DECIPHERING ECTOPIC CALCIFICATION: CONTRIBUTION OF THE RARE, INHERITED DISORDER PSEUDOXANTHOMA ELASTICUM

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ABSTRACT

Soft connective tissue calcification is still an intriguing problem due to the high number of genes, proteins, and enzymes involved in the process. Numerous epidemiological and experimental studies of the ectopic calcification associated with metabolic, inflammatory, and degenerative disorders have been performed. Moreover, in the last decade, great efforts have been made in studying the genetic disorders leading to soft connective tissue calcification, trying to understand the imbalance between pro and anti-calcifying factors in the different disorders, and why calcification occurs only in certain body regions (which often differ between the various genetic defects). The rare, inherited disorder pseudoxanthoma elasticum (PXE), which is caused by mutations in the *ABCC6* gene, is an interesting model because the gene responsible is mainly expressed in the liver, whereas calcification affects peripheral soft connective tissues. It has been suggested that liver deficiency of the protein encoded by *ABCC6* directly induces peripheral calcification, although, in contrast, several studies both in humans and in transgenic mice indicate that peripheral mesenchymal cells might be permanently involved in PXE calcification. In this review, the author suggests that early in development PXE cells may undergo epigenetic changes and acquire a permanent pro-calcific signature. However, given the complexity of the calcification process and the metabolic inter-exchanges among the different calcific genetic disorders, a bioinformatic approach analysing data ranging from genes to functional proteins and clinical features may complete the puzzle and provide new therapeutic perspectives in PXE, as well as in other calcific disorders.

Keywords: Soft connective tissue calcification, ectopic calcification, extracellular matrix calcification, fibroblast gene expression, fibroblast epigenetic changes, calcification genetic disorders, pseudoxanthoma elasticum.

SCIENTIFIC BACKGROUND

Soft connective tissue calcification is frequently observed in metabolic or degenerative/inflammatory processes, such as diabetes, chronic kidney disease, and atherosclerosis,[1-3] or within necrotic materials, such as in advanced atherosclerosis and tuberculosis.[4,5] The process has also been induced in animals and in cells *in vitro* via treatment with chemicals[6,7] or excess vitamins[8,9] in order to understand the metabolic pathways involved. Interestingly, mesenchymal cells were shown to switch to pro-osteogenic gene expression under the influence of various stimuli.[8,10,11] In the previous decade, great advances have been derived from studies on genetic disorders characterised by soft connective tissue calcification.

THE CALCIFICATION PROCESS AND ITS CONTROL

Calcification is due to the precipitation of minerals, the most abundant of which is calcium phosphate, followed by magnesium phosphate, and then calcium carbonate and magnesium carbonate.[12-14] Therefore, ectopic calcification is mainly due to hydroxyapatite accumulation. Within cells, free calcium works as a metabolic controller and its low

concentration (about 100-200 nmol/dm^3) is finely regulated by specific calcium-binding proteins.[15-17] Moreover, mitochondria and the endoplasmic reticulum can actively accumulate an excess of calcium in order to maintain intracellular ion homeostasis.[15,16,18] In contrast, calcium concentration in the extracellular space is physiologically very high (about 1 mmol/dm^3),[16,18] and it would form hydroxyapatite in the presence of phosphate, the level of which is, hopefully, finely regulated.[19] Therefore, the concentration of free phosphate can be considered as the limiting factor for extracellular matrix calcification.

Despite the various historical names, there are two main types of calcification: 'passive' and 'active'. Passive calcification is a consequence of metabolic, hormonal alterations,[1-3,7,12] or, more frequently, of cell degeneration and death.[5,20,21] In these cases, the intracellular and extracellular concentrations of free calcium and phosphate become high enough to overcome their solubility, with the formation of the rather insoluble form of calcium phosphate. Very high intracellular ion concentrations can be reached by alteration of the cell membrane barrier[12] due to deficiency in cellular ion-binding proteins and compartments,[16,18] or by the enzymatic liberation of ions, including phosphate derived from nucleic acids and the membrane phospholipids of dead cells.[22] High extracellular ion concentrations may be reached in kidney and hormonal disorders, or by erroneous diets.[1-3,7,8] Moreover, similar to calcification associated with atheroma or tuberculosis,[1,4,5] vessels are scarce in necrotic areas and ions remain sequestered for a long time, which favours the formation of large mineral precipitates. However, modification of the expression of genes involved in the homeostatic control of ion solubility has also been reported in cases of 'passive calcification'.[9,10,23,24] This is not surprising because months or even years are necessary in order to produce clinically relevant calcification, and time-dependent, adaptable gene expression of surviving cells can occur.

Active calcification is a dynamic process that occurs in the extracellular space of soft connective tissues in the absence of metabolic, inflammatory, or necrotic events. This process is very often age-associated and depends on the imbalance of proteins and enzymes that maintain the homeostatic control of calcium and phosphate ion concentrations within the extracellular space. Interestingly, some molecules exhibit opposing functions within bone and in soft connective tissues, as they seem to favour calcification within bone and yet inhibit calcium precipitation in the non-bone connective tissues.[25-29] Interestingly, calcification of soft connective tissues *in vivo* is often associated with, or dependent upon, osteoporotic bone decalcification.[25]

GENES AND PROTEINS INVOLVED IN ECTOPIC CALCIFICATION

Exhaustive reviews describing proteins involved in ectopic calcification have been published recently.[24,26,27] Some of these proteins, such as osteopontin[28] and fetuin,[29] mainly function within the extracellular fluids and inhibit calcification by interfering with crystal growth. Other proteins are involved in the regulation of extracellular ion concentrations at a local level, such as ectoenzyme nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), progressive ankylosis protein (ANK), tissue non-specific alkaline phosphatase (TNAP), matrix Gla protein (MGP), and many others. While all of these proteins and enzymes have specific functions, they also form a functional network whose overall result depends on the polymorphisms and expression of their encoding genes, which are often influenced by environmental factors such as the surrounding matrix. This network is complex and only partially known, with inhibition of the expression of one gene inducing over or under-expression of other genes also involved in the calcification process.[25-27,30]

CONTRIBUTION OF GENETIC DISORDERS TO THE COMPREHENSION OF ECTOPIC CALCIFICATION

In recent years, studies on rare genetic disorders characterised by soft connective tissue calcification have greatly improved our knowledge of the mineralisation process.[26,31-33] For the majority of these diseases, the causative gene is known and transgenic mice are available, often recapitulating, at least in part, the clinical features present in humans. However, even when calcification is a direct consequence of a gene defect, the tissue-specific 'interpretation' of each single gene, either normal or mutated, is far from being completely understood.

A typical form of soft connective tissue calcification occurs in generalised arterial calcification of infancy (GACI), which is a rare genetic disorder caused

by loss of function in the gene encoding the enzyme ENPP1 that hydrolyses adenosine triphosphate to adenosine monophosphate and pyrophosphate (PPi). GACI is therefore characterised by systemic deficiency of the strong anti-calcifying factor PPi within the extracellular space.[34-36] This induces severe calcification of peri-articular tissues and arteries, which leads to heart failure, even possibly within the first months of life.[35,36] It is noteworthy that PPi inhibits the expression of pro-calcifying genes[37] and inhibits calcification because it binds hydroxyapatite and hinders crystal growth.[38] The strong effect of PPi deficiency in GACI suggests that, among the various anti-calcifying molecules, PPi must play a fundamental role, at least in vessels, peri-articular tissues, and cartilage. Recently, the important role of PPi in calcification has been confirmed by the observation that surviving GACI patients may present clinical and histological features typical of another rare genetic disorder, pseudoxanthoma elasticum (PXE), which is characterised by skin, retina, and vessel calcification,[39] and in which matrix calcification is associated with, or dependent upon, a series of peripheral cell metabolic alterations, among which is high TNAP activity and consequent deficiency of extracellular PPi.[40-42] Apart from PPi, TNAP liberates phosphate from a variety of organic molecules, including ATP, phospholipids, and even the DNA of necrotic cells.[43] The expression of TNAP is complex, is regulated by circulating and local factors, and its activity produces high levels of pro-calcific free phosphate.[44,45]

Within the extracellular matrix, PPi and inorganic phosphate are always in a dynamic equilibrium determined by the activity of enzymes, kidney function, hormones, and diet. Generalised calcification due to a high level of phosphate is present in chronic kidney disorders,[46] with alteration of gene expression towards bone-specific phenotypes.[48,49] Interestingly, administration of PPi is able to reduce calcification in haemodialysed patients, uraemic mice, and *in vitro* cells.[49,50] Therefore, the level of PPi must be kept relatively high in peripheral soft connective tissues in order to avoid calcification. This is also influenced by the activity of ANK, which transports PPi from within cells to the extracellular space.[51] The low level of extracellular PPi present in cases of ANK deficiency induces calcification of the articular cartilage and dysregulation of osteoblastic/osteoclastic differentiation, as seen in humans and in transgenic mice.[52,53] ANK deficiency further confirms the importance of PPi as an anti-calcifying factor in the extracellular matrix, but also stresses the importance of tissue-specific gene expression for the maintenance of appropriate levels of PPi within the extracellular milieu of different body regions.

Another important anti-calcifying molecule is the mature form of MGP. MGP deficiency, both in humans[54] and in transgenic mice,[31] has demonstrated the importance of glutamic-acid-rich proteins within the extracellular space.[55] In particular, MGP seems to prevent calcium precipitation within vessel walls and within peripheral soft connective tissues due to its ion-binding capability[56] and also due to its interaction with extracellular matrix molecules that have a relevant role in calcification.[57] As shown in both humans and transgenic mice, mutations in the *MGP* gene may lead to a loss of expression or deficient maturation of the protein, which causes a decrease in its anti-calcifying potential. MGP is actively produced by fibroblasts and smooth muscle cells, and seems to regulate calcification at a local level, although it is present within the systemic circulation.[58] Moreover, the activity of MGP depends on its post-transcriptional maturation, i.e. γ-carboxylation of glutamic acid residues and phosphorylation of serine residues.[59] These events are governed by specific genes and may also depend on exogenous factors such as vitamin K.[60] Indeed, a study on another human genetic disorder characterised by severe calcification of skin (vitamin K epoxide reductase [VKOR] deficiency), showed that MGP deficiency may be due to impairment of the vitamin K cycle, which produces the reduced form of vitamin K necessary for γ-carboxylation and maturation of MGP.[61] In addition, calcification in humans may also depend on the ability of the microRNA molecule miR-133a to regulate expression of VKOR complex subunit 1.[62] These data once again illustrate the complex interdependence of genes and environmental factors in the calcification process.

Low amounts of mature, γ-carboxylated MGP have also been observed in PXE, which is characterised by calcification of elastic fibres within soft connective tissues.[63,64] Fibroblasts isolated from the dermis of PXE patients produce a low amount of γ-carboxylated MGP *in vitro*.[65] Interestingly, this immature form of MGP is strongly associated with calcified elastic fibres in the dermis of PXE patients.[66] Low levels of MGP were also observed

in the ATP-binding cassette, subfamily C member 6 (*Abcc6*) −/− model of PXE.[67] Therefore, MGP seems to play an important role in the calcification process caused by ABCC6/*Abcc6* deficiency. Given the importance of vitamin K to MGP maturation, this vitamin was thought to be directly involved in PXE calcification and this was supported by the low level of vitamin K measured in PXE patients.[68] However, studies on transgenic mice[69] and on fibroblasts *in vitro*[70] failed to confirm a role for vitamin K in PXE-associated MGP maturation. Interestingly, data from PXE fibroblasts *in vitro* showed that vitamins K1 and K2 increase cellular protein carboxylation, with the exception of MGP.[71] However, given the recent observation that vitamin K administration reduces mineralisation in zebrafish models of PXE and GACI,[72] the role of vitamin K and of vitamin K-dependent MGP expression and maturation should be investigated further in both humans and animal models.

CONTRIBUTION OF PSEUDOXANTHOMA ELASTICUM TO THE UNDERSTANDING OF ECTOPIC CALCIFICATION

The current review describes data derived from studies on plasma and *in vitro* fibroblasts isolated from the dermis of PXE patients obtained in our laboratory through the use of various techniques, from molecular biology and gene expression to metabolic and structural analyses. PXE is an interesting model for studying the calcification process. It is a recessive inherited disorder caused by mutations in the gene *ABCC6*, which encodes a protein that is a member of the ATP-binding cassette (ABC) family of membrane transporters, mainly expressed in the liver and kidney and much less in other tissues.[73] Its role is to export substances directly or indirectly involved in calcification out of hepatocytes. PXE is characterised by progressive calcification of elastic fibres, mainly in the medium dermis (Figure 1), in vessel walls, and in Bruch's membrane within the retina (Figure 2).[39,40,63,64] These organs are located far from the liver, kidney, and other major gene-expressing tissues,[73] and therefore it is reasonable to accept that plasma from PXE patients is deficient in anti-calcific molecules produced and secreted by the liver,[71] or that peripheral cells, such as smooth muscle cells and fibroblasts, modify their metabolism towards a more pro-calcifying phenotype as a consequence of liver ABCC6 deficiency.[40,41,74]

The substrates of the protein encoded by *ABCC6* are still under investigation: it has been suggested that leukotriene C4, organic anions, and glutathione conjugates, as well as synthetic compounds, could be substrates.[75,76] Data obtained through molecular docking and virtual-screening approaches indicate that lipids are also potential substrates for ABCC6.[77] Recent data suggest that ABCC6 mediates the release of ATP from hepatocytes,[78] with mutations in the transporter leading to low plasma levels of ATP-derived, anti-calcific PPi. Interestingly, a deficiency in circulating ATP would be in agreement with the markers of oxidative stress observed in the plasma of PXE patients[79] and in *Abcc6* −/− mice,[80] as well as with the low peripheral level of anti-calcifying PPi due to the low level of substrate available for ENPP1.[34-36] Less clear, and more indirect, is the effect of plasma ATP deficiency on proteoglycan,[81,82] lipoprotein, and triglyceride[83,84] alterations that we and other groups have observed in PXE patients, as well as in *Abcc6* −/−transgenic mice.

Deficiency in the poorly identified circulating factors produced by the liver is suggested to favour peripheral calcification in PXE.[71] Indeed, we and other laboratories have observed that the serum of PXE patients has an abnormal composition compared with age-matched controls because it has a redox potential lower than normal, high levels of oxidised proteins and lipids,[79] and an abnormal amount and quality of glycosaminoglycans,[82] lipoproteins, triglycerides,[84] and proteins that may directly interfere with calcium precipitation, such as fetuin A.[29,85,86] These alterations *in vivo* may depend on the specific genotype of the PXE patient. However, higher levels of cholesterol and triglycerides, as well as reduced levels of fetuin A, have been measured in transgenic mice that differ only in the ablation of the *Abcc6* gene.[83,86] Therefore, all of the plasma alterations observed in PXE patients, with the majority being confirmed in *Abcc6* −/− transgenic mice, may be the result of an age-dependent adjustment of a series of interdependent genes whose modified expression is due to a loss of function in ABCC6. Moreover, as PXE is a genetic disorder, these alterations would probably start during embryogenesis and therefore profoundly and permanently influence mesenchymal cell metabolism and differentiation.[87]

A B

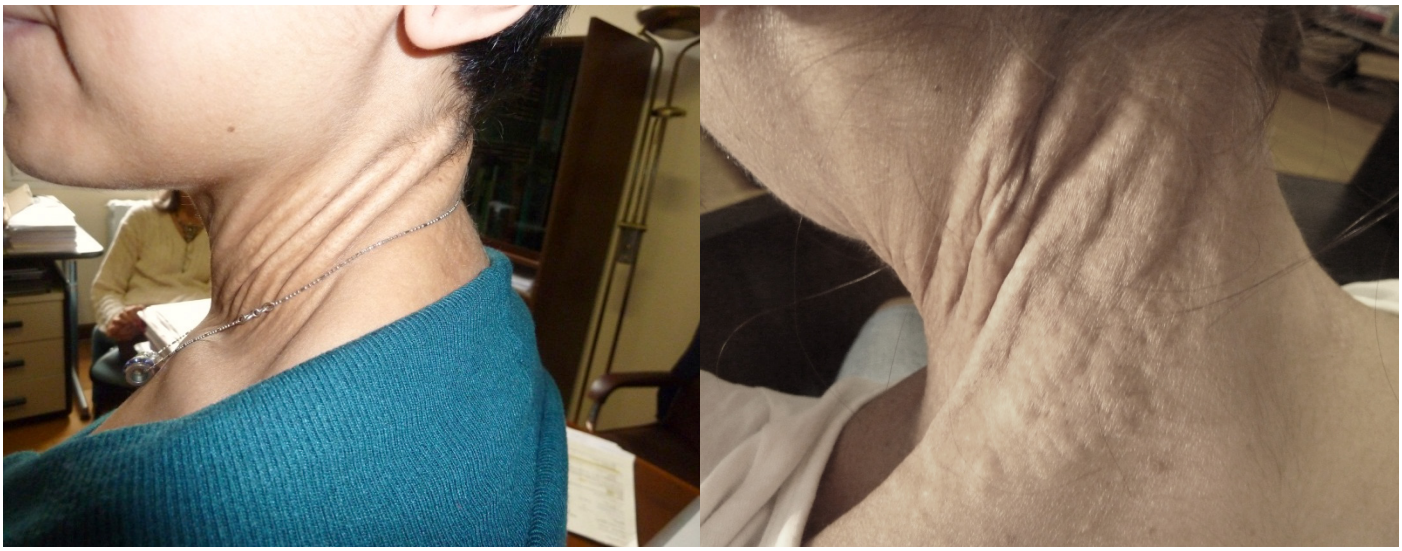

Figure 1: Skin alterations in the neck of a 17-year-old girl (A) and a 35-year-old woman (B) affected by pseudoxanthoma elasticum.
The elastic fibre calcification within the dermis produces confluent papules and redundant skin in the affected areas.

A B

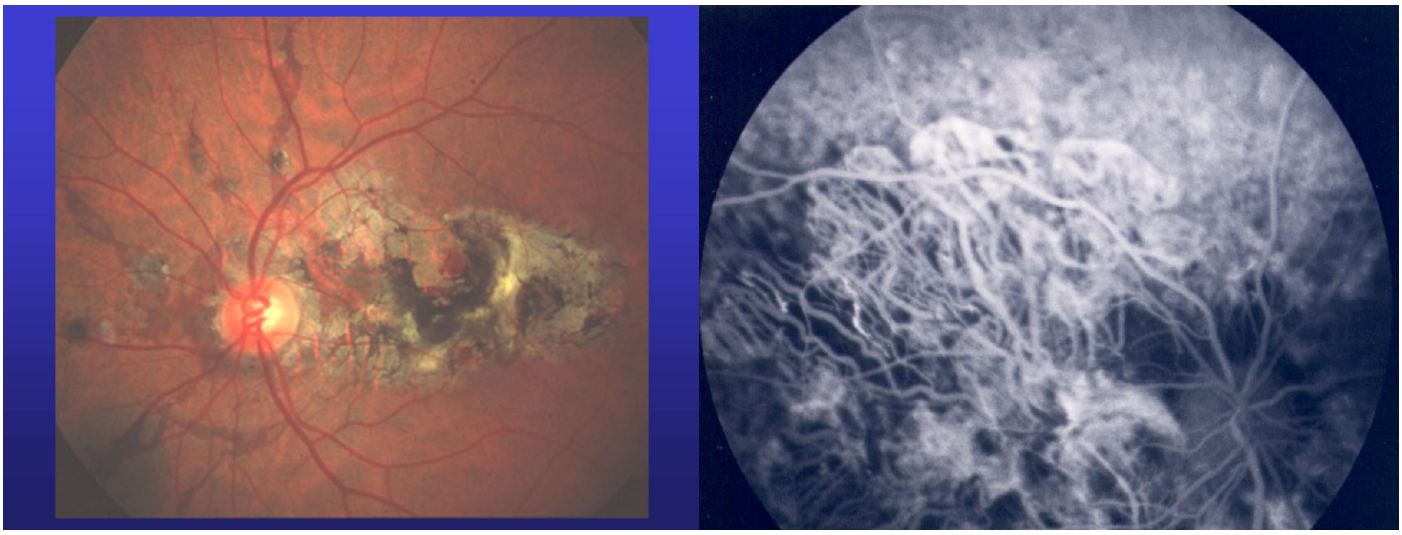

Figure 2: The retina of a 45-year-old man (A) and a 45-year old woman (B) affected by pseudoxanthoma elasticum.
Displaying great heterogeneity between patients, calcification of the elastin in Bruch's membrane induces angioid streaks, followed by neovascularisation, haemorrhages, and scarring.

Several studies have shown that fibroblasts isolated from the dermis of PXE patients exhibit metabolic behaviour and gene expression that is different from sex and age-matched controls. Some alterations are evident only in the absence of fetal calf serum,[84] suggesting that PXE fibroblast metabolism *in vivo* is continuously influenced by circulating factors.[79,80,82,83,85,86] However, several of these metabolic alterations have been shown in PXE fibroblasts grown in optimal culture conditions *in vitro*, which suggests an intrinsic and permanent abnormal gene expression in these cells.[41,65,66,70,74,81,84,88-92] Moreover, some of these metabolic alterations have also been observed

in *Abcc6* −/− transgenic mice,[67,80] confirmed in fibroblasts isolated from these animals and which exhibit a pro-calcific phenotype *in vitro*,[93] and have also been shown in ABCC6-knockdown HepG2 cells.[94] Therefore, data from patients and from animal models of PXE, as well as data from fibroblasts isolated from patients and from transgenic mice, strongly indicate that ABCC6 deficiency induces generalised and local metabolic alterations that are largely independent from circulating factors, and that are permanent and transmissible to subsequent generations because they can be observed up to the 8th passage *in vitro*. In contrast, the onset and severity of clinical manifestations would seem to depend on the genetic background of the patients and mice,[95-97] on the interplay among membrane transporter genes and their promoters,[76,98,99] and on unrelated metabolic disorders.[100-102]

CONCLUSION AND PERSPECTIVES

Studies on calcifying genetic disorders have largely contributed to solving the puzzle of identifying the genes and proteins involved in calcification. In some cases, calcification is due to the mutation of genes controlling the production of anti-calcifying molecules, such as PPi in GACI due to *ENPP1* mutations, or carboxylated MGP in gamma-glutamyl carboxylase or VKOR deficiencies. The case of PXE is more complex, involving calcification of peripheral soft connective tissues,[63,64,73] whereas the protein encoded by the mutated gene (whose function is still not clear) is mainly expressed in the liver. Recent data indicate ATP as the substrate exported by the ABCC6 transporter from within hepatocytes into the circulation.[78] This finding would explain some of the plasma alterations we and others have observed in PXE, but it would not explain the permanent metabolic alterations observed in isolated fibroblasts in the presence of normal calf serum, such as deficient MGP carboxylation,[65-68] redox imbalance,[70,91,92] altered proteoglycan metabolism,[81,82] higher matrix degradation,[88,89] membrane transporter alterations,[90] altered lipoprotein and cholesterol metabolism,[83,84] and expression of a pro-calcific phenotype.[41,42,65,68,74,93]

In conclusion, the data on PXE patients, isolated fibroblasts, and animal models seem to indicate that: i) Extracellular matrix calcification in PXE depends on *ABCC6* mutations (causative), genetic background (affecting onset and severity), and environmental factors (nature of surrounding matrix, diet, age); ii) PXE fibroblasts maintained in optimal culture conditions *in vitro* exhibit a permanent pro-calcific gene expression profile without expressing genes involved in osteogenesis,[41] which is also confirmed in fibroblasts isolated from *Abcc6* −/− mice;[93] iii) This peculiar and permanent gene expression profile of PXE fibroblasts could depend on permanent epigenetic modifications of genes, promoters, or histones, as a consequence of *ABCC6* mutations during embryogenesis or early in development. This hypothesis is supported by the fact that epigenetic regulation of *ABCC6* gene expression has already been observed,[98,99] and that *in vitro* fibroblasts isolated from normal and *Abcc6* −/− mice with identical genetic background show different pro-calcific phenotypes;[93] iv) The 'epigenetic' proposal for the 'pro-calcific' signature of PXE fibroblasts could explain the peculiar organ distribution of calcification, as it has been shown that fibroblasts very quickly differentiate depending on the area of migration and maintain the acquired differentiation throughout their lifespan;[87,103] v) Given the great influence of environmental factors on epigenetic events, this hypothesis may also explain the different clinical phenotypes and severities among PXE patients, even in the presence of identical *ABCC6* mutations and a similar/identical genetic background, as in identical twins; vi) Identification of epigenetic modulation of gene expression in PXE peripheral cells could open new therapeutic perspectives for PXE and other calcification disorders. To conclude, the enormous amount of data derived from studies on PXE and on other inherited calcific disorders strongly indicate that a bioinformatic approach ranging from genes to proteins and to clinical features could help to identify the crucial, and probably common, steps towards calcification upon which therapeutic approaches can be based.

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