infants can present with neonatal heart failure, arterial hypertension and death within the first six months of life [33]. To date, more than 40 different causative mutations in ENPP1 have been identified in GACI patients accounting for approximately 70% of the affected cases [33]. Based on the number of reported pathogenic variants in NHLBI ESP6500 (16 carriers in 6021 individuals) a carrier frequency of one in 376 individuals (0.27%), and a disease frequency of one in 566,000 individuals can be estimated [34**].

The cardiovascular phenotype of the disease is quite variable [35] and can even vary to a great extent between siblings carrying the same mutations: of two Taiwanese siblings with identical genotype, one developed extensive arterial calcification and severe hypertension, and died of heart failure at the age of 6 weeks, while the other

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**Figure 1**

Diffuse arterial calcifications visualized by CT-scan in the lower limb arteries of a patient with pseudoxanthoma elasticum (PXE).
sibling had an uncomplicated clinical course [36]. The phenotype of the disease is recapitulated in the so-called tiptoe-walking (ttw/ttw) mouse associated with articular cartilage and peri-skeletal ligament calcification and also with aortic calcification [37,38]. In these mice, connective tissue calcification progresses to hyperostotic joints and spine ankylosis leading to the ‘tiptoe-walking’ phenotype. A comparable phenotype can be found in \textit{asilaj} mice, which carry the V246D missense mutation [39,40] and in \textit{Enpp1} knockout mice [41,42,43]. Intriguingly, deficiency of ENPP1 has also been shown to exert protective effects against obesity and diabetes in mice [44], however, the metabolic phenotype has yet to be assessed in GACI patients, and warrants future investigation.

Hypophosphataemia can compensate for the GACI phenotype and this might reflect a physiologic compensation mechanism rather than a primary defect [33]. However, several mutations in the \textit{ENPP1} gene can result in the phenotype of autosomal-recessive hypophosphatemic rickets (ARHR) without any arterial calcification [45,46], suggesting a different pathway involved in the generation of ARHR linked to direct renal P_{3}'-handling functions of ENPP1. Treatment with synthetic analogues of pyrophosphate, namely bisphosphonates seems to significantly increase survival in patients with GACI [33], however, spontaneous regression of the calcifications can occur [47], and studies on the exact long term natural history of the disease are pending. Based on the finding that mutations in \textit{ENPP1} can also cause PXE and that mutations in \textit{ABCC6} can also cause GACI it has become obvious that an overlap of genotype and phenotype in GACI and PXE exists [48]. This has led to the hypothesis of a shared pathogenic principle in GACI and PXE [49], which finally held to be true. Most recently, subcutaneous administration of an ENPP1–Fc fusion protein was shown to prevent the mortality, vascular calcifications and sequelae of disease in the \textit{asilaj} mouse model of GACI [50]. This very promising preclinical study may pave the way to clinical trials with enzyme replacement therapy in patients with this rare disorder carrying mutations in \textit{ENPP1}.

\textbf{Arterial calcification due to deficiency of CD73 (ACDC)}
Calcification of joints and arteries (arterial calcification due to deficiency of CD73 (ACDC); OMIM# 211800), was originally described by Magnus-Levy [51] and recently shown to be caused by deficiency of CD73, encoded by the NT5E gene [52,53]. Clinical features include calcified large vessels and periarticular calcifications in the joints of the hands and feet. CD73 has 5’ exonuclease activity that converts AMP to adenosine and P_{1} [54]. Receptor binding of adenosine triggers a downstream intracellular signalling cascade that results in inhibition of TNAP activity [55]. Thus, it has been proposed that increased TNAP activity is central to the mechanism underpinning ACDC.

\textbf{Hutchinson–Gilford progeria syndrome}
The rare premature aging disorder Hutchinson–Gilford progeria syndrome (HGPS) is characterized by excessive atherosclerosis and both blood vessel and aortic valve calcification [56–58]. HGPS patients express progerin, a mutant form of lamin A. Progerin expression results in
abnormal nuclear membrane architecture causing abnormal higher-order chromatin organization [59,60]. Knock-in mice expressing progerin exhibit reduced circulating PPi levels similar to mice lacking ENPP1 [61]. Interestingly this low PPi is the result of increased TNAP activity and decreased extracellular ATP due to mitochondrial dysfunction. Consistent with this observation of supressed PPi levels, these mice show excessive aortic calcification which is ameliorated by exogenous PPi administration [61]. These findings suggest connective tissue calcification in HGPS shares key features of PXE (decreased ATP), GACI (low PPi) and ACDC (increased TNAP activity), further demonstrating the overlap between these CTCs.

**Keutel syndrome**

Keutel syndrome (KTLS) (OMIM#245150) is an extremely rare autosomal recessive disorder that manifests during the early childhood of patients predominantly from the Middle East. Since its first description in 1971 by Keutel and colleagues in two consanguineous siblings [62], less than 30 patients have been reported to date.

Foremost clinical characteristics of KTLS include abnormal calcification in laryngal, tracheobronchial, auricular, nasal and rib cartilage, brachytelephalangism and facial abnormalities such as mid-facial hypoplasia, depressed nasal bridge and reduced alae nasi [62–67]. Further symptoms include mild to severe unilateral or bilateral hearing loss, multiple peripheral pulmonary artery stenosis, mental retardation and respiratory conditions, including dyspnoea and cough, that lead to hospitalization and incidentally to diagnosis [62–68]. Additionally, long-term follow-up studies have revealed that, KTLS patients develop skin lesions, typically after 30 years of age [69] and suffer chronic and progressive respiratory disease caused by gradual laryngotracheobronchial calcification and stenosis [65,69]. Subsequent post-mortem examination of the youngest sibling originally described has also uncovered calcification of pulmonary, coronary, hepatic, renal, meningeal and cerebral arteries [65–68].

It is well established that KTLS is due to loss-of-function mutations in the *MGP* gene [66,67,70–72], encoding MGP, a potent local mineralization inhibitor predominantly expressed by chondrocytes and VSMCs [73]. Post-translational modifications of MGP, such as vitamin K-dependent glutamate carboxylation and serine phosphorylation, have been shown to trigger these inhibitory properties [74]. Mice lacking *Mgp* develop abnormal cartilage calcification and extensive vascular calcification that leads to premature death due to aortic dissection [73]. Although all of the seven mutations reported in humans predict absent or non-functional MGP, they result in variable phenotypes with cardinal and secondary features of variable penetrance [66,67,70–72]. Moreover, in contrast to *Mgp*-deficient mice, with the exception of one clinical case [65,67], vascular calcification has not been observed in KTLS patients. Interestingly, measurements of circulating MGP species have highlighted high levels of phosphorylated MGP [67]. This suggests that phosphorylation-dependent residual MGP activity may contribute to the absence of arterial calcification and more generally, that the variable phenotypic features observed clinically in KTLS could be explained by altered levels of the different MGP species.

Systemic hypertension is frequently found in KTLS patients before adulthood. Therefore, under the control of standard anti-hypertensive medication [69], KTLS has a good prognosis. Nevertheless, life expectancy of patients primarily depends on the severity of the associated respiratory complications. Indeed, symptomatic treatment with corticosteroids or bronchodilators is not always effective [65,67,69]. Interestingly, a recent study reported the efficacy of BMP inhibitors in reducing vascular calcification and improving survival in *Mgp*<sup>−/−</sup> mice [75**]. Besides targeting vascular calcification, which is very rare in KTLS, BMP inhibition strategies could also be employed as a pharmaceutical approach to reduce abnormal cartilage calcification. This would have particularly high therapeutic value if successfully developed to treat tracheobronchial tree calcification, which initiates respiratory distress and complications, and ultimately determines the quality and duration of life of KTLS patients.

**Fibrodysplasia ossificans progressiva (FOP)**

Fibrodysplasia ossificans progressiva (FOP; OMIM#135100) is a devastating rare disease, characterized by the progressive heterotopic endochondral ossification (HOE) of skeletal muscle, fascia, tendons, and ligaments [76]. The range of joint motion in FOP patients becomes gradually and progressively limited by HOE. Indeed, this ossification is so diffuse that it is commonly referred to as a second skeleton [76]. HOE occurs in flare ups and is most commonly triggered by muscle injury, as also observed in *Abca6*<sup>−/−</sup> mice [77], and viral infection. Unlike other CTCs discussed in this review, FOP is not the result of a deficiency but rather a gain of function mutation. All patients with the typical FOP phenotype have a substitution of the Arg206 residue for a His residue in Activin A receptor type I (ACVR1) which is a type I Bone Morphogenetic Protein Receptor (BMPR) [78]. This A206H ACVR1 exhibits ligand free activation of its BMP signalling pathways and a greatly enhanced response to BMP [79,80]. Studies suggest that FKBP1A (a protein that negatively regulates BMP type I R) has reduced binding capabilities to A206H ACVR1, preventing it from modulating its activity [79–81]. Knock-in mice that express the A206H ACVR1 recapitulate the phenotype of FOP patients [82], with data from these mice and other models suggesting that TIE2<sup>+</sup> mesenchymal cells are the progenitors of the HOE through their response to
tissue inflammation [83–85]. Currently, management of FOP symptoms is limited to glucocorticoids and non-steroidal anti-inflammatories to minimize flare ups and pain [76], with no therapeutic strategy currently available to inhibit or prevent the HOE associated with this rare CTC. Given the positive effects of BMP inhibitors in Mgp-deficient mice, it would be beneficial to recapitulate these studies in the FOP mouse model as a first step to a pharmaceutical approach.

**Rare CTCs of the brain**

There are a number of disorders demonstrating connective tissue calcification in the brain, including coeliac disease with Epilepsy and Cerebral calcifications (CEC; OMIM# 226810). To date, less than 200 CEC patients have been reported in the literature [86]. The suspected Mendelian basis of this disease is characterized by three primary pathologies. The first is epilepsy, most commonly occipital epilepsy. Drug resistance is also common for this epilepsy with evolution towards epileptic encephalopathy. The second pathology is cerebral calcification, with bilateral occipital calcifications most frequently observed. The third pathology is coeliac disease, although in older patients (>2 years) bowel symptoms are less common [87,88]. Whilst folate deficiency has been proposed as the cause of CEC [89], recent studies suggest a possible autoimmune component to the disease with the identification of autoantibodies to transglutaminase isoenzyme 6 in the serum of a CEC patient [90]. Current CEC treatment regimens

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Figure 3

Model of a functional network revealed by rare CTCs (GACI due to ENPP1 deficiency, PXE due to ABCC6 deficiency, KTLS due to MGP deficiency, ACDC due to CD73 deficiency, HGPS due to abnormal progerin expression and FOP due to ACVR1 gain of function). This model describes how ENPP1, ABCC6, MGP, CD73, progerin and ACVR1 serve as components in a network of factors, concurrent with established mechanisms of matrix vesicle regulated HA crystal formation, consequentially exerting balanced effects to promote and suppress connective tissue calcification. ENPP1 generates AMP and PPi from ATP, CD73 (ecto-5’-nucleotidase) hydrolyses AMP to generate adenosine and Pi, TNAP hydrolyses PPi into two Pi molecules. PPi suppresses hydroxyapatite deposition and inhibits connective tissue calcification. Adenosine signalling suppresses TNAP expression. Pi is a component of hydroxyapatite crystal deposition. A206H ACVR1 exhibits an enhanced response to BMP signalling. The roles of ABCC6 and MGP have yet to be fully defined. Progerin causes increased TNAP activity and decreased ATP concentrations due to mitochondrial dysfunction, combined these effects of progerin result in decreased PPi.
include folate supplementation and gluten free diet, the efficacy of which has been shown to directly correlate with how early treatment is implemented [87,88].

Idiopathic Basal Ganglia Calcification (IBGC; OMIM# 213600) is characterized by calcification of the basal ganglia as well as the thalamus and cerebellum [91]. Patients display a range of neuropsychiatric and movement disorders including dementia, psychosis, Parkinsonism, dystonia, and migraine. Mutations in the gene for type III sodium-dependent P1 co-transporter 2 (PiT-2) leading to impaired or loss of function have been identified in several patient cohorts [92–96]. Direct evidence for a role for PiT-2 in IBGC was first provided by studies investigating the phenotype of mice lacking PiT-2. These mice develop calcification predominantly in the thalamus but also in the basal ganglia and brain cortex [97]. Of particular interest, histological analyses of these mice suggest that calcification initiates in or around the vasculature which closely mimics the phenotype observed in IBGC patients [91]. Mutations in the gene for platelet derived growth factor receptor B (PDGFRB) have also been described in IBGC patients [98]. PDGFRB regulates PiT-1 in VSMCs [99,100] and a role for this molecule in IBGC would be consistent with a vascular origin of the calcification. Considering IBGC patients have normal circulating P1 levels [91] any role PiT-2 and/or PDGFRB may play in calcification would be local to the affected tissue. This highlights the heterogeneity of P1 regulation in different tissues which should be an important consideration when planning pharmaceutical interventions for CTCs.

Future directions
Much of our understanding of the potential mechanisms underpinning CTCs (Figure 3) has arisen through the use of rodent models. However, significant differences between physiology, anatomy, and pathology exist between mice and men. In contrast, large animal models can show markedly greater similarity to humans [101]. The recent explosion of precise and efficient genome editing techniques through CRISPR/Cas9 technology permits the generation of tailored models for translational research [102]. These novel systems provide huge potential for large animal models for future investigations into the regulatory factors and molecular pathways that contribute to rare inherited forms of CTCs in vivo.

At present, only very limited pharmaceutical strategies exist to inhibit connective tissue calcification. Further pre-clinical and clinical studies are required to examine new approaches such as targeting mechanisms common to different CTCs (e.g. PPi, regulation) and/or enzyme replacement therapy (e.g. ENPP1). These investigations may bring to fruition the first comprehensive treatment for both inherited and acquired CTCs.

Conflict of interest statement
Nothing declared.

Acknowledgements
This work was supported by an Institute Strategic Programme Grant (BR/ J004316/1) from the Biotechnology and Biological Sciences Research Council (BBSRC) (VEM, NAR), the French Society of Vascular Medicine (GL), the PXE-France french patients’ association (GL), PXE-International (GL), Université de Lorraine (HK), Centre National de la Recherche Scientifique (CNRS) (HK), Institut National de la Santé et de la Recherche Médicale (INSERM) (HK) and Deutsche Forschungsgemeinschaft (DFG) (FR) and The Hungarian Scientific Research Fund (OTKA) (AV).

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:
* of special interest
** of outstanding interest


This study demonstrates that osteopontin (Opn) is elevated in Phospho1 knock out mice and that concurrent knockout of Opn with Phospho1 rescues the skeletal phenotype.


This study identifies matrix vesicles as exosomes and demonstrates that factors that can increase exosome release promote vascular calcification.
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This study identifies matrix vesicles as exosomes and demonstrates that factors that can increase exosome release promote vascular calcification.


This paper demonstrates that autophagy is dependent on oxidative stress and that inhibiting it increases calcification, whilst inducing autophagy decreases calcification.


This study reveals that the factor which normally prevents PXE is pyrophosphate, which is provided to the circulation in the form of nucleoside triphosphates.


This is the first report to demonstrate that the liver is the main source of circulating ATP and that this is mediated by ABC6C.


This study demonstrated for the first time that ENPP1 is expressed both in osteocytes and osteoclasts and further characterizes the ENPP1 allele phenotype.


This is the first report to demonstrate the efficacy of exogenous recombinant ENPP1 to treat GACI.


This study demonstrates that inhibiting BMP signalling decreases vascular calcification in MGP−/− mice and that some of the features of MGP are dependent on BMP signalling.


This paper provides a current indelth review of large animal models relevant to cardiovascular disease.


This review describes the development and applications of Cas9 for a variety of research and translational applications.